NUTRITION AND MANAGEMENT OF ANIMALS WE KEEP AS COMPANIONS

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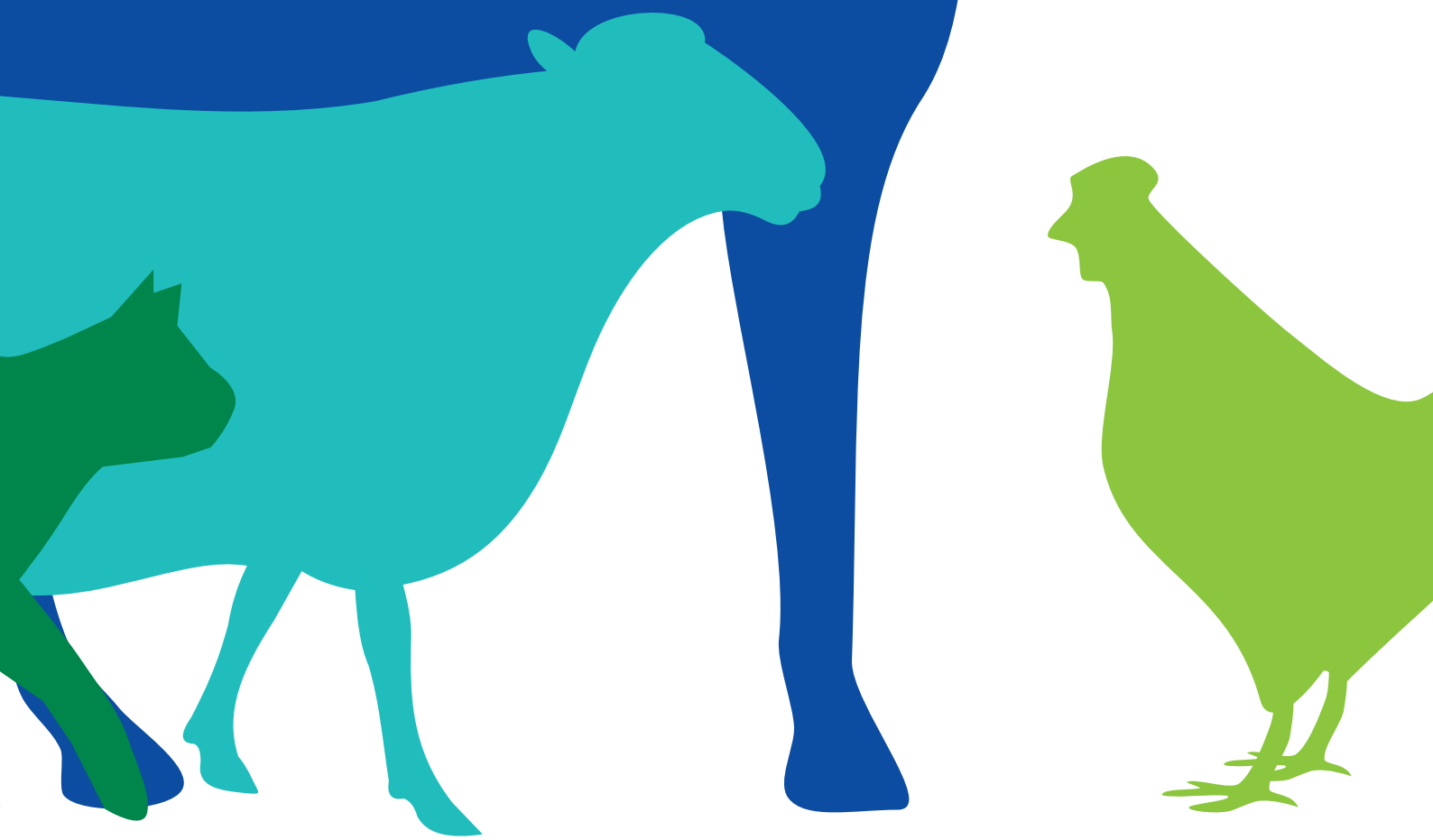

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NUTRITION AND MANAGEMENT OF ANIMALS WE KEEP AS COMPANIONS

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EDITORIAL
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Editorial: Nutrition and Management of Animals We Keep as Companions

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Editorial on the Research Topic

Nutrition and Management of Animals we Keep as Companions

SUMMARY AND COMMENTARY

Pet ownership has recently grown at an unprecedented rate, with The Association of Pet Products Manufacturing Association (APPMA) (1) reporting that pet ownership has increased 3% in the last year, with an estimated 70% of U.S. households owning at least one pet. The APPA also reports that pet owners also increased spending and, largely due to the COVID pandemic, shifted where they buy food with direct-to-consumer purchasing on the rise, with 86% of pet owners purchasing pet food online in 2020, up from 72% in the previous year. Most recently, the millennial generation has now taken over as the largest pet-owning generation, followed by boomers, then GenX. While the growth of the pet food industry is partly driven by ownership, there is also growth stimulated by the growing appreciation of the value of pets to human health and well-being. Indeed, growing evidence suggests physical and mental benefits resulting in positive health outcomes for pet-owning humans. As our bond with companion animals grows, we need to support their role in our lives by better understanding the effects of nutrition, and other parts of the environment that we share with them, on the metabolism, health, and well-being of our companion animals. This special issue adds to the primary literature concerning canine and feline metabolism, nutrition, and behavior.

With a growing global human population and increased demand for food, there is a need to identify novel or alternative food ingredients and processes for pet foods that increase nutrient availability. These ingredients may replace ingredients that can be used in the human food sector, reducing the competition for resources across the food chain. Physical, chemical, or thermal processes that improve digestion and nutrient availability can improve digestion and nutrient delivery to the organism resulting in a reduced need for overall food intake. The food format most commonly used for companion animals is extrusion and as such, cereal grains are widely used in pet food to supply carbohydrates, but also to support optimum physical kibble characteristics. However, dietary carbohydrates are associated with a post-prandial blood glucose response, and ingredients that elicit a large glycemic response have been implicated in weight gain and obesity. Teixeira et al. found the partial replacement of corn with sorghum and with and without hydrolysable tannin extract did not elicit differences in glycemic response, total tract apparent digestibility of dry matter, organic matter, and macronutrients among treatments. These data help to support the inclusion of sorghum instead of corn. Similarly, von Shaumburg et al. examined the inclusion of white and red sorghum grains on the gastrointestinal health of cats and found that both sorghum ingredients were well-tolerated by cats and, for the most part, did not differ from the control that included corn. These data further support the inclusion of these ingredients.

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Reilley et al. added to our available ingredients with the investigation of legumes (garbonzo beans, green lentils, peanut flour) and yeast, and all in contrast to poultry by-product meal on palatability and digestibility in adult dogs. The inclusion of plant-based proteins did not affect apparent total tract digestibility of macronutrients, yeast increased fecal branched-chain fatty acid, fecal butyrate concentrations, and total fecal short-chain fatty acid concentrations. Yeast fed dogs also had greater β-diversity. As such, the researchers concluded that all ingredients are potential ingredients in canine diets and may support beneficial shifts in the microbiome. Similarly, but for the purpose of providing dietary fiber, Finet et al. examined the novel fiber source miscanthus grass, comparing it to beet pulp + tomato pomace and cellulose. While cats fed beet pulp had the greatest concentration of short-chain fatty acids, with the exception of butyrate, which was similar, the cats fed miscanthus grass has greater α-diversity than cats fed beet pulp. Overall, these data suggest that miscanthus grass fiber and fiber blends are good alternatives to the commonly used dietary fiber sources such as beet pulp and cellulose. The research into alternative ingredients and how to appropriately allocate these across the food chain will be integral to the future sustainability of the pet food industry and impact our ability to produce high nutritional quality foods, and we will continue to see investment in this area of research.

The option of diet formats is also increasing in propensity and indeed, raw meat-based diets have been touted to improve metabolic health, but little data exists to support these assertions. Moore et al. compared serum metabolites in client-owned Staffordshire bull terriers with canine atopic dermatitis and fed either a raw meat-based diet (RMBD) or a kibble. Dogs fed kibble had greater concentrations of methionine than dogs fed RMBD, but lower concentrations of carnitine and creatine. The dogs with atopic dermatitis experienced a larger metabolic shift in response to diet, but diet did not change the severity of canine atopic dermatitis. Building on those outcomes, Antuaniemi et al. sought to understand how the skin transcriptome is affected by atopic dermatitis and the feeding of raw diets in contrast to kibble. At the end of feeding a median of 137 days of one of these dietary treatments, 149 genes were found to be differentially expressed in transcripts between atopic and healthy dogs, and all genes were upregulated in the raw fed dogs in contrast to the kibble dogs. Interestingly, atopic dermatitis was largely associated with alterations in lipid and keratinocyte metabolism and angiogenesis. Further, the transcriptome in raw-fed dogs suggests enhanced innate immunity and lower oxidative stress and provides further support for this format of feeding. However, a key obstacle to feeding raw diets is understanding the nutrient content of those ingredients to enable precise food formulation based on nutrient targets of the final product. One feeding format that is being increasingly used to feed domestic cats is whole-prey diets, a version of raw meat-based diets. Owens et al. measured the crude protein, moisture content, and amino acid concentrations in fresh and frozen ground rabbits with or without gastrointestinal tracts (GIT). Focusing on the indispensable amino acids, fresh rabbits with GIT had greater concentrations of methionine, lysine, histidine, and arginine, and freezing resulted in lower methionine in the ground rabbit without the GIT. Most importantly, all indispensable amino acids exceeded recommendations of regulatory agencies except for taurine, which was below recommendations and suggests that taurine may need to be provided to cats if consuming a whole prey diet based on rabbit. Taken together, raw meat-based diets have a place in our feeding management of companion animals, but we need more data on not only the effects of feeding these diets, but the characterization of raw ingredients to support product development.

A key concern for diet management is the effect of early nutrition on the onset of disease later in life and diet format results in a different dietary chemical profile that may be a significant determinant. Hemida et al. investigated the effect of early life dietary, environmental, demographic, and genetic variables and whether these factors are associated with inflammatory bowel disease in dogs. Interestingly, and in opposition to current recommendations for pets, this cross-sectional questionnaire-based study suggested that feeding a high fat, low carbohydrate, non-processed meat-based diet resulted in significantly less inflammatory bowel disease later in life than when an ultra-processed, carbohydrate-based diet was fed. Not surprisingly, a normal body condition early in life supported a lower incidence of IBD, while greater body condition scores were related with a greater prevalence of IBD in dogs. Along the same vein, dietary transition is commonly reported to cause gastrointestinal distress and numerous dietary solutions are being investigated, of which yeast products could hold promising bioactivity. Lin et al. found that supplementing a yeast cell wall fraction supplement tended to result in greater *C. perfringens* and a rapid diet transition to a canned diet or a high fiber diet did not alter fecal pH, dry matter, calprotectin, and *E. coli,* but the inclusion of yeast during this dietary transition tended to result in greater fecal IgA. While a subtle change, the greater IgA suggests enhanced intestinal immune function, and as such, yeast products may help to support gastrointestinal health after dietary transition. Diet change is not the only thing that may disrupt the gastrointestinal environment, high-intensity exercise may also challenge this environment and dietary fiber optimization was studied in a study by Thornton et al. Sled dogs were fed diets containing kibble with a 4:1 or 3:1 insoluble:soluble fiber ratio and also had increasing amounts of exercise over a 9-week period. When sled dogs received the 3:1 diet they experienced greater fecal short-chain fatty acids and a lower internal body temperature than dogs who received the 4:1 but experienced no differences in body composition or respiratory rate. Separately, after the period of step-wise increases in exercise, dogs had an average 7% increase in lean and a 3.5% decrease in fat mass. With sporting dogs, an improvement in the soluble fiber delivery may improve gut health and will not impact performance and indeed, exercise, albeit higher intensity than most pet dogs would receive, does produce favorable shifts in body composition and we need to consider appropriate exercise for our dogs and cats to support optimum metabolism.

There has been recent interest in how diet affects cardiac health in dogs and a suggestion that the inclusion of legumes or elimination of grains may underpin the increased reporting

of dilated cardiomyopathy in particular. Reis et al. examined the inclusion of fava beans in a moderate protein diet and compared that to two commercial diets, one with normal protein (25% crude protein and containing grains) and high protein (41% crude protein containing no grains) and largely using fava beans as a dietary protein ingredient. Similar to Teixeira et al. high-tannin and fermentation were also included in the design. There are a myriad of differences between the treatments of high protein and low protein and high tannin and low tannin, but interestingly, dogs fed normal protein for 7 days experienced greater left ventricular end-systolic volume and cardiac output than dogs fed high protein, but there were no differences among the fava bean fed dogs. These data suggest that fava beans are suitable for inclusion in dog diets, especially if fermented, and that protein intake may play a role in cardiovascular health. In a similar study from the same laboratory, Quilliam et al. sought to investigate whether the fiber brought in by the inclusion of pulses would decrease glycemic response, decrease the rate of digestion, and decrease the bioavailability of macronutrients. Dogs were fed six diets formulated at an inclusion level of 20% available carbohydrates using different ingredients, including legumes, for 7 days. The inclusion of ingredients that possess greater amylose and dietary fiber decreased total tract apparent digestibility, but this was not due to increased bile acid losses. Overall, the authors noticed this decrease in digestibility and the glycemic response was largely due to a lower animal protein content, and that this could put dogs at risk for chronic deficiencies of taurine and may result in the etiology of dilated cardiomyopathy, and that longer-term studies are warranted. In a similar vein, Gizzarelli et al. investigated different carbohydrates sources and their effects on plasma and whole blood taurine status. With diets formulated to provide similar essential nutrients, there was no impact on plasma or whole blood taurine confirming that indeed diets supply nutrients and the ingredients, when used properly, do not affect taurine status. The research into the etiology of dilated cardiomyopathy will continue to be investigated and the characterization of these diets, including detailed nutrient and anti-nutrient content, will continue as the research community and pet food companies continue to pursue new world ingredients as described above.

While cardiac disease may have been recently in the news, overweight and obesity remain the number one nutritionally related issue in our pet population. Niese et al. examined pets and owners both undergoing a simultaneous weight loss program. Overall, the combined results of both human and dog weight loss studies suggest that when either dog or human are actively attempting to lose weight, that it results in passive weight loss in the other and supports using an integrative approach to supporting human and pet weight loss. This opens a new opportunity for physicians and veterinarians to work together and offers a unique integrative or One Health approach touted by the authors. Overall, we expect to see emerging approaches to managing health in our companion animals and the collaboration among human and animal health communities to advance this area of research.

While pet nutrition is riddled with the problems of current formulas, companion animal researchers stay focused on understanding a myriad of arenas regarding nutrient sufficiency. Ruggeiro and Backus compared supplementing cats with either vitamin D2 or 25(OH)D2. Overall, supplementation with 25(OH)D2 was superior to supplementation of vitamin D2 to increase vitamin D status as measured by the presence of vitamin D epimers and may suggest that we need to consider multiple epimeric forms of vitamin D when considering what to supplement in diets. In feline nutrition, we also see a move toward the desire by owners to feed plant-based products, and Dodd et al. report on a case study where a young male cat presents with chronic feline lower urinary tract disease (FLUTD) and was placed on a home-made plant-based diet with the hopes of increasing water intake and promoting an acidic, dilute urine. Indeed, this dietary treatment reduced urinary saturation, and the FLUTD resolved and no subsequent FLUTD was reported by the owner, promoting the use of plant-based diets as an option for the treatment of FLUTD. Indeed, pet owners will continue to drive the industry through their unique consumer trends and, while not always supported by scientists and health care professionals, we need to be open to the communities we serve to understand the perspectives that they hold and the demands they have for the products that companion animal research supports.

AUTHOR CONTRIBUTIONS

AS and GB had primary responsibility for the final content. All authors read and approved the final manuscript.

REFERENCES

1. American Pet Products Manufacturers Association (APPMA). *2021-2022 National Pet Owners Survey*. Greenwich: American Pet Products Manufacturers Association (2016). Available online at: http://www.americanpetproducts.org/pubs_survey.asp (accessed July 26, 2021).

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ORIGINAL RESEARCH
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The Effect of Atopic Dermatitis and Diet on the Skin Transcriptome in Staffordshire Bull Terriers

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Canine atopic dermatitis (CAD) has a hereditary basis that is modified by interactions with the environment, including diet. Differentially expressed genes in non-lesional skin, determined by RNA sequencing before and after a dietary intervention, were compared between dogs with naturally occurring CAD ($n = 4$) and healthy dogs ($n = 4$). The dogs were fed either a common commercial heat-processed high carbohydrate food (kibble diet) ($n = 4$), or a non-processed high fat food (raw meat-based diet) ($n = 4$). At the end of the diet intervention, 149 differentially expressed transcripts were found between the atopic and healthy dogs. The main canonical pathways altered by the dysregulation of these genes were angiopoietin signaling, epidermal growth factor signaling, activation of angiogenesis, and alterations in keratinocyte proliferation and lipid metabolism. On the other hand, 33 differently expressed transcripts were found between the two diet groups, of which 8 encode genes that are annotated in the current version of the dog genome: immunoglobulin heavy constant mu (*IGHM*), immunoglobulin lambda-like polypeptide 5 (*IGLL5*), B-cell antigen receptor complex-associated protein beta chain (*CD79B*), polymeric immunoglobulin receptor (*PIGR*), cystathionine β-synthase (*CBS*), argininosuccinate synthase 1 (*ASS1*), secretory leukocyte peptidase inhibitor (*SLPI*), and mitochondrial ribosome recycling factor (*MRRF*). All genes were upregulated in the raw diet group. In conclusion the findings of this study suggest alterations in lipid and keratinocyte metabolism as well as angiogenesis in the skin of atopic dogs. Additionally, a possible enhancement of innate immunity and decrease in oxidative stress was seen in raw food fed dogs, which could have an important role in preventing hypersensitivities and disturbed immunity at young age.

Keywords: atopic dermatitis, canine, diet, gene expression, RNAseq, skin, kibble diet, raw meat-based diet

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INTRODUCTION

Atopic dermatitis (AD) in humans is a complex hereditary disease characterized by its genetic pre- disposition, as well as how it causes immunologic hyperreactivity and a defective skin barrier. An impaired epidermal barrier is one of the typical features of AD (1–3). Also, environmental factors play a role in its pathogenesis (4). Canine atopic dermatitis (CAD) shares genetic,

phenotypic, and histological similarities with human AD (5). CAD is estimated to affect 10–15% of the canine population and seems to be increasing over time (5, 6). It has been reported that pure bred dogs are more likely to develop atopy and allergic dermatitis than mixed breeds (7). Staffordshire bull terriers and similar breeds (bull terrier, American Staffordshire terrier, and pit bull terrier) have previously been shown to be particularly susceptible to CAD (8–13).

Diet has profound effects on the whole body, including its metabolism. People with skin conditions often modify their diet to influence their clinical symptoms (14–16), as do dog owners for their dogs (17). It has been previously suggested that clinical manifestations of CAD can be modulated by diet (18–21). It has been reported that certain diets can potentially affect skin symptoms positively both in humans (22–24) and in dogs (25–27). Research on how diets impact skin gene expression in dogs is still scarce. However, dietary changes are shown to change the gene expression in the skin of mice (28), rats (29), and humans (30, 31). The impact that the elimination diet has on the skin of dogs with cutaneous adverse food reactions has been studied using quantitative PCR (qPCR) (32). The diets influence on gene expression in the liver (33), skeletal muscle (34), adipose tissue (35), colonic mucosa (36), and brain tissue (37) of dogs has also been studied.

Previously published studies that have focused on altered gene expression in atopic canine skin have used mRNA microarrays (38–40) and (qPCR) (41–50). The objective of this study was to find differentially expressed genes between atopic and healthy dogs, as well as to compare the effects of two different diet types on the gene expression of the skin using RNA sequencing (RNA-Seq). As alterations in skin function of atopic patients are already well recognized, our hypothesis was that there would be differences in the gene expression between atopic and healthy dogs. In addition, we hypothesized that two different dietary choices would subsequently affect skin gene expression differently. Eight Staffordshire bull terriers were used, both atopic and healthy, equally distributed between diet groups. To our best knowledge, neither previous studies using RNA-Seq to compare atopic and healthy dogs, or studies regarding the effect of diet on canine skin gene expression have been conducted.

METHODS

Animals and Sample Collection

The study protocol was approved by the Animal Experiment Board in Finland (ELLA) (permit number: ESAVI/3244/04.10.07/2013). All owners filled in and signed a written consent form. Dogs were living in their home environment during the diet intervention trial. The eight study dogs used in the present study were part of a larger diet intervention trial studying CAD. The study was conducted at the department of equine and small animal medicine at the University of Helsinki.

At the baseline visit, dogs were evaluated by a veterinarian and blood samples were collected. Non-lesional skin biopsies were taken from the axillary area under anesthesia using an 8 mm biopsy punch, and samples were immediately stored at −80°C.

TABLE 1 | Basic information of the eight dogs used in RNA-Sequencing analyses.

Dog ID	Age (years)	Gender	Diet group	Diagnosis
55	13	Female	KD	Healthy
47	4	Male	KD	Healthy
66	4	Male	KD	CAD
33	4	Female	KD	CAD
65	3	Female	RMBD	Healthy
37	5	Female	RMBD	Healthy
40	6	Female	RMBD	CAD
34	2	Male	RMBD	CAD

KD, kibble diet; RMBD, raw meat-based diet; CAD, canine atopic dermatitis.

The animals were sedated using dexmedetomidine (Dexdomitor, Orion Pharma) 5–10 μg/kg intramuscularly and intravenous propofol (PropoVet, Orion Pharma) as needed. Skin biopsies were taken only from dogs that had not taken oral glucocorticoids and cyclosporine for 4 weeks, or oral antihistamines, topical glucocorticoids, and medicated shampoos for 2 weeks prior to sample collection.

After the baseline visit, the dogs were divided into two diet groups, and fed either a commercial heat-processed, high carbohydrate (kibble) diet [Hill's Science PlanTM Canine Adult Sensitive Skin with Chicken (KD), detailed composition of food shown in **Supplementary Table 1**], or a commercial non-processed, high fat (raw meat-based) diet [MUSH BARF Vaisto® Pork-Chicken-Lamb and/or MUSH BARF Vaisto® Beef-Turkey-Salmon (RMBD), detailed compositions shown in **Supplementary Table 2**]. The diets differed by their fat and carbohydrate content, their ingredients (KD having chicken as a main animal protein source and RMBDs having three different animal protein sources each) and their manufacturing methods (the KD was heat-processed and the RMBDs were only ground and frozen). Owners were asked to feed their dogs at least 99.9% trial diet, giving portions as recommended by the manufacturer, adjusting if necessary, to maintain normal body weight. Water was allowed *ad libitum*. At the end visit, the same protocol was followed as the baseline visit, and non-lesional skin biopsies were again obtained from the same area and immediately stored at −80°C.

Skin biopsies were obtained from client-owned Staffordshire bull terriers ($n = 8$). They were stratified between diet cohorts by age, sex, and health status when possible, since the number of healthy dogs was very small. The dogs diagnosed with naturally occurring CAD ($n = 4$) were fed either the KD ($n = 2$) or RMBD ($n = 2$). The healthy control dogs were fed either the KD ($n = 2$) or the RMBD ($n = 2$). This allowed comparisons to be made between KD-fed ($n = 4$) and RMBD-fed ($n = 4$) dogs, as well as between atopic ($n = 4$) and healthy ($n = 4$) dogs. Information regarding the dogs chosen for RNA-Seq analyses ($n = 8$) are shown in **Table 1**.

RNA Extraction

Prior to RNA extraction, the skin biopsy samples were transferred to RNAlater®-ICE Frozen Tissue Transition Solution

(Life Technologies, Carlsbad, CA, USA) and allowed to thaw overnight at −20°C. Subcutaneous fat was carefully trimmed from the skin biopsies, and the samples were homogenized using a tissue homogenizer (TissueRuptor, Qiagen, Hilden, Germany). Total RNA was extracted using Qiagen miRNAeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's recommended protocol. After RNA extraction, DNAse treatment using RNase-free DNase I (Thermo Fisher Scientific, Waltham, MA, USA) was performed. The total RNA concentration of samples was analyzed using a 260 nm ultraviolet spectrophotometer (NanoDrop ND-1000, Thermo Fisher Scientific, Wilmington, DE, USA). The integrity and quality of the RNA was analyzed using an Agilent 2100 Bioanalyzer (Agilent Biotechnologies Inc., Santa Clara, CA, USA) and only samples with RIN >7 and RNA amount higher than 1 μg were sent for analysis. Prior to being sent to the sequencing facility, the samples were stored at −80°C.

RNA-Seq and Data Analysis

Next generation sequencing was performed on all of RNA skin samples of the dogs, taken at the baseline ($n = 8$) and end of the diet intervention ($n = 8$). One microgram (μg) of total RNA was ribodepleted and an RNA-Seq library was created using a ScriptSeq v2™ Complete kit for human/mouse/rat (Illumina, Inc., San Diego, CA, USA). Paired-end library creation and transcriptome sequencing were completed at the Institute for Molecular Medicine Finland (FIMM). Libraries were quality controlled by High Sensitivity chips by Agilent Bioanalyzer (Agilent) before being sequenced on an Illumina HiSeq platform (HiSeq 2000, Illumina, Inc., San Diego, CA, USA).

The bioinformatic analysis was performed at the Plataforma Andaluza de Bioinformática (University of Málaga, Spain). Quality control and initial pre-processing was performed using SeqTrimNext (v. 2.0.53), where low quality, ambiguous, low complexity stretches, adaptor, organelle DNA, polyA/polyT tails, and contaminating sequences were removed (51). Mapping was performed with restrictive conditions of bowtie2 (v.2.2.2), including parameters for rejecting discordant alignments and optimized for paired-end reads (52). Samtools (v.0.1.19) quantified known transcripts (count reads per transcripts) (53), and transcripts were annotated using Full-Lengther-Next (54). Statistical comparisons of transcript expression between diet groups and between atopic and healthy dogs were performed using DEgenes-Hunter (v.2.0.11) (55), a tool that imputes raw read counts generated by Bowtie2/Samtools into the EdgeR (56) and DESeq2 (57) algorithms. Fold change (FC) ≥2, and a false discovery rate (FDR) corrected $p < 0.05$ were set as thresholds. Differentially expressed transcripts common to both analyses (EdgeR and DESeq) were also identified. RNA-Seq can detect a higher percentage of differentially expressed genes compared to expression arrays, especially genes with low abundance. Typically, in many controlled experiments which utilize RNA-Seq, the number of biological samples is a limiting factor (57) which ultimately restricts the statistical inference to the largest gene expression differences between the groups. Specific algorithms have been developed to improve statistical inference in RNA-Seq datasets with small sample sizes (56–58).

TABLE 2 | Descriptive data and statistical analysis of the atopic and healthy dogs used in the study comparing the skin gene expression profiles ($n = 8$).

		Atopic dogs	Healthy dogs	*p*-value
Dogs	*N*	4	4	
Age	Years	3.5 (±1.7)	6.0 (±4.7)	0.468
Gender	% male	50	25	0.500
Duration	Days, median	129	146	0.058
Weight	Baseline, Kg	17.3 (±2.9)	16.1 (±1.8)	0.773
	End visit, Kg	17.7 (±2.7)	16.7 (±1.7)	0.564

Data are mean ± SD. Analyzed using Mann-Whitney U-test, Fisher's Exact test and Wilcoxon signed ranks test.

TABLE 3 | Descriptive data and statistical analysis of the kibble diet (KD) and raw meat-based diet (RMBD) fed dogs used in the study comparing the skin gene expression profiles ($n = 8$).

		KD group	RMBD group	*p*-value
Dogs	*N*	4	4	
Age	Years	5.9 (±4.8)	3.6 (±1.8)	0.663
Gender	% male	50	25	0.500
Duration	Days, median	144	132	0.465
Weight	Baseline, Kg	16.3 (±1.4)	17.1 (±3.3)	0.773
	End visit, Kg	16.7 (±1.2)	17.7 (±2.9)	0.564

Data are mean ± SD. Analyzed using Mann-Whitney U-test, Fisher's Exact test and Wilcoxon signed ranks test.

EdgeR and DESeq2 are shown to have the highest sensitivity to detect true differences between group means in read count datasets produced by RNA-Seq methods (57, 58). These tools utilize different approaches in calculation of dispersion estimates for read count datasets, and algorithms are differently affected by outlying observations (58).

The comparisons related to dogs' characteristics between the two diet groups were analyzed using the Mann-Whitney U test, Fisher's Exact test and the Wilcoxon signed ranks test. SPSS software (version 25, IBM SPSS Statistics. Chicago, Ill., USA) was used for the statistical analyses. The statistical significance threshold was set at $P < 0.05$.

RESULTS

Clinical Findings

There were no statistical differences between the comparison groups ($n = 8$) in any of the recorded descriptive data of the dogs (**Tables 2**, **3**). The mean weight of the dogs in the atopic ($n = 4$) and healthy cohorts ($n = 4$) did not change significantly during the trial ($p = 0.180$ and 0.141, respectively; **Table 2**), or in the KD-fed and RMBD-fed cohorts ($p = 0.285$ and 0.102, respectively; **Table 3**). The diet intervention lasted 84–147 days (median 137 days).

Sequencing Overview

An average of 52.2 million sequencing reads were obtained per sample, ranging from 40.7 to 69.4 million reads. Mean

TABLE 4 | Differentially expressed genes in the skin of atopic dogs ($n = 4$) compared to healthy dogs ($n = 4$) at the baseline visit.

Gene	FDR	*P*-value	Log_2FC	Algorithm
PKHD1	0.0242	6.00E-06	−2.6	EdgeR
KRT4	0.0013	1.57E-07	−4.8	EdgeR
LYZF2*	0.0032	5.92E-07	−4.6	EdgeR
AHDC1	0.0168	5.35E-06	−0.73	DESeq2
DEDD	0.0259	9.89E-06	−0.60	DESeq2
SGOL2	0.0146	2.05E-06	0.66	DESeq2
LEPR*	0.0295	4.60E-05	−1.1	DESeq2
DUSP1	0.0297	1.31E-05	−0.87	DESeq2

*RNA-sequensing was used. *Multiple transcripts were found, only the one with the highest FC are shown. FDR, false discovery rate corrected p-value; PKHD1, polycystic kidney and hepatic disease 1; KRT4, keratin 4; LYZF2, lysozyme C; AHDC1, AT-hook DNA-binding motif-containing protein 1; DEDD, death effector domain containing, SGOL2, shugoshin 2; LEPR, leptin receptor; DUSP1, dual specificity phosphatase 1.*

read length obtained from sequencing was 93.8 bp after pre-processing quality trimming. An average of 93.47% of the reads were mapped to the canine reference genome. The obtained read counts were then uploaded into the DEgenes-Hunter tool, where comparisons using two different software (DESeq2 and EdgeR) were performed.

Differential Gene Expression Between Atopic and Healthy Dogs

At the baseline visit, three downregulated genes were found in atopic dogs compared to healthy dogs by EdgeR. Four downregulated genes and one upregulated gene were found by DESeq2 in atopic dogs compared to healthy dogs (**Table 4**).

After the diet intervention, EdgeR found 200 transcripts, and DeSeq found 451 transcripts that were differentially expressed between atopic and healthy groups after the diet intervention (S3), of which 149 differentially expressed transcripts between groups were found by both EdgeR and DESeq2 (**Figure 1A**; **Supplementary Table 3**). According to the analyses of their biological function, 69 of the differentially expressed transcripts were involved in dermatological and inflammatory conditions, of which 8 were associated with atopic dermatitis based on the analysis of biological functions (**Figure 1B**). Signaling pathways affected by gene dysregulation in the atopic group included angiopoietin signaling, epidermal growth factor signaling, AMP-activated protein kinase signaling, retinoid X receptor (RXR)/farnesoid X receptor (FXR) signaling, and leptin signaling in obesity. Also, upregulation of EGF, AKT3, KLB, ANGPT1, and TEK led to the predicted activation (z-score = 2.23) of the IL-8 inflammatory pathway (**Figure 1C**).

Comparison Between the Two Diet Groups

Altogether 33 differently expressed transcripts were found between the diet groups, of which 8 genes are annotated in the current version of the dog genome: immunoglobulin heavy constant mu (*IGHM*), immunoglobulin lambda-like polypeptide 5 (*IGLL5*), B-cell antigen receptor complex-associated protein beta chain (*CD79B*), polymeric immunoglobulin receptor (*PIGR*), cystathionine β-synthase (*CBS*), arginosuccinate synthase 1 (*ASS1*), secretory leukocyte peptidase inhibitor (*SLPI*), and mitochondrial ribosome recycling factor (*MRRF*). These genes ($n = 8$) were all upregulated in the RMBD group compared to the KD group (**Table 5**). They were then studied regarding their biological implication. Activation of ASS1 and CBS in the raw diet group indicated upregulation of cystein biosynthesis and methionine degradation, the citrulline-nitric oxide cycle, the urea cycle, and arginine biosynthesis (**Figure 2**). Biological functions activated by CBS included for example concentration of glutathione, conversion of homocysteine, and concentration of phospholipids, and inhibited by CBS included for example oxidative stress, hyperkeratosis, and accumulation of reactive oxygen species (**Figure 3**). In addition, the expression of IGHM, IGLL5, and CD79B activates the differentiation of B lymphocytes and the memory immune response, determines the quantity of different immunoglobulins, inhibits hypoplasia of the lymphoid organ, determines the quantity and secretion of autoantibody, inflammation of secretory structure, and lesioning of skin (**Figure 4**).

The activation of immunological pathways such as IL-7 signaling, and B-cell related pathways were also found. Upregulation of (natural) IgM antibodies can result from enhanced IL-7 production by epithelial cells in the gastro-intestinal tract, where they are then shuttled across the epithelium into the lumen as a result of upregulated poly Ig receptor expression (59).

DISCUSSION

To our best knowledge, the present study is the first RNA-Seq study of altered skin gene expression between dogs fed two different diets. Although the sample sizes in this study was limited, differences were found both between the atopic and healthy groups and between the diet groups. There were many more differentially expressed genes found between the same atopic and healthy dogs at the end of the diet intervention than at baseline. This suggests that the diet is an important background factor which should be considered when studying the gene expression of animals with skin diseases.

In dogs suffering from CAD, the defective skin barrier is believed to facilitate the penetration of allergens into the skin. This then leads to sensitization against environmental allergens and subsequent cutaneous inflammation, which then further aggravates the impairment of the skin barrier (60). Lipids in the skin are important to its barrier function as they help prevent transepidermal water loss (TEWL) (61). Expression of galectin 12 (*LGALS12*), hydroxycarboxylic acid receptor 1 (*HCAR1*) and ATP Binding Cassette Subfamily D Member 2 (*ABCD2*) were upregulated in the atopic dogs in the present study. *LGALS12* and *HCAR1* suppress lipolysis (62, 63). As part of the barrier formation process, keratinocytes secrete lipids through lipolysis (64). Very-long chain fatty acids (VLCFA) act as a water barrier and are important in forming a cornified envelope, both of which help maintain the integrity and functionality of the skin (65, 66). ABCD2 is suggested to have a role in a transporting VLCFAs to

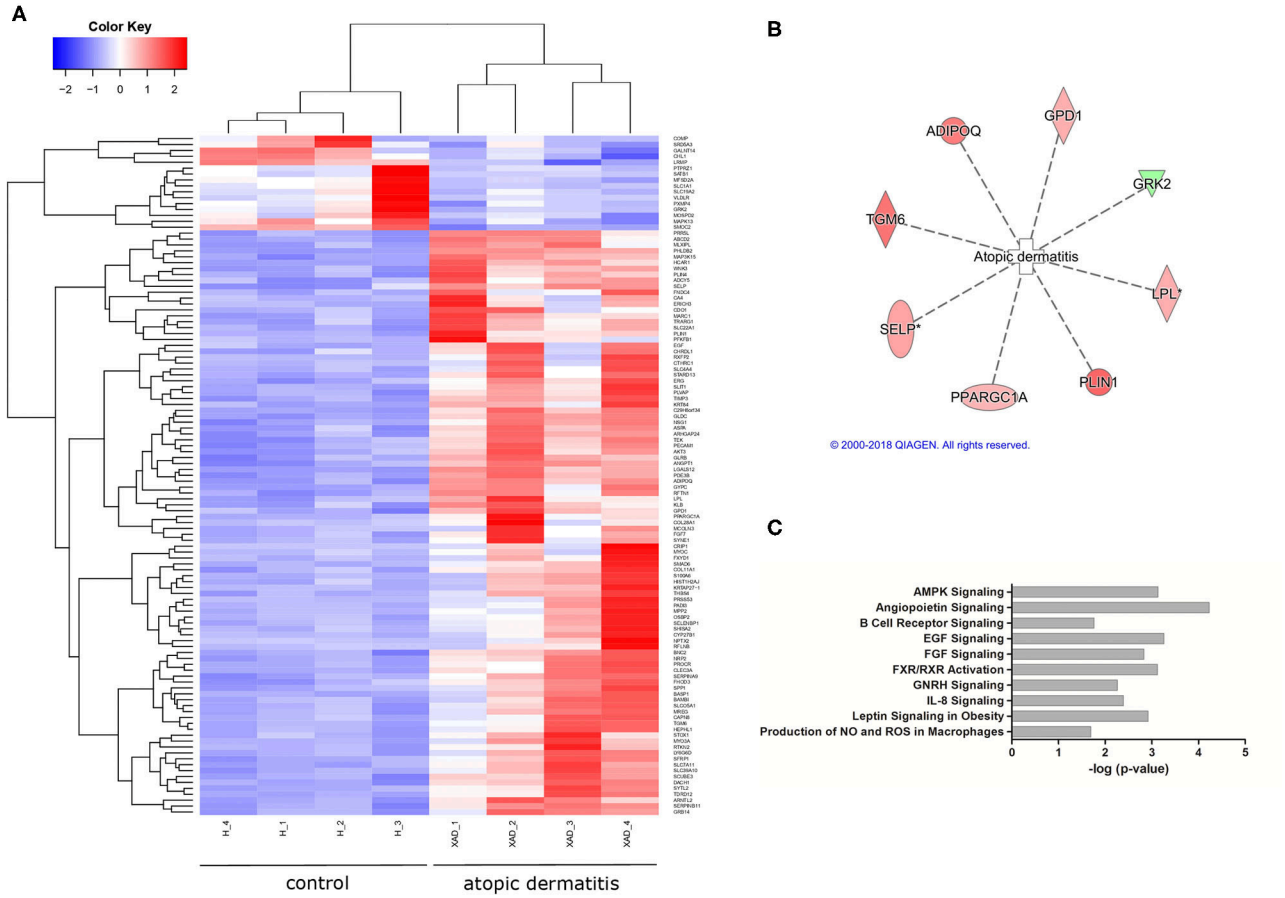

FIGURE 1 | Differential gene expression in atopic vs. healthy dogs ($n = 8$). **(A)** A heatmap showing differentially expressed genes in atopic and healthy dogs (downregulated in blue, upregulated in red). **(B)** A total of eight genes associated with atopic dermatitis were found differentially expressed in atopic dogs after the diet trial (green indicates downregulation, red indicates increased expression). **(C)** Signaling pathways affected by gene dysregulation in dogs with atopic dermatitis compared to healthy dogs.

peroxisomes for degradation by β-oxidation (67). These results suggest that the lipid metabolism in the skin of atopic dogs might have been impaired, detrimentally affecting skin barrier function.

The cornified envelope formation of the skin also requires the differentiation of keratinocytes (61, 68). This differentiation is regulated by the epidermal differentiation complex (EDC). Another gene cluster located in the EDC is the S100 gene family. A higher expression of S100 calcium binding protein A6 (*S100A6*) was found in the non-lesional skin of atopic dogs compared to healthy dogs. One member of the S100 gene family, *S100A8*, has been previously shown to be associated with lesional skin of atopic dogs (38–50). A recent *in vitro* study showed that the overexpression of *S100A6* results in a less differentiated keratinocyte phenotype and thus disturbs the differentiation process (69). Additionally, one of the epigenetic regulators needed for the terminal differentiation of keratinocytes, special AT-rich sequence-binding protein 1 (*SATB1*) (70), was downregulated in the skin of atopic dogs in the present study. These results indicate that the differentiation of keratinocytes in the skin of atopic dogs might be dysfunctional. Altered gene expression of keratins (KRTs) are found in both the skin of atopic dogs (39, 40, 71) and human AD patients (39, 72). In the present study, a transcript of the *KRT4* gene was highly downregulated, and the *KRT84* gene was overexpressed in all atopic dogs compared to healthy dogs at the diet intervention baseline. An association of *KRT84* with CAD has not been previously reported.

In the present study, angiopoietin signaling was the most upregulated canonical pathway found in the atopic dogs. Angiogenesis has been reported to play a role in atopic dermatitis (73–75). In the present study AKT serine/threonine kinase 3 (*AKT3*), angiopoietin 1 (*ANGPT1*), tyrosine receptor kinase (*TEK*), and secreted phosphoprotein 1 (*SPP1*) were upregulated in the skin of atopic dogs, all of which are known to regulate angiogenesis (76–78). *ANGPT1* has been shown to be upregulated in the skin of the AD mouse model (75). Together with *TEK, ANGPT1* has also been reported to be upregulated in psoriatic skin in humans (79). Elevated expression of *SPP1* in the skin of psoriatic patients has been reported, but the expression of *SPP1* in lesional skin of AD patients was not observed (80).

TABLE 5 | Differentially expressed genes in raw meat-based diet fed dogs compared to kibble diet fed dogs after the diet intervention ($n = 8$).

Gene name	FDR	P-value	Log_2FC	Algorithm
PIGR	0.0225	2.48E-05	6.4	EdgeR
SLPI	0.0487	6.11E-05	5.4	EdgeR
IGHM*	1.28E-05	7.83E-10	5.3	ER/ DS
IGLL5*	0.00287	1,56E-06	5.3	
CD79B	0.00564	5.09E-06	4.8	
ASS1	0.00564	5.17E-06	2.1	ER/ DS
CBS*	0.0408	4.74E-05	1.8	ER/ DS
MRRF	0.0360	2.26E-05	0.70	DESeq2

*Only genes that are annotated in the dog genome are shown. *Multiple transcripts were found, only the one with the highest FC are shown. FDR, false discovery rate; FC, fold change; PIGR, polymeric immunoglobulin receptor; SLPI, secretory leukocyte peptidase inhibitor; IGHM, immunoglobulin heavy constant mu; ASS1, argininosuccinic synthase 1; CBS, cystathionine β-synthase; MRRF, mitochondrial ribosome recycling factor; IGLL5, immunoglobulin lambda-like polypeptide 5; CD79B, B-cell antigen receptor complex-associated protein beta chain; ER, EdgeR; DS, DESeq2.*

Hence the upregulated angiogenesis found in the present study comports with previous research.

The upregulation of several immunity-related genes was found in the RMBD fed dogs at the end of the diet intervention. The *IGHM* gene encodes the C region of the mu heavy chain, which defines the IgM isotype. As immunoglobulin M's (IgM's) are the first antibodies to be produced in an ongoing immune response to infection or immunization (81), the upregulation of *IGHM* in the RMBD fed group may indicate activation of humoral immune mechanisms. IgM antibodies are generally polyspecific and have low binding affinities and reflect an increased innate immune defense. Together with *IGLL5*, *IGHM* regulates both the quantity of different immunoglobulins and inhibits the hypoplasia of lymphoid organs. Together with *CD79B, IGHM* increases lymphoid tissue quantity. *CD79B* inhibits biological functions such as skin lesioning and inflammation of secretory structures. Activation of these three genes also increases the proliferation of B lymphocytes. Playing an important role in the mucosal immune system, *PIGR* transports polymeric immunoglobulins to the apical surfaces of epithelia (82). While IgM and IgA antibodies only have a limited antigen specificity, they generally show a large bystander response. Thus, secretory IgMs and IgAs in the gastrointestinal tract are polyreactive against primarily commensal bacteria and most of these "natural" anti-commensal secretory Igs (sIgs) are made through T cell-independent B cell responses (83). Secretions collected at mucosal surfaces contain significant proportions of IgA due to passive transudation, reflecting the degree of mucosal inflammation. The sIgA is generated at the cleavage site of PIGR and acts as an inhibitory factor against bacteria on the skin surface. In human AD, abnormalities in sIgA have been reported (84). Previous studies have indicated that *PIGR* and its secretory component have an anti-inflammatory role in inflammatory skin diseases (85–87).

In the citrulline-nitric oxide (NO) cycle, found in many cells, ASS1 is a rate-limiting enzyme for nitric oxide synthesis (88). The expression of *ASS1* in macrophages and neutrophils can be upregulated in response to bacterial lipopolysaccharides, and hence also contributes to the innate immune defense (89). Although bacteria present in the RMBD may have enhanced the expression of *ASS1*, its expression is also necessary for optimal control of persistent pathogens (90), which may be beneficial to dogs suffering from CAD. NO functions as an antimicrobial (91) and can initiate human keratinocyte differentiation (92), which is necessary for the proper development of a functional skin barrier (93). It has been suggested that high concentrations of NO are functionally important for the resolution of chronic inflammatory processes (42, 94).

SLPI, which is a serine protease inhibitor and an antimicrobial peptide, was upregulated in the RMBD group. Lancto et al. (42) found a lower expression of *SLPI* both in the lesional and non-lesional skin of atopic dogs compared to healthy dogs. Considering the roles of *IGHM, PIGR, ASS1,* and *SLPI*, the RMBD may have both enhanced the dogs' innate immunity and improved barrier function of the skin. Consumption of certain dietary constituents found in the RMBD, namely water-soluble vitamins and amino acids, have been previously shown to positively affect the skin barrier by decreasing TEWL in dogs (93). It remains unclear whether the effect seen in the present study was due to the quantity of certain nutrients, or due to the differences in fat or moisture content of the diets.

CBS catalyzes a reaction in which serine and L-homocysteine (Hcy) are condensed to cystathionine and subsequently converted to cysteine, which is the limiting reagent in the production of glutathione, an important antioxidant (95–97). Inflammatory processes cause alterations to this pathway (97) and reduced glutathione production is associated with an increased vulnerability to oxidative stress (96). Elevated oxidative stress and immune dysfunction, which eventually leads to skin damage, appears to also play a role in the pathophysiology of atopic dermatitis. Clinical symptoms of atopic dermatitis can thus be mitigated by increasing antioxidant levels, as they help reduce oxidative damage. A study recently reported that cystathionine and cysteine inhibit the upregulation of proinflammatory mediators in human keratinocytes (98). Additionally, *MRRF* depletion results in elevated ROS production and cellular dysfunction (99). In our study, the expression of *CBS* and *MRRF* suggests that the RMBD may have inhibited ROS production in skin cells. Oxidative stress plays an important role in the pathogenesis of atopic dermatitis in mammals (100, 101), and the induction of oxidative stress is related to both excessive levels of ROS and to deficiencies within the antioxidant system (102). The possible effects of increased *CBS* and *MRRF* activity might offer therapeutic value to atopic dogs, since ROS production is increased in canine atopic dermatitis during the inflammatory process (103).

Anderson et al. (104) studied gene expression profiles of peripheral blood mononuclear cells from dogs fed either a kibble diet ($n = 8$) or a raw red meat diet ($n = 7$) using Agilent Canine 4 × 44 k microarrays. Their results indicated that a short-term (3 week) diet influenced gene expression at the system level, and that the kibble diet was proinflammatory and the raw red meat diet had anti-inflammatory effects. The

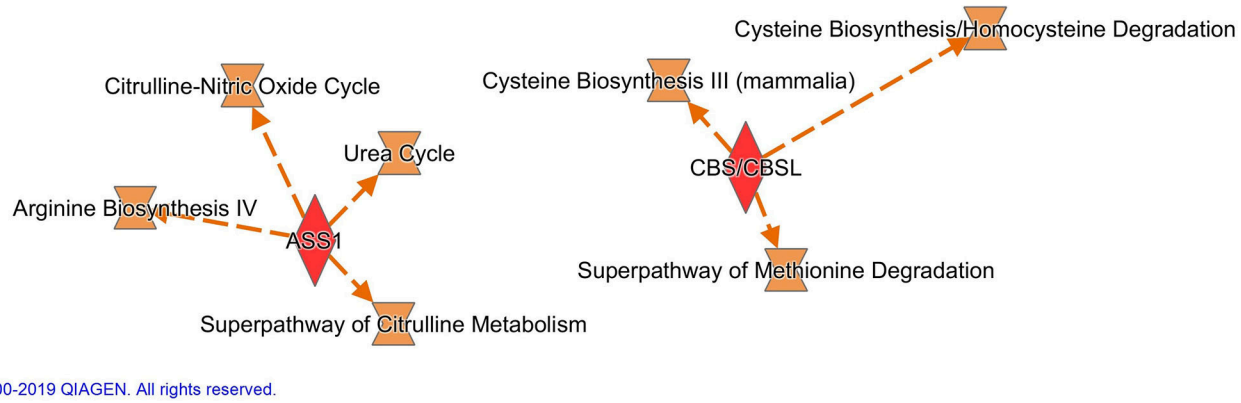

FIGURE 2 | Canonical pathways activated by the expression of ASS1 and CBS in the raw meat-based diet fed dogs. ASS1, argininosuccinate synthase 1; CBS, cystathionine β-synthase.

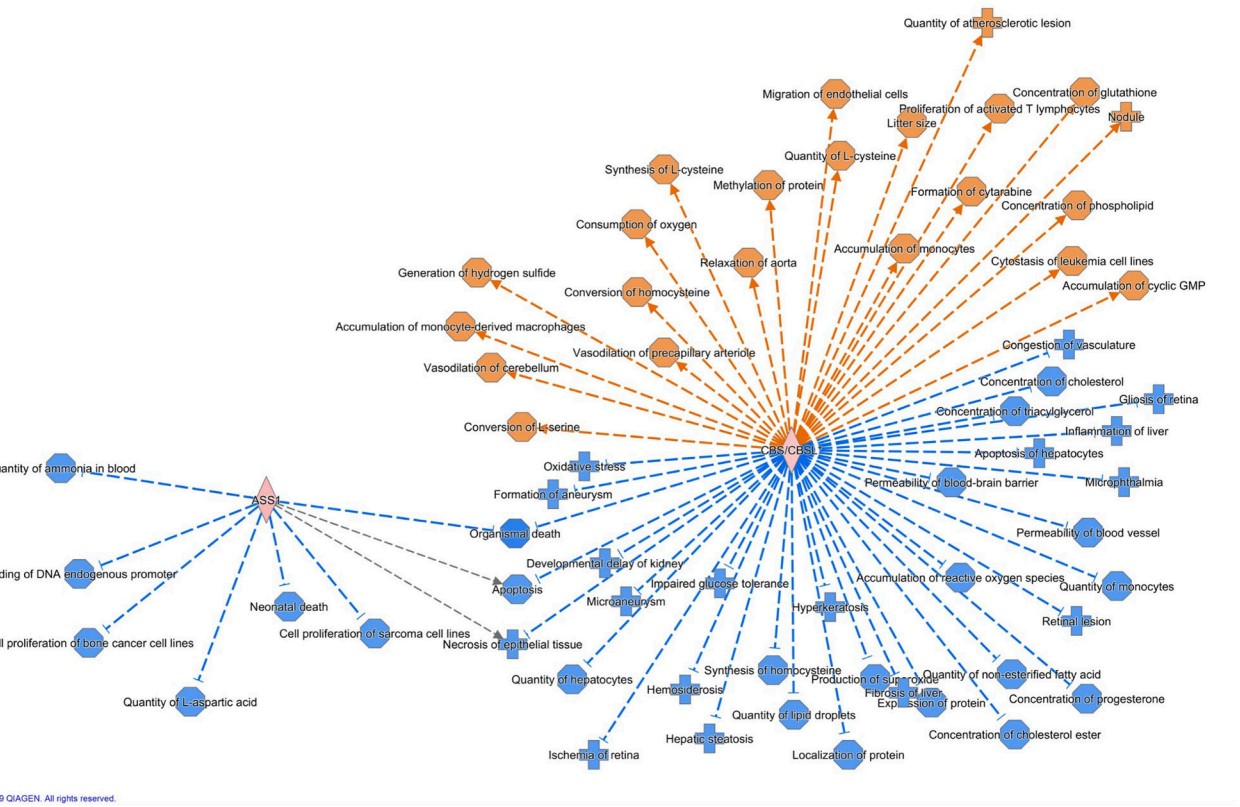

FIGURE 3 | Biological functions activated (in orange) or inhibited (in blue) by the expression of ASS1 and CBS in the raw meat-based diet fed dogs. ASS1, argininosuccinate synthase 1; CBS, cystathionine β-synthase.

comparison of the RMBD and KD in the present study showed that the differentially expressed genes mainly related to immune function, where *CBS* and *PIGR* also have anti-inflammatory effects when upregulated. Our findings support the results of Anderson et al. (104), demonstrating a similar effect over a longer diet intervention. However, more research is needed to verify this observation.

In the present study, genes upregulated in the skin of RMBD fed dogs were found to be related to innate immune function, inflammation and antioxidants, possibly indicating that their innate immunity was enhanced, and that there was less oxidative stress. Thus, RMBDs may have an important role in preventing hypersensitivities and disturbed immunity in puppyhood (105). Since the raw food was served raw, it may have enhanced

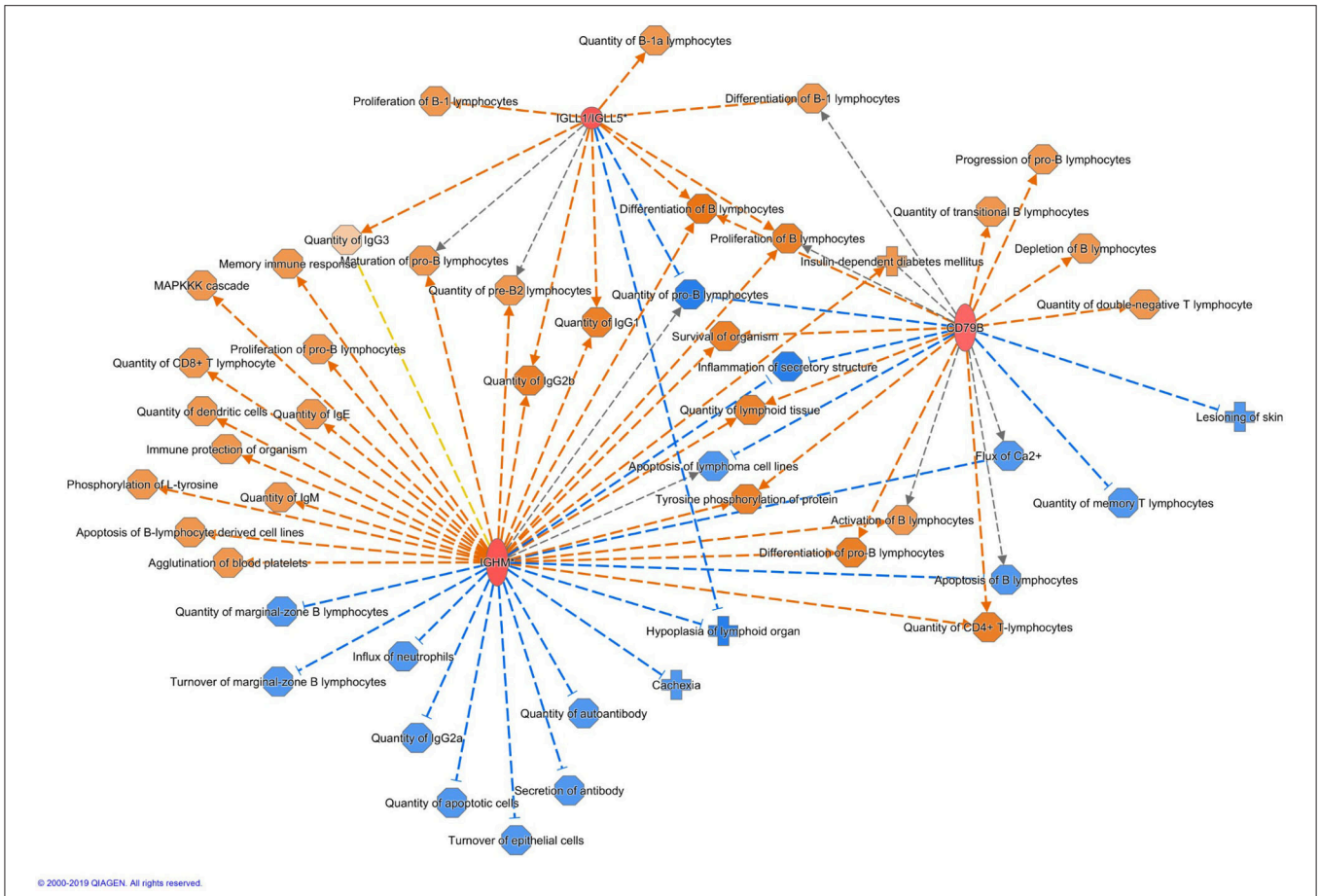

FIGURE 4 | Biological functions activated (in orange) or inhibited (in blue) by the expression of IGHM, IGLL5, and CD79B in the raw meat-based diet fed dogs. IGHM, immunoglobulin heavy constant mu; IGLL5, immunoglobulin lambda-like polypeptide 5; CD79B, B-cell antigen receptor complex-associated protein beta chain.

the passive innate immunity more than the more sterile and processed KD. This has been suggested as a reason for the protective effect of raw meat-based diets in canine *Toxocara canis* infections (106). High-protein diets have been shown to be anti-inflammatory in the skin of mice and they might also prove to be beneficial for dogs suffering from allergic skin conditions (107). Secondary skin infections are common in atopic dogs, and antibiotics are often used when treating them. Antibiotic resistant bacteria are an increasing problem in veterinary medicine (108, 109). If the immunity of the skin could be enhanced through diet at a young age, it might decrease the frequency of antibiotic treatments, although further research is warranted.

Since this was a pilot study, the sample size was limited. To counteract this, two different algorithms were used to analyze the data. However, our results might still in part reflect the individual genetic differences between the dogs. Because these dogs were client-owned, it cannot be ruled out that different environmental factors may have affected the results. The KD and RMBDs had both very different macronutrient profiles and ingredients and their comparison was performed intentionally as a test between two common types of canine diet. Although this complicates the interpretation of the results it nevertheless shows the differential effect that diets had on gene expression. A larger sample size with more controlled diets should be used to validate the results of this study.

CONCLUSIONS

The present study showed that lipid metabolism and differentiation of keratinocytes were possibly altered in the skin of atopic dogs. Additionally, compared to the KD fed group, the gene transcription profile of dogs induced by the RMBD in this study is consistent with an enhancement of innate immunity and decreased oxidative stress and may have an important role in preventing hypersensitivities and a disturbed immunity. As there were two major factors differentiating the diets, processed vs. non-processed and high carbohydrate vs. high fat, further studies must be conducted to determine which, or to what extent these factors influenced the results seen in the present study.

DATA AVAILABILITY STATEMENT

The RNA-Seq data can be found in the SRA database under accession number SRP110851.

ETHICS STATEMENT

This animal study was reviewed and approved by Animal Experiment Board in Finland (ELLA) (permit number: ESAVI/3244/04.10.07/2013). Written informed consent was obtained from the owners for the participation of their animals in this study.

AUTHOR CONTRIBUTIONS

JA, AH-B, and KE contributed conception and design of the study. JA, SZ-L, and KE did laboratory work. JA, SZ-L, KE, AH-B, and HS analyzed and interpreted the data. JA wrote the first draft of the manuscript. SZ-L, KE, AH-B, and HS wrote sections of the manuscript. All authors contributed to manuscript revision, read, and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2020.552251/full#supplementary-material

Supplementary Table 1 | Composition and analytical constituent of the study kibble diet Hill's Science Plan Canine adult sensitive skin with chicken.

Supplementary Table 2 | Composition and analytical constituent of the study raw meat-based food Mush Vaisto diets.

Supplementary Table 3 | Differentially expressed transcripts, biological functions, and pathways after the diet intervention in atopic vs. healthy dogs.

REFERENCES

1. Marsella R, Olivry T, Carlotti DN, International Task Force on Canine Atopic Dermatitis. Current evidence of skin barrier dysfunction in human and canine atopic dermatitis. *Vet Dermatol*. (2011) 22:239–248. doi: 10.1111/j.1365-3164.2011.00967.x
2. Ramms L, Fabris G, Windoffer R, Schwarz N, Springer R, Zhou C, et al. Keratins as the main component for the mechanical integrity of keratinocytes. *Proc Natl Acad Sci USA*. (2013) 110:18513–8. doi: 10.1073/pnas.1313491110
3. Santoro D, Marsella R, Pucheu-Haston CM, Eisenschenk MN, Nuttall T, Bizikova P. Review: pathogenesis of canine atopic dermatitis: skin barrier and host-micro-organism interaction. *Vet Dermatol*. (2015) 26:84–e25. doi: 10.1111/vde.12197
4. Barnes KC. An update on the genetics of atopic dermatitis: scratching the surface in 2009. *J Allergy Clin Immunol*. (2010) 125:16–29. doi: 10.1016/j.jaci.2009.11.008
5. Marsella R, Girolomoni G. Canine models of atopic dermatitis: a useful tool with untapped potential. *J Invest Dermatol*. (2009) 129:2351–7. doi: 10.1038/jid.2009.98
6. Hillier A, Griffin CE. The ACVD task force on canine atopic dermatitis (I): incidence and prevalence. *Vet Immunol Immunopathol*. (2001) 81:147–51. doi: 10.1016/S0165-2427(01)00296-3
7. Bellumori TP, Famula TR, Bannasch DL, Belanger JM, Oberbauer AM. Prevalence of inherited disorders among mixed-breed and purebred dogs: 27,254 cases (1995-2010). *J Am Vet Med Assoc*. (2013) 242:1549–55. doi: 10.2460/javma.242.11.1549
8. Anturaniemi J, Uusitalo L, Hielm-Björkman A. Environmental and phenotype-related risk factors for owner-reported allergic/atopic skin symptoms and for canine atopic dermatitis verified by veterinarian in a Finnish dog population. *PLoS ONE*. (2017) 12:e0178771. doi: 10.1371/journal.pone.0178771
9. Mazrier H, Vogelnest LJ, Thomson PC, Taylor RM, Williamson P. Canine atopic dermatitis: breed risk in Australia and evidence for a susceptible clade. *Vet Dermatol*. (2016) 27:167–e42. doi: 10.1111/vde.12317
10. Nødtvedt A, Egenvall A, Bergvall K, Hedhammar A. Incidence of and risk factors for atopic dermatitis in a Swedish population of insured dogs. *Vet Rec*. (2006) 159:241–6. doi: 10.1136/vr.159.8.241
11. Oberbauer AM, Belanger JM, Bellumori T, Bannasch TL, Famula TR. Ten inherited disorders in purebred dogs by functional breed groupings. *Canine Genet Epidemiol*. (2015) 2:9. doi: 10.1186/s40575-015-0021-x
12. Picco F, Zini E, Nett C, Naegeli C, Bigler B, Rüfenacht S, et al. A prospective study on canine atopic dermatitis and food-induced allergic dermatitis in Switzerland. *Vet Dermatol*. (2008) 19:150–5. doi: 10.1111/j.1365-3164.2008.00669.x
13. Zur G, Ihrke PJ, White SD, Kass PH. Canine atopic dermatitis: a retrospective study of 266 cases examined at the University of California, Davis, 1992-1998. Part I Clinical features and allergy testing results. *Vet Dermatol*. (2002) 13:89–102. doi: 10.1046/j.1365-3164.2002.00285.x
14. Afifi L, Danesh MJ, Lee KM, Beroukhim K, Farahnik B, Ahn RS, et al. Dietary Behaviors in Psoriasis: Patient-Reported Outcomes from a U.S. National Survey. *Dermatol Ther*. (2017) 7:227–42. doi: 10.1007/s13555-017-0183-4
15. Johnston GA, Bilbao RM, Graham-Brown RA. The use of dietary manipulation by parents of children with atopic dermatitis. *Br J Dermatol*. (2004) 150:1186–9. doi: 10.1111/j.1365-2133.2004.05888.x
16. Nosrati A, Afifi L, Danesh MJ, Lee K, Yan D, Beroukhim K, et al. Dietary modifications in atopic dermatitis: patient-reported outcomes. *J Dermatolog Treat*. (2017) 28:523–38. doi: 10.1080/09546634.2016.1278071
17. Dell DL, Griffin CE, Thompson LA, Griffies JD. Owner assessment of therapeutic interventions for canine atopic dermatitis: a long-term retrospective analysis. *Vet Dermatol*. (2012) 23:228–e47. doi: 10.1111/j.1365-3164.2012.01054.x
18. Bizikova P, Pucheu-Haston CM, Eisenschenk MN, Marsella R, Nuttall T, Santoro D. Review: role of genetics and the environment in the pathogenesis of canine atopic dermatitis. *Vet Dermatol*. (2015) 26:95–e26. doi: 10.1111/vde.12198
19. Kawano K, Oumi K, Ashida Y, Horiuchi Y, Mizuno T. The prevalence of dogs with lymphocyte proliferative responses to food allergens in canine allergic dermatitis. *Pol J Vet Sci*. (2013) 16:735–9. doi: 10.2478/pjvs-2013-0104
20. Nødtvedt A, Bergvall K, Sallander M, Egenvall A, Emanuelson U, Hedhammar A. A case-control study of risk factors for

canine atopic dermatitis among boxer, bullterrier and West Highland white terrier dogs in Sweden. *Vet Dermatol*. (2007) 18:309–15. doi: 10.1111/j.1365-3164.2007.00617.x
21. Popa I, Pin D, Remoué N, Osta B, Callejon S, Videmont E, et al. Analysis of epidermal lipids in normal and atopic dogs, before and after administration of an oral omega-6/omega-3 fatty acid feed supplement. A pilot study. *Vet Res Commun*. (2011) 35:501–9. doi: 10.1007/s11259-011-9493-7
22. Jaffary F, Faghihi G, Mokhtarian A, Hosseini SM. Effects of oral vitamin E on treatment of atopic dermatitis: a randomized controlled trial. *J Res Med Sci*. (2015) 20:1053–7. doi: 10.4103/1735-1995.172815
23. Kim G, Bae JH. Vitamin D and atopic dermatitis: a systematic review and meta-analysis. *Nutrition*. (2016) 32:913–20. doi: 10.1016/j.nut.2016.01.023
24. Mohajeri S, Newman SA. Review of evidence for dietary influences on atopic dermatitis. *Skin Therapy Lett*. (2014) 19:5–7.
25. Klinger CJ, Hobi S, Johansen C, Koch HJ, Weber K, Mueller RS. Vitamin D shows in vivo efficacy in a placebo-controlled, double-blinded, randomised clinical trial on canine atopic dermatitis. *Vet Rec*. (2018) 182:406. doi: 10.1136/vr.104492
26. Plevnik Kapun A, Salobir J, Levart A, Tavčar Kalcher G, Nemec Svete A, Kotnik T. Vitamin E supplementation in canine atopic dermatitis: improvement of clinical signs and effects on oxidative stress markers. *Vet Rec*. (2014) 175:560. doi: 10.1136/vr.102547
27. Saevik BK, Bergvall K, Holm BR, Saijonmaa-Koulumies LE, Hedhammar A, Larsen S, et al. A randomized, controlled study to evaluate the steroid sparing effect of essential fatty acid supplementation in the treatment of canine atopic dermatitis. *Vet Dermatol*. (2004) 15:137–45. doi: 10.1111/j.1365-3164.2004.00378.x
28. Kaikiri H, Miyamoto J, Kawakami T, Park SB, Kitamura N, Kishino S, et al. Supplemental feeding of a gut microbial metabolite of linoleic acid, 10-hydroxy-cis-12-octadecenoic acid, alleviates spontaneous atopic dermatitis and modulates intestinal microbiota in NC/nga mice. *Int J Food Sci Nutr*. (2017) 68:941–51. doi: 10.1080/09637486.2017.1318116
29. Wang P, Sun M, Ren J, Aslam MN, Xu Y, Fisher GJ, et al. Dietary fish oil supplementation enhances expression of genes involved in cornified cell envelope formation in rat skin. *J Invest Dermatol*. (2018) 138:981–3. doi: 10.1016/j.jid.2017.11.019
30. Fabbrocini G, Bertona M, Picazo Ó, Pareja-Galeano H, Monfrecola G, Emanuele E. Supplementation with Lactobacillus rhamnosus SP1 normalises skin expression of genes implicated in insulin signalling and improves adult acne. *Benef Microbes*. (2016) 7:625–30. doi: 10.3920/BM2016.0089
31. Scott JF, Das LM, Ahsanuddin S, Qiu Y, Binko AM, Traylor ZP, et al. Oral vitamin D rapidly attenuates inflammation from sunburn: an interventional study. *J Invest Dermatol*. (2017) 137:2078–86. doi: 10.1016/j.jid.2017.04.040
32. Veenhof EZ, Knol EF, Schlotter YM, Vernooij JC, Rutten VP, Willemse T. Characterisation of T cell phenotypes, cytokines and transcription factors in the skin of dogs with cutaneous adverse food reactions. *Vet J*. (2011) 187:320–4. doi: 10.1016/j.tvjl.2010.02.005
33. Kil DY, Vester Boler BM, Apanavicius CJ, Schook LB, Swanson KS. Age and diet affect gene expression profiles in canine liver tissue. *PLoS ONE*. (2010) 5:e13319. doi: 10.1371/journal.pone.0013319
34. Middelbos IS, Vester BM, Karr-Lilienthal LK, Schook LB, Swanson KS. Age and diet affect gene expression profile in canine skeletal muscle. *PLoS ONE*. (2009) 4:e4481. doi: 10.1371/journal.pone.0004481
35. Swanson KS, Belsito KR, Vester BM, Schook LB. Adipose tissue gene expression profiles of healthy young adult and geriatric dogs. *Arch Anim Nutr*. (2009) 63:160–71. doi: 10.1080/17450390902733934
36. Kil DY, Vester Boler BM, Apanavicius CJ, Schook LB, Swanson KS. Gene expression profiles of colonic mucosa in healthy young adult and senior dogs. *PLoS ONE*. (2010) 5:e12882. doi: 10.1371/journal.pone.0012882
37. Swanson KS, Vester BM, Apanavicius CJ, Kirby NA, Schook LB. Implications of age and diet on canine cerebral cortex transcription. *Neurobiol Aging*. (2009) 30:1314–26. doi: 10.1016/j.neurobiolaging.2007.10.017
38. Merryman-Simpson AE, Wood SH, Fretwell N, Jones PG, McLaren WM, McEwan NA, et al. Gene (mRNA) expression in canine atopic dermatitis: microarray analysis. *Vet Dermatol*. (2008) 19:59–66. doi: 10.1111/j.1365-3164.2008.00653.x
39. Plager DA, Torres SM, Koch SN, Kita H. Gene transcription abnormalities in canine atopic dermatitis and related human eosinophilic allergic diseases. *Vet Immunol Immunopathol*. (2012) 149:136–42. doi: 10.1016/j.vetimm.2012.06.003
40. Theerawatanasirikul S, Sailasuta A, Thanawongnuwech R, Suriyaphol G. Alterations of keratins, involucrin and filaggrin gene expression in canine atopic dermatitis. *Res Vet Sci*. (2012) 93:1287–92. doi: 10.1016/j.rvsc.2012.06.005
41. Klukowska-Rötzler J, Chervet L, Müller EJ, Roosje P, Marti E, Janda J. Expression of thymic stromal lymphopoietin in canine atopic dermatitis. *Vet Dermatol*. (2013) 24:54–9. doi: 10.1111/j.1365-3164.2012.01096.x
42. Lancto CA, Torres SM, Hendrickson JA, Martins KV, Rutherford MS. Altered expression of antimicrobial peptide genes in the skin of dogs with atopic dermatitis and other inflammatory skin conditions. *Vet Dermatol*. (2013) 24:414–21. doi: 10.1111/vde.12034
43. Mullin J, Carter S, Williams N, McEwan N, Nuttall T. Transcription of canine toll-like receptor 2, β-defensin 1 and β-defensin 103 in infected atopic skin, non-infected atopic skin, healthy skin and the CPEK cell line. *Vet Microbiol*. (2013) 162:700–6. doi: 10.1016/j.vetmic.2012.09.017
44. Nuttall TJ, Knight PA, McAleese SM, Lamb JR, Hill PB. Expression of Th1, Th2 and immunosuppressive cytokine gene transcripts in canine atopic dermatitis. *Clin Exp Allergy*. (2002) 32:789–95. doi: 10.1046/j.1365-2222.2002.01356.x
45. Roque JB, O'Leary CA, Kyaw-Tanner M, Duffy DL, Shipstone M. Real-time PCR quantification of the canine filaggrin orthologue in the skin of atopic and non-atopic dogs: a pilot study. *BMC Res Notes*. (2011) 4:554. doi: 10.1186/1756-0500-4-554
46. Santoro D, Marsella R, Ahrens K, Graves TK, Bunick D. Altered mRNA and protein expression of filaggrin in the skin of a canine animal model for atopic dermatitis. *Vet Dermatol*. (2013) 24:329–36. doi: 10.1111/vde.12031
47. Schamber P, Schwab-Richards R, Bauersachs S, Mueller RS. Gene expression in the skin of dogs sensitized to the house dust mite *Dermatophagoides farinae*. *G3 (Bethesda)*. (2014) 4:1787–95. doi: 10.1534/g3.114.013003
48. Schlotter YM, Rutten VP, Riemers FM, Knol EF, Willemse T. Lesional skin in atopic dogs shows a mixed Type-1 and Type-2 immune responsiveness. *Vet Immunol Immunopathol*. (2011) 143:20–6. doi: 10.1016/j.vetimm.2011.05.025
49. van Damme CM, Willemse T, van Dijk A, Haagsman HP, Veldhuizen EJ. Altered cutaneous expression of beta-defensins in dogs with atopic dermatitis. *Mol Immunol*. (2009) 46:2449–55. doi: 10.1016/j.molimm.2009.05.028
50. Wood SH, Clements DN, Ollier WE, Nuttall T, McEwan NA, Carter SD. Gene expression in canine atopic dermatitis and correlation with clinical severity scores. *J Dermatol Sci*. (2009) 55:27–33. doi: 10.1016/j.jdermsci.2009.03.005
51. Falgueras J, Lara AJ, Fernández-Pozo N, Cantón FR, Pérez-Trabado G, Claros MG. SeqTrim: a high-throughput pipeline for pre-processing any type of sequence read. *BMC Bioinformatics*. (2010) 11:38. doi: 10.1186/1471-2105-11-38
52. Hoeppner MP, Lundquist A, Pirun M, Meadows JR, Zamani N, Johnson J, et al. An improved canine genome and a comprehensive catalogue of coding genes and non-coding transcripts. *PLoS ONE*. (2014) 9:e91172. doi: 10.1371/journal.pone.0091172
53. Li H, Handsaker B, Wysoker A, Fennell T, Ruan J, Homer N et al. The sequence alignment/map format and SAMtools. *Bioinformatics*. (2009) 25:2078–9. doi: 10.1093/bioinformatics/btp352
54. Supercomputing and Bioinnovation Center (SBC) (2018). Available online at: http://www.scbi.uma.es/site/scbi/downloads/313-full-lengthernext (accessed February 15, 2018).
55. González Gayte I, Moreno RC, Zonjic RS, Claros MG. DEgenes Hunter - a flexible r pipeline for automated RNA-seq studies in organisms without reference genome. *Genomics Comput Biol*. 2365–7154. doi: 10.18547/gcb.2017.vol3.iss3.e31
56. Love MI, Huber W, Anders S. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biol*. (2014) 15:550doi: 10.1186/s13059-014-0550-8
57. Zhou X, Lindsay H, Robinson MD. Robustly detecting differential expression in RNA sequencing data using observation weights. *Nucleic Acids Res*. (2014) 42:e91. doi: 10.1093/nar/gku310

58. Spencer J, Sollid LM. The human intestinal B-cell response. *Mucosal Immunol*. (2016) 9:1113. doi: 10.1038/mi.2016.59
59. Boguniewicz M, Leung DY. Atopic dermatitis: a disease of altered skin barrier and immune dysregulation. *Immunol Rev*. (2011) 242:233–46. doi: 10.1111/j.1600-065X.2011.01027.x
60. Candi E, Schmidt R, Melino G. The cornified envelope: a model of cell death in the skin. *Nat Rev Mol Cell Biol*. (2005) 6:328–40. doi: 10.1038/nrm1619
61. Cai TQ, Ren N, Jin L, Cheng K, Kash S, Chen R, et al. Role of GPR81 in lactate-mediated reduction of adipose lipolysis. *Biochem Biophys Res Commun*. (2008) 377:987–91. doi: 10.1016/j.bbrc.2008.10.088
62. Harrison WJ, Bull JJ, Seltmann H, Zouboulis CC, Philpott MP. Expression of lipogenic factors galectin-12, resistin, SREBP-1, and SCD in human sebaceous glands and cultured sebocytes. *J Invest Dermatol*. (2007) 127:1309–17. doi: 10.1038/sj.jid.5700743
63. Yang RY, Yu L, Graham JL, Hsu DK, Lloyd KC, Havel PJ, et al. Ablation of a galectin preferentially expressed in adipocytes increases lipolysis, reduces adiposity, and improves insulin sensitivity in mice. *Proc Natl Acad Sci USA*. (2011) 108:18696–701. doi: 10.1073/pnas.1109065108
64. Mizutani Y, Mitsutake S, Tsuji K, Kihara A, Igarashi Y. Ceramide biosynthesis in keratinocyte and its role in skin function. *Biochimie*. (2009) 91:784–90. doi: 10.1016/j.biochi.2009.04.001
65. Fourcade S, Ruiz M, Camps C, Schlüter A, Houten SM, Mooyer PA, et al. A key role for the peroxisomal ABCD2 transporter in fatty acid homeostasis. *Am J Physiol Endocrinol Metab*. (2009) 296:E211–21. doi: 10.1152/ajpendo.90736.2008
66. Fürstenberger G, Epp N, Eckl KM, Hennies HC, Jørgensen C, Hallenborg P, et al. Role of epidermis-type lipoxygenases for skin barrier function and adipocyte differentiation. *Prostaglandins Other Lipid Mediat*. (2007) 82:128–34. doi: 10.1016/j.prostaglandins.2006.05.006
67. Lorand L, Graham RM. Transglutaminases: crosslinking enzymes with pleiotropic functions. *Nature Rev Mol Cell Biol*. (2003) 4:140–56. doi: 10.1038/nrm1014
68. Graczyk A, Leśniak W. S100A6 expression in keratinocytes and its impact on epidermal differentiation. *Int J Biochem Cell Biol*. (2014) 57:135–41. doi: 10.1016/j.biocel.2014.10.007
69. Fessing MY, Mardaryev AN, Gdula MR, Sharov AA, Sharova TY, Rapisarda V, et al. p63 regulates Satb1 to control tissue-specific chromatin remodeling during development of the epidermis. *J Cell Biol*. (2011) 194:825–39. doi: 10.1083/jcb.201101148
70. Fuchs E. Keratins and the skin. *Annu Rev Cell Dev Biol*. (1995) 11:123–53. doi: 10.1146/annurev.cb.11.110195.001011
71. Sugiura H, Ebise H, Tazawa T, Tanaka K, Sugiura Y, Uehara M, et al. Large-scale DNA microarray analysis of atopic skin lesions shows overexpression of an epidermal differentiation gene cluster in the alternative pathway and lack of protective gene expression in the cornified envelope. *Br J Dermatol*. (2005) 152:146–9. doi: 10.1111/j.1365-2133.2005.06352.x
72. Zhang Y, Matsuo H, Morita E. Increased production of vascular endothelial growth factor in the lesions of atopic dermatitis. *Arch Dermatol Res*. (2006) 297:425–9. doi: 10.1007/s00403-006-0641-9
73. Buroker NE, Ning XH, Zhou ZN, Li K, Cen WJ, Wu XF, et al. AKT3, ANGPTL4, eNOS3, and VEGFA associations with high altitude sickness in Han and Tibetan Chinese at the Qinghai-Tibetan Plateau. *Int J Hematol*. (2012) 96:200–13. doi: 10.1007/s12185-012-1117-7
74. Chen L, Marble DJ, Agha R, Peterson JD, Becker RP, Jin T, et al. The progression of inflammation parallels the dermal angiogenesis in a keratin 14 IL-4-transgenic model of atopic dermatitis. *Microcirculation*. (2008) 15:49–64. doi: 10.1080/10739680701418416
75. Varricchi G, Granata F, Loffredo S, Genovese A, Marone G. Angiogenesis and lymphangiogenesis in inflammatory skin disorders. *J Am Acad Dermatol*. (2015) 73:144–53. doi: 10.1016/j.jaad.2015.03.041
76. Kuroda K, Sapadin A, Shoji T, Fleischmajer R, Lebwohl M. Altered expression of angiopoietins and Tie2 endothelium receptor in psoriasis. *J Invest Dermatol*. (2001) 116:713–20. doi: 10.1046/j.1523-1747.2001.01316.x
77. Puxeddu I, Berkman N, Ribatti D, Bader R, Haitchi HM, Davies DE, et al. Osteopontin is expressed and functional in human eosinophils. *Allergy*. (2010) 65:168–74. doi: 10.1111/j.1398-9995.2009.02148.x
78. Thomas M, Augustin HG. The role of the angiopoietins in vascular morphogenesis. *Angiogenesis*. (2009) 12:125–37. doi: 10.1007/s10456-009-9147-3
79. Buommino E, Tufano MA, Balato N, Canozo N, Donnarumma M, Gallo L, et al. Osteopontin: a new emerging role in psoriasis. *Arch Dermatol Res*. (2009) 301:397–404. doi: 10.1007/s00403-009-0939-5
80. Petrušić V, Zivković I, Stojanović M, Stojićević I, Marinković E, Inić-Kanada A, et al. Antigenic specificity and expression of a natural idiotope on human pentameric and hexameric IgM polymers. *Immunol Res*. (2011) 51:97–107. doi: 10.1007/s12026-011-8236-8
81. Asano M, Komiyama K. Polymeric immunoglobulin receptor. *J Oral Sci*. (2011) 53:147–56. doi: 10.2334/josnusd.53.147
82. Turula H, Wobus CE. The role of the polymeric immunoglobulin receptor and secretory immunoglobulins during mucosal infection and immunity. *Viruses*. (2018) 10:237. doi: 10.3390/v10050237
83. Imayama S, Shimozono Y, Hoashi M, Yasumoto S, Ohta S, Yoneyama K, et al. Reduced secretion of IgA to skin surface of patients with atopic dermatitis. *J Allergy Clin Immunol*. (1994) 94:195–200. doi: 10.1053/ai.1994.v94.a55222
84. Nihei Y, Maruyama K, Zhang JZ, Kobayashi K, Kaneko F. Secretory component (polymeric immunoglobulin receptor) as an intrinsic inhibitor of biological functions of interferon gamma in keratinocytes. *Arch Dermatol Res*. (1995) 287:546–52. doi: 10.1007/BF00374074
85. Fernandez MI, Pedron T, Tournebize R, Olivo-Marin J-C, Sansonetti PJ, Phalipon A. Anti-inflammatory role for intracellular dimeric immunoglobulin A by neutralization of lipopolysaccharide in epithelial cells. *Immunity*. (2003) 18:739–49. doi: 10.1016/S1074-7613(03)00122-5
86. Husson A, Brasse-Lagnel C, Fairand A, Renouf S, Lavoinne A. Argininosuccinate synthetase from the urea cycle to the citrulline-NO cycle. *Eur J Biochem*. (2003) 270:1887–99. doi: 10.1046/j.1432-1033.2003.03559.x
87. Nihei Y, Maruyama K, Endo Y, Sato T, Kobayashi K, Kaneko F. Secretory component (polymeric immunoglobulin receptor) expression on human keratinocytes by stimulation with interferon-gamma and differences in response. *J Dermatol Sci*. (1996) 11:214–22. doi: 10.1016/0923-1811(95)00444-0
88. Hattori Y, Campbell EB, Gross SS. Argininosuccinate synthetase mRNA and activity are induced by immunostimulants in vascular smooth muscle. Role in the regeneration or arginine for nitric oxide synthesis. *J Biol Chem*. (1994) 269:9405–8.
89. Qualls JE, Subramanian C, Rafi W, Smith AM, Balouzian L, DeFreitas AA, et al. Sustained generation of nitric oxide and control of mycobacterial infection requires argininosuccinate synthase 1. *Cell Host Microbe*. (2012) 12:313–23. doi: 10.1016/j.chom.2012.07.012
90. Nathan C. Nitric oxide as a secretory product of mammalian cells. *FASEB J*. (1992) 6:3051–64. doi: 10.1096/fasebj.6.12.1381691
91. Liebmann J, Born M, Kolb-Bachofen V. Blue-light irradiation regulates proliferation and differentiation in human skin cells. *J Invest Dermatol*. (2010) 130:259–69. doi: 10.1038/jid.2009.194
92. Watson AL, Fray TR, Bailey J, Baker CB, Beyer SA, Markwell PJ. Dietary constituents are able to play a beneficial role in canine epidermal barrier function. *Exp Dermatol*. (2006) 15:74–81. doi: 10.1111/j.0906-6705.2005.00385.x
93. Bruch-Gerharz D, Schnorr O, Suschek C, Beck KF, Pfeilschifter J, Ruzicka T, et al. Arginase 1 overexpression in psoriasis: limitation of inducible nitric oxide synthase activity as a molecular mechanism for keratinocyte hyperproliferation. *Am J Pathol*. (2003) 162:203–11. doi: 10.1016/S0002-9440(10)63811-4
94. Bogdan C. Nitric oxide and the immune response. *Nat Immunol*. (2001) 2:907–16. doi: 10.1038/ni1001-907
95. Miles EW, Kraus JP. Cystathionine beta-synthase: structure, function, regulation, and location of homocystinuria-causing mutations. *J Biol Chem*. (2004) 279:29871–4. doi: 10.1074/jbc.R400005200
96. Prudova A, Bauman Z, Braun A, Vitvitsky V, Lu SC, Banerjee R. S-adenosylmethionine stabilizes cystathionine beta-synthase and modulates redox capacity. *Proc Natl Acad Sci USA*. (2006) 103:6489–94. doi: 10.1073/pnas.0509531103
97. Rosado JO, Salvador M, Bonatto D. Importance of the trans-sulfuration pathway in cancer prevention and promotion. *Mol Cell Biochem*. (2007) 301:1–12. doi: 10.1007/s11010-006-9389-y

98. Lee E, Kim HJ, Lee M, Jin SH, Hong SH, Ahn S, et al. Cystathionine metabolic enzymes play a role in the inflammation resolution of human keratinocytes in response to sub-cytotoxic formaldehyde exposure. *Toxicol Appl Pharmacol*. (2016) 310:185–94. doi: 10.1016/j.taap.2016.09.017
99. Rorbach J, Richter R, Wessels HJ, Wydro M, Pekalski M, Farhoud M, et al. The human mitochondrial ribosome recycling factor is essential for cell viability. *Nucleic Acids Res*. (2008) 36:5787–5579. doi: 10.1093/nar/gkn576
100. Niwa Y, Sumi H, Kawahira K, Terashima T, Nakamura T, Akamatsu H. Protein oxidative damage in the stratum corneum: evidence for a link between environmental oxidants and the changing prevalence and nature of atopic dermatitis in Japan. *Br J Dermatol*. (2003) 149:248–54. doi: 10.1046/j.1365-2133.2003.05417.x
101. Tsuboi H, Kouda K, Takeuchi H, Takigawa M, Masamoto Y, Takeuchi M, et al. 8-hydroxydeoxyguanosine in urine as an index of oxidative damage to DNA in the evaluation of atopic dermatitis. *Br J Dermatol*. (1998) 138:1033–5. doi: 10.1046/j.1365-2133.1998.02273.x
102. Valko M, Leibfritz D, Moncol J, Cronin MTD, Mazur M, Telser J. Free radicals and antioxidants in normal physiological functions and human disease. *Int J Biochem Cell Biol*. (2007) 39:44–84. doi: 10.1016/j.biocel.2006.07.001
103. Kapun AP, Salobir J, Levart A, Kotnik T, Svete AN. Oxidative stress markers in canine atopic dermatitis. *Res Vet Sci*. (2012) 92:469–70. doi: 10.1016/j.rvsc.2011.04.014
104. Anderson RC, Armstrong KM, Young W, Maclean P, Thomas DG, Bermingham EN. Effect of kibble and raw meat diets on peripheral blood mononuclear cell gene expression profile in dogs. *Vet J*. (2018) 234:7–10. doi: 10.1016/j.tvjl.2018.01.005
105. Ji H, Li X-K. Oxidative stress in atopic dermatitis. *Oxid Med Cell Longev*. (2016) 2016:2721469. doi: 10.1155/2016/2721469
106. Nijsse R, Mughini-Gras L, Wagenaar JA, Ploeger HW. Recurrent patent infections with *Toxocara canis* in household dogs older than six months: a prospective study. *Parasit Vectors*. (2016) 9:531. doi: 10.1186/s13071-016-1816-7
107. Cui X, Kim E. Dual effects of high protein diet on mouse skin and colonic inflammation. *Clin Nutr Res*. (2018) 7:56–68. doi: 10.7762/cnr.2018.7.1.56
108. Hensel N, Zabel S, Hensel P. Prior antibacterial drug exposure in dogs with meticillin-resistant *Staphylococcus pseudintermedius* (MRSP) pyoderma. *Vet Dermatol*. (2016) 27:72–8e20. doi: 10.1111/vde.12292
109. Hillier A, Lloyd DH, Weese JS, Blondeau JM, Boothe D, Breitschwerdt E, et al. Guidelines for the diagnosis and antimicrobial therapy of canine superficial bacterial folliculitis (Antimicrobial Guidelines Working Group of the International Society for Companion Animal Infectious Diseases). *Vet Dermatol*. (2014) 25:163–75. doi: 10.1111/vde.12118

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Targeted Metabolomics With Ultraperformance Liquid Chromatography–Mass Spectrometry (UPLC-MS) Highlights Metabolic Differences in Healthy and Atopic Staffordshire Bull Terriers Fed Two Different Diets, A Pilot Study

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Background: While anecdotal evidence has long claimed that a raw meat–based diet (RMBD) improves the metabolic health of canines, no rigorous scientific study has clarified this issue. Canine atopic dermatitis (CAD) has also been linked to metabolic health, but its relation to diet remains poorly understood. This study investigates whether dietary choice is linked to metabolic health in healthy and CAD-diagnosed canines via targeted serum and urine metabolomic analysis of polar, non-ionic metabolites, as well as whether the underlying CAD condition modulates the response to nutritional intake.

Materials and Methods: Serum metabolites of client-owned Staffordshire bull terriers, divided into CAD-diagnosed ($n = 14$) and healthy ($n = 6$) cohorts, were studied. Urine metabolites of a subset of the CAD-diagnosed canines ($n = 8$) were also studied. The canines were split into two cohorts based on diet. The first cohort were fed a commercially available high-fat, moderate-protein, low-carbohydrate RMBD ($n = 11$, CAD diagnosed $n = 8$, healthy $n = 3$). Those in the second cohort were fed a commercially available moderate-fat, moderate-protein, high-carbohydrate kibble diet (KD) ($n = 9$: CAD diagnosed $n = 6$, healthy $n = 3$). The diet intervention period lasted approximately 4.5 months (median 135 days). Statistical analyses of the serum profiles across all dogs ($n = 20$) and the urine profiles of the CAD-diagnosed subset ($n = 8$) were performed.

Results and Discussion: The KD cohort was found to have higher concentrations of methionine than the RMBD cohort, both in serum (all dogs, $p < 0.0001$) and in urine (CAD-only cohort, $p < 0.0002$), as well as cystathionine and 4-pyridoxic acid. Methionine plays important roles in homocysteine metabolism, and elevated levels have been implicated in various pathologies. The CAD ($n = 14$) cohort dogs showed starker

metabolic changes in response to diet regarding these pathways compared to the healthy ($n = 6$) cohort. However, there was no significant change in CAD severity as a result of either diet. Likely due to the higher meat content of the RMBD, higher concentrations of several carnitines and creatine were found in the RMBD cohort. Citrulline was found in higher concentrations in the KD cohort. Our findings provide insight into the relationship between diet and the serum and urine metabolite profiles of canines. They also suggest that neither diet significantly affected CAD severity.

Keywords: raw meat-based diet, targeted metabolomics, canine atopic dermatitis, canine health, kibble diet, diet intervention

INTRODUCTION

With the recent advancements in the field of metabolomics, an emerging approach to studying mammalian health, it has become easier to study and understand the relationship between an individual's metabolome and environmental factors (1). As the key concept of metabolomics is that the metabolic state of an organism represents the "overall physiological status of the organism" (2), the nascent field of canine nutritional metabolomics holds potential for both improving our understanding of canine disease risk factors and the underlying causes behind those risks (3). Here, we incorporate metabolomics as a novel approach to understanding the links between canine disease and diet. Study of the effects of nutritional intake on a canine's blood serum biochemistry can be complemented with the simultaneous analysis of the metabolomic profile of the urine. An excess of a polar metabolite's concentration in blood above the needs of an organism's normal function can be seen as an increase in the metabolite concentration in the urine as it exceeds the renal threshold for that compound (4). Through the use of combined media (blood serum and urine) in this study, we examine the extent to which the homeostasis of quantified blood metabolites is maintained and its relationship with food intake (4, 5).

The majority of domesticated dogs in the developed world eat a kibble diet (KD). According to the recently embraced NOVA food classification (6–8), kibble is an "ultraprocessed" product. Kibble is a mixture of ultraprocessed grains, such as wheat corn, and rice, mixed with ultraprocessed animal by-product meal and enriched with chemical additives, including synthetic vitamins, minerals, trace elements, preservatives, coloring agents, and palatability enhancers (9, 10). The raw meat–based diet (RMBD), in contrast, consists of raw animal parts. Complete and balanced commercial RMBDs also contain small amounts of raw vegetal matter as a source of fiber. The popularity of RMBDs is particularly high in Finland (11), but has also increased throughout the industrialized world (12). The possible health benefits of feeding dogs with RMBDs remain understudied in comparison to its popularity (13). In a recent review regarding the subject of raw feeding and its health effects (14), the authors concluded that there was insufficient evidence to evaluate the risks and benefits of RMBDs with regard to canine health. The NOVA classification of RMBDs is currently under debate. Although the raw ingredients themselves are minimally processed (8) (chopped, mixed, and frozen), minerals and vitamins are often added. The processing of the individual ingredients used to produce kibble may significantly alter their nutritional value and the overall health of the dog, although the reasons for this remain poorly understood (15, 16). The KD macronutrient profile differs remarkably from the RMBD profile. In terms of percent dry matter, a KD usually consists of a "protein:fat:carbohydrate" (PFC) macronutrient ratio 16–38%:6–18%:40–60%, whereas the PFC ratio of RMBD is typically %45:50%:0–10% (17).

Canine atopic dermatitis (CAD), part of the atopic complex, is a common systemic disease in canines and is considered a form of chronic inflammation or pruritus of the skin that manifests as an allergic response to an environmental factor (18). Clinical protocols for CAD diagnoses include the CADESI-4 scale and Favrot's criteria (19, 20). The development of CAD has been suggested to be genetically predisposed in canines, as well as further modulated by epigenetic factors (18). Phenotypically, the disease manifests itself differently in each individual (21), although there is a relatively consistent trait of elevated concentrations of the antibody immunoglobulin E across both atopy types and species (22, 23). Atopic dermatitis (AD) has been associated with several of the classic markers of metabolic syndrome (MetS) found both in humans (24) and in canines (25). This relationship, likely mediated via inflammatory markers, is not fully understood (26). The relationship between skin inflammation and oxidative stress markers in humans as a result of MetS has been studied (27), and several pathophysiological disease mechanisms that combine AD and MetS have been proposed (21, 28). Nutrition has been shown to have a vital role in determining the development of MetS through modulating metabolic pathways that have been attributed to the development of AD (22). CAD typically comprises both food-induced AD and non-food-induced AD (23). Although physiologically indistinguishable (19), they can be differentiated with the diet-restriction provocation trial (20). The link between metabolic health and CAD remains poorly studied. Most attention has focused on metabolic processes in the skin, especially in relation to fatty acids and lipids (29–32). It has long been known that the immune system of animals can be modulated by metabolites derived from nutrition (33). In canines, for example, vitamin D

Abbreviations: KD, kibble diet; RMBD, raw meat–based diet; %ME, percent metabolizable energy; PFC, Protein: fat: carbohydrate; CAD, canine atopic dermatitis; AD, Atopic dermatitis; MetS, Metabolic syndrome; CADESI(-4), Canine Atopic Dermatitis Extent and Severity.

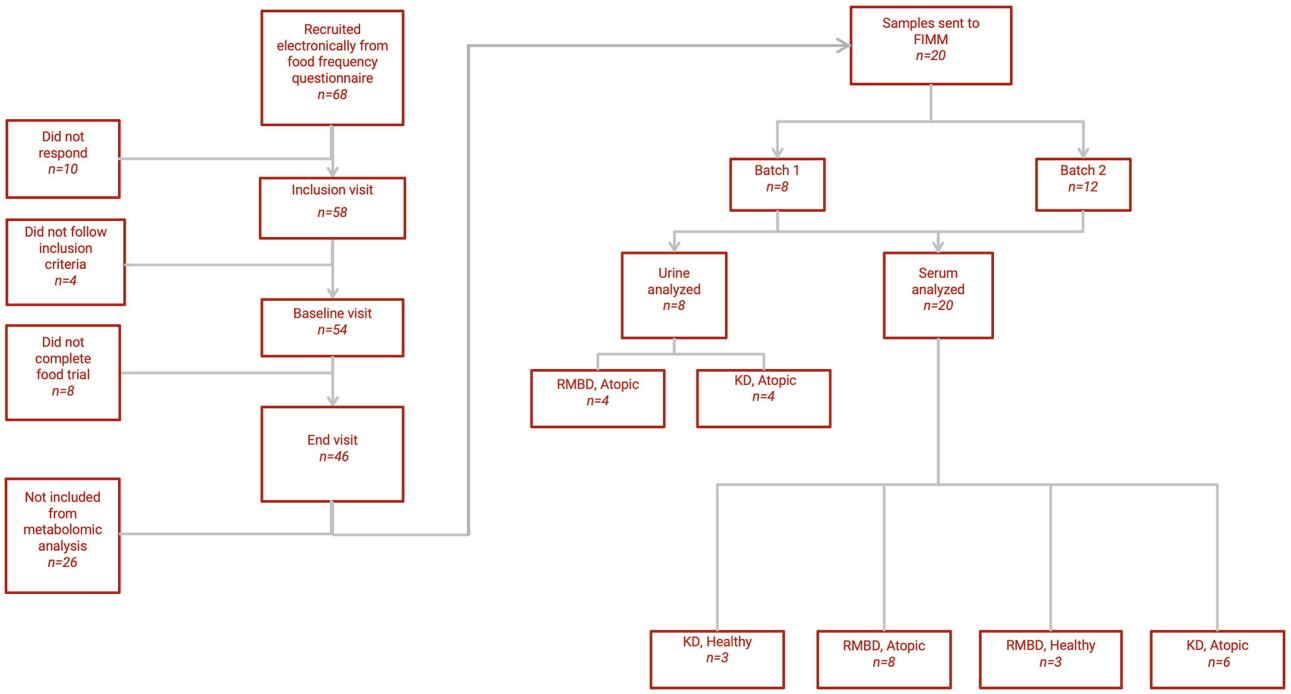

FIGURE 1 | Flowchart of study. A flowchart depicting the selection process of the Staffordshire bull terriers used for the metabolomic analysis ($n = 20$) and how they resulted in the cohorts based on diet (KD, kibble diet; RMBD, raw meat–based diet) and health status (CAD, canine atopic dermatitis).

(34) and fatty acid supplementations (35–37) have been shown to have a protective effect against allergic pruritic responses.

Improving the length of pet health span remains a long-term goal in research of the health–nutrition axis. To achieve this, most research focuses on practical solutions, for example, improving diet to treat chronic disease in canines (38). Most research on dog diet and nutrition deals with improving food palatability (39, 40), modifying stool quality and nutrient absorption (41), all while meeting the daily caloric requirements. Little consideration of disease prevention has been reported in the literature (42). It has been well-established that a healthy diet in humans contributes to an increased health span and that an unhealthy diet increases the risk of many pathologies (43–45). In canines, studies to see whether certain diets help treat chronic diseases have mainly involved observing whether certain types of diets and functional foods appear to have a protective or therapeutic effect against chronic ailments (46–48).

This study is the metabolomics portion of a "nutriomics" research project, which has already been performed using a larger subset of samples from pet Staffordshire bull terriers by the DogRisk group (49, 50). The aim of our pilot study was to use targeted metabolomics to understand how nutrition relates to CAD and how nutrition and the CAD condition are related to canine blood and urine metabolite concentrations and canine metabolic health in general.

MATERIALS AND METHODS

Design and Animals

A flowchart of the diet intervention is shown in **Figure 1**. In this diet intervention study, initiated in 2013, client-owned pet Staffordshire bull terriers were first studied with nutrigenomic (49) and hematological (50) approaches. The family history of the dogs has been reported elsewhere (49). The diet intervention included inclusion, baseline, and end visits during the diet trial. No special inclusion diet was required prior to baseline, although the diet of each dog prior to their baseline visit was determined using a food frequency questionnaire. Of the original cohort of Staffordshire bull terriers that underwent the whole study and fulfilled all criteria of the diet trial ($n = 46$), only a subset ($n = 20$) was selected for serum metabolomic analysis due to high running costs. The subset ($n = 20$) was stratified based on owner-reported diets prior to baseline, as well as their diet during the study. All dogs analyzed for this study were fed solely kibble (KD) or raw food (RMBD) over a diet intervention period of 3 to 5 months (median = 135 days), i.e., forming a KD cohort ($n = 9$) and an RMBD cohort ($n = 11$). The dogs included in the analysis ($n = 20$) were also split into cohorts based on whether they were CAD-diagnosed ($n = 14$) or healthy ($n = 6$). For analysis that considered diet and health condition, the dogs were divided into four cohorts, healthy-KD ($n = 3$), CAD-KD ($n = 6$), healthy-RMBD ($n = 3$), CAD-RMBD ($n = 8$). Urine metabolomic analysis of samples collected at the end of the diet intervention was performed for a subset ($n = 8$) of only CAD-diagnosed individuals, also due to high costs of analysis. The baseline samples were collected in during September and October, and the end samples were all collected between February and April. The winter months were chosen for the diet intervention due to the seasonality of the disease, as CAD symptoms have been reported to be exacerbated as a result of pollen and blooming plant exposure (51, 52). Because of unrelated circumstances (pregnancy of the study coordinator),

TABLE 1 | Percent metabolizable energy (%ME) of the kibble (Hill's Science Plan) and two raw meat–based diets (MUSH BARF Vaisto, pork–chicken–lamb, beef–turkey–salmon).

Macronutrient	Hill's Science Plan™ canine, adult sensitive skin with chicken (%ME)	MUSH Vaisto (pork–chicken–lamb) (%ME)	MUSH Vaisto (beef–turkey–salmon) (%ME)	MUSH diets combined average (%ME)
Protein	23.28	23.84	28.09	25.96
Fat	35.76	76.16	71.91	74.04
Carbohydrate	40.95	0.00	0.00	0.00

%ME, % metabolizable energy.
The values are calculated using the modified Atwater factors as suggested by the National Research Council (53).

TABLE 2 | Overview of the experimental setup of diet intervention, including division of Staffordshire bull terriers into diet cohorts (diet overview in **Table 1**), gender, health status, disease phenotype, diet intervention length, and age.

Batch	1		2		1 and 2	
Diet cohort	**RMBD**	**KD**	**RMBD**	**KD**	**RMBD**	**KD**
Dogs (total) (*n*)	4	4	7	5	11	9
Gender (male/female)	4/0	2/2	3/4	3/2	7/4	5/4
Sterilized (yes/no)	2/2	3/1	2/5	1/4	4/7	4/5
Blood serum analyzed	Yes	Yes	Yes	Yes	Yes	Yes
Urine analyzed	Yes	Yes	No	No	No	No
Atopy (total) (*n*)	4	4	4	2	8	6
NFIAD/FIAD	3/1	3/1	4/0	2/0	7/1	5/1
Healthy (*n*)	0	0	3	3	3	3
Mean diet intervention length (days) (SD)	126 (35.3)	141 (26.6)	137 (27.0)	136 (29.7)	133 (29.0)	139 (26.7)
Mean CADESI score at BL (SD)	CAD: 13.5 (9.0)	CAD: 19.0 (10.8)	CAD: 12.5 (8.7) Healthy: 3.3 (1.2)	CAD: 18.5 (16.3) Healthy: 2.7 (1.2)	CAD: 13 (8.3) Healthy: 3.3 (1.2)	CAD: 18.8 (11.1) Healthy: 2.7 (1.2)
Age at BL (months; mean, SD)	44.7 (34.9)	56.2 (31.7)	60.8 (35.9)	75.2 (46.1)	54.9 (34.7)	66.8 (39.3)

RMBD, raw meat–based diet; KD, kibble diet; NFIAD, non–food-induced atopic dermatitis; FIAD, food-induced atopic dermatitis; SD, standard deviation; BL, baseline.

the trial ended later than planned. Seasonality possibly affected the disease phenotype, as the end visit was delayed in some cases to spring, when plants already started blooming in Finland.

The canines were evaluated before and after the diet intervention by a dermatologist, who used Favrot's criteria (19), the Canine Atopic Dermatitis Extent and Severity Index (CADESI-4) scale (20), and biochemical and hematological tests. The threshold for whether a canine suffered from CAD required a fulfillment of 5 of 8 of Favrot's criteria. The severity of the CAD was diagnosed using the CADESI-4 scale, which categorizes CAD severity as follows: 0–10 = in remission, 11–33 = mild CAD, 34–59= moderate CAD, ≥60 = severe CAD. All CAD-diagnosed canines in this study hence suffered from mild CAD. Owner-reported data regarding CAD severity as a visual analog scale to evaluate the level of pruritus at 2-week intervals from baseline to end was also collected. The owner-reported pruritus conflicted with the dermatologist's CAD severity evaluation in some cases. However, for clarity, only the diagnosis reported by the dermatologist was used in this study.

The diets used in the study were a commercial KD and two commercial RMBDs. The RMBDs used in this study had an average PFC macronutrient ratio of 26:74:0 percent metabolizable energy (% ME). The KD diet used in this study had a PFC macronutrient ratio of 23:36:41% ME (**Table 1**).

The commercial dry diet used in this study was Hill's Science Plan™ Canine Adult Sensitive Skin With Chicken (detailed composition shown in **Supplementary Table 1A**). The two commercial raw meat diets used in this study were MUSH Vaisto® Pork–Chicken–Lamb and MUSH Vaisto® Beef–Turkey–Salmon (detailed compositions shown in **Supplementary Table 1B**). For the RMBDs, owners were free to either choose one or combine both diets. According to manufacturer claims, both the KD and the two RMBD options were "complete diets." Owners were asked to feed their dogs 99.9% with the trial food using amounts recommended by the manufacturer, adjusting amounts if their dog's body weight would start to fall or rise. Owners reported the adherence to diet using a food diary. Water was allowed *ad libitum*.

Samples

The metabolomic analysis of blood and urine samples were performed in two batches, i.e., batches 1 and 2. Both batches are described in **Table 2**. For batch 1, blood serum samples, collected at baseline and end, and urine samples collected only at end, from atopic dogs ($n = 8$) were used. For batch 2, only blood serum

samples collected at baseline and end from a cohort of both atopic and healthy dogs were used (atopic $n = 6$, healthy $n = 6$). For analysis of serum, batches 1 and 2 were combined (atopic $n = 14$, healthy $n = 6$) for several of the analyses described below.

Blood samples were collected from the jugular vein using Vacuette® 3 mL EDTA, 3 mL lithium heparin, and 6 mL plain serum tubes by a closed method (Vacutainer® Safety-Lok™ blood collection sets, Becton Dickinson, Meylan, France). Serum samples were allowed to clot at room temperature for 30 min before centrifugation (2,100 × g for 15 min). Urine samples were collected into factory-clean specimen jars and frozen after collection in 5 mL tubes. All samples were fasting samples collected in the morning. After collection they were stored at −80°C.

The targeted metabolomic analyses of the dogs' serum samples at baseline and end of the diet intervention (all dogs $n = 20$, healthy $n = 6$, atopic $n = 14$) were performed at the Finnish Institute of Molecular Medicine (FIMM) using targeted liquid chromatography (LC) mass spectrometry (MS). As targeted metabolomics of canine samples had not been performed before the first batch (batch 1, $n = 8$) was sent to FIMM to test the method. As the results were interpretable, more samples (batch 2, $n = 12$) were sent. Common polar, non-ionic metabolites ($n = 102$) were targeted with nanomolar accuracy (±0.005 μM) using the BioCrates p180 kit as standards for isotopic quantification, including amino acids, bile acids, nucleobases, nucleosides, choline metabolites, carbohydrates, and enzyme cofactors. A full list of the targeted metabolites used in the standard mixture is included in **Supplementary File 20**. A labeled internal standard mixture (10 μL) was added to 100 μL of serum or urine samples, which were all run in triplicate to ensure reliability. Metabolites were extracted by adding four parts (1:4, sample: extraction solvent) of the 100% acetonitrile + 1% formic acid solvent. The collected extracts were dispensed into Ostro™ 96-well plates (Waters Corporation, Milford, USA) and filtered by applying vacuum at a delta pressure of 300 to 400 mbar for 2.5 min using a robotic vacuum station. The filtrate was transferred to a 96-well collection plate, which was placed under the Ostro™ plate. The collection plate was sealed with the well cap mat and placed in the auto-sampler of the LC system for injection. Samples were analyzed using high-throughput targeted quantitative metabolic profiling using the ACQUITY ultraperformance LC–tandem MS (UPLC-MS/MS) instrument (Waters), with a 1.7-μm BEH amide HILIC column for chromatography.

Data Preprocessing

Sample preparation for UPLC-MS/MS, as well as raw spectral data processing, was carried out on site by FIMM personnel. Subsequent concentration data were provided for each metabolite, along with comments regarding their reliability. The raw spectral data were acquired with MassLynx 4.1, and TargetLynx software. Detailed information regarding the raw spectrum metabolomics analysis, including chemicals and reagents, metabolite extraction protocol, and serum sample preparation, can be found elsewhere (54). All metabolomics instrumentation used for analysis was owned by and located in the FIMM metabolomics unit in Biomedicum (Metabolomics Unit, Finnish Institute for Molecular Medicine FIMM, Helsinki, Finland).

Based on LC-MS raw data processing, for batch 1, 80 of the original 102 targeted metabolites in serum samples (**Supplementary Table 2A**), and 80 of the original 102 metabolites in urine samples, were used in the statistical analysis (**Supplementary Table 2B**). The raw data from batch 2 were considerably better, and only one of the 102 metabolites, spermidine, had to be omitted from analysis. For the combined batch serum analysis, 79 of the original 102 metabolites were used for the statistical analysis (**Supplementary Table 2C**).

Original metabolite values in the serum and urine datasets were reported in μmol/L. Urine metabolite values were normalized to their respective creatinine concentrations. Urine metabolite values used in data analysis were adjusted to metabolite (μmol)/creatinine (mmol). Creatinine-adjusted urine metabolite values were used in the analysis that combines serum and urine datasets. Only usable metabolite concentration values found in both datasets were used. In summary, 72 of the original 102 metabolite values were used in the analysis that combines serum and urine metabolite values.

Statistical Analysis

Statistical analysis was performed with the R package MetaboAnalystR (55). Source code for the statistical analysis workflow was documented as R-generated analysis reports (**Supplementary Files 1–19**). Targeted metabolites that were unreliably quantified or contained >50% missing values were removed with Excel prior to data processing with R. The integrity of all serum samples and urine samples were checked with R prior to data analysis. As metabolite concentrations fluctuate greatly, the raw concentration values in both serum and urine were log transformed using a generalized logarithm function, allowing the concentrations to assume a more normal distribution for subsequent analysis. To improve the sample size and hence statistical power for downstream analysis, batch correction for the end-of-diet time points of batches 1 and 2 serum data was performed using the ComBat empirical Bayes method developed by Johnson et al. (56) in order to combine the two cohorts as there was significant variation due to batch effect. Combined-batch analysis of serum concentrations from batches 1 and 2 used values generated with the K-nearest neighbor algorithm prior to their combination to estimate any remaining missing values. The similarity between batches 1 and 2 end values was analyzed with principal component analysis. A two-dimensional (2-D) principal component analysis plot of both pre-correction and post-correction is attached in **Supplementary Figure 1**. Each metabolite included in the combined-batch analysis was tested to see whether there was a significant difference between batches after batch correction using a *t*-test. No significant differences were observed due to batch after the batch correction was performed. In all of the metabolite datasets used in this study, the K-nearest neighbor algorithm was used to compute missing metabolite values for metabolites that were missing <50% of the values within each cohort.

For the results of statistical analysis, the cutoff for significance was set at false discovery rate (FDR) <0.05 (also referred to

as the FDR-adjusted p-value or q-value in some tables). In all statistical analyses, p-values are reported. As a general rule for metabolomics analysis, the reporting of FDR values are recommended to ensure that results are statistically significant as the number of parameters tested is far greater than the number of samples (57). In essence, the FDR "controls the expected proportion of falsely rejected hypotheses" (58).

Univariate Analysis of Baseline and End of Diet Intervention

Univariate analysis of baseline serum values from batches 1 and 2, as well as the combined batch dataset with respect to diet cohorts and health status cohorts, was performed to confirm whether there were any significant metabolite concentration differences between either cohort at the baseline of the diet intervention. Analyses of diet and health were first performed separately. For both the baseline and end of diet intervention, a general linear model (GLM) and parametric t-tests were used to observe statistically significant fold changes between the RMBD and KD cohorts in batch 1 serum and urine samples, in batch 2 serum samples, and in the combined batch serum samples, i.e., analysis of all dogs in the study. Univariate analysis reports were created for each test between diet cohorts and health status both at the baseline and end of the diet intervention and can be found in **Supplementary File 21**.

Univariate Analysis of CADESI-4 Score, Weight, and Age With Diet

The change in CADESI-4 scores between diet cohorts was determined by testing the change (end timepoint minus baseline) to see whether diet correlated with change in phenotype. The same was done for weight and age. Changes in CADESI-4 scores were also compared within dietary cohorts between gender, as well as neutering status.

Analysis Between Sample Media and Dietary Cohorts at End of Diet Intervention

A two-way analysis of variance (ANOVA) was performed between sample media (blood or urine) and dietary cohorts (KD or RMBD). Hierarchical clustering was then combined with the results from the two-way ANOVA to generate heatmap visualizations of the significantly different metabolites between diet cohorts and sample type in the serum and urine data. The differences in variance between cohorts are also reported as F-values.

Fold-change comparisons combined with t-tests were used to identify significant differences between serum and urine metabolite concentrations. The GLM was then used to perform correlation analysis between samples and identify which significant metabolites correlate with diet. To visualize how the samples within cohorts contributed to significant metabolite differences observed with the GLM, heatmap visualizations of significant metabolites (FDR < 0.05) within individual batches, as well as combined batch results from Fisher least significant difference (LSD) test were created.

To further explore the results seen from t-tests and the ANOVA, a supervised multivariate regression-based analysis, partial least squares–discriminant analysis (PLS-DA), was used to test the significance between sample media and diet cohorts. This was performed to determine the extent to which the linear combination of the metabolite values for a given sample can predict the diet cohort of the dog. For each component, each metabolite was assigned a variable importance in projection (VIP) score. The VIP score signifies the relative contribution a given metabolite has to discriminating the cohorts that are compared in the model and is dependent on the percentage variation explained by the component vectors used in the model.

To observe the risk of overfitting when using PLS-DA, cross-validation using the leave-one-out approach (LOOCV) was used to determine the accuracy, R2 and Q2 values of each respective component, where Q2 values have been computed to resemble the scale used for R2 and accuracy scores ($0< x <1$). Loading plots for the components 1 and 2 (the two components that explain the most variation between cohorts) were visualized to show the relative contributions metabolites had to the creation of their respective component vector.

Analysis Between Diet and Atopy at End of Diet Intervention

Analysis of diet and health combined for batch 2 and combined batch datasets to test for interaction was also performed with a two-way ANOVA. As all dogs in batch 1 were diagnosed with atopy, no analysis with regard to health status was performed. For the combined batch dataset, the results from the end of the diet intervention were studied with a two-way ANOVA between diet and atopy and their interactions. Results were visualized with a heatmap. To further explore the results seen from t-tests and the ANOVA, PLS-DA was used to identify the extent to which the diet and atopy cohorts differed.

RESULTS

Univariate Analysis of Baseline and End of Diet Intervention

By controlling for baseline bias, mildly significant concentration differences of arginine, histidine, and threonine between the two diet cohorts ($p < 0.05$, FDR > 0.05) were found (**Supplementary Table 4**). No significant metabolite concentration differences between atopic and healthy individuals were observed either at baseline or at the end of the diet intervention.

For all dogs' serum samples in the study ($n = 20$), the metabolites that significantly differ (FDR ≤ 0.05) between diet cohorts at the end of the diet intervention are presented in **Table 3**. A more comprehensive table of all dogs at the end of the diet intervention, significant differences between the diet cohorts of the batches separately, and only urine metabolites from the individuals of batch 1 ($n = 8$), as well as serum metabolites from only atopic dogs ($n = 14$), are included in **Supplementary Tables 5–9**).

At the end of the diet intervention, hexanoylcarnitine (FDR $= 0.015$, $p = 0.0015$), decanoylcarnitine (FDR $= 0.016$, $p = 0.0018$), octanoylcarnitine (FDR $= 0.052$, $p = 0.01$), acetylcarnitine (FDR $= 0.086$, $p = 0.021$), creatine (FDR $= 0.03$, $p = 0.005$), and

TABLE 3 | Comparison of significantly different metabolite concentrations in all dog's serum samples between kibble diet (KD, $n = 9$) and raw meat–based diet (RMBD, $n = 11$) at end of diet intervention.

Metabolite	Mean (SD) of KD cohort	Mean (SD) of RMBD cohort	*p*	*q* (FDR)	Fold change	In KD cohort
Methionine	6.686 (0.294)	5.697 (0.305)	<0.0001	0	1.17	Up
4-Pyridoxic acid	−8.830 (0.460)	−11.025 (0.804)	<0.0001	0	−1.25	Up
Citrulline	5.659 (0.204)	4.654 (0.507)	<0.0001	0.0011	1.22	Up
Cytosine	−4.146 (0.790)	−5.964 (0.930)	0.0002	0.0026	−1.44	Up
Proline	7.965 (0.406)	7.099 (0.403)	0.0002	0.0026	1.12	Up
Cystathionine	3.154 (1.292)	0.152 (1.004)	0.0002	0.0026	20.78	Up
Taurochenodeoxycholic acid	−0.898 (0.762)	−3.255 (1.357)	0.0002	0.0026	−3.62	Up
Hexanoylcarnitine	−7.033 (0.484)	−5.937 (0.760)	0.0015	0.0148	1.18	Down
Decanoylcarnitine	−6.414 (0.485)	−5.443 (0.661)	0.0018	0.0156	1.18	Down
Glycine	8.629 (0.299)	8.049 (0.407)	0.0023	0.018	1.07	Up
Creatine	4.155 (0.616)	5.176 (0.753)	0.0043	0.0297	−1.25	Down
Kynurenine	0.849 (0.513)	0.242 (0.319)	0.0045	0.0297	3.51	Up
Dimethylglycine	2.369 (0.511)	1.606 (0.575)	0.0062	0.0374	1.48	Up
Trimethylamine-N-oxide	−3.100 (11.157)	1.534 (0.830)	0.0074	0.042	0.49	Down

SD, standard deviation; KD, kibble diet; RMBD, raw meat–based diet; FDR < 0.05, false discovery rate <0.05.
Mean serum concentrations are presented as the natural log of the original metabolite concentration.

creatinine (FDR = 0.15, $p = 0.041$) concentrations were higher in serum of the RMBD cohort than in the KD cohort (all dogs, $n = 20$). Higher serum concentrations of urea-cycle metabolites citrulline (FDR = 0.001, $p < 0.0001$) and proline (FDR = 0.002, $p = 0.0002$) and the nucleobase cytosine (FDR = 0.0026, $p = 0.0002$) were observed in all of the dogs of the KD cohort. Higher concentrations of the primary bile acid taurochenodeoxycholic acid (FDR = 0.0026, $p = 0.0002$) and taurocholic acid were found in the KD cohort relative to the RMBD cohort (1.87-fold higher concentration, FDR = 0.112, $p = 0.028$). Serum methionine concentrations were higher in the KD-fed dogs (FDR <0.0001, $p < 0.0001$), as well as cystathionine (FDR = 0.0026, $p = 0.0002$), dimethylglycine (FDR = 0.037, $p = 0.0062$), and 4-pyridoxic acid (FDR <0.0001, $p < 0.0001$). There were higher urine concentrations of betaine, the precursor to dimethylglycine, in the RMBD-fed cohort (FDR = 0.0022, $p = 0.0008$), as well as a trend in serum of all dogs (FDR = 0.086, $p = 0.02$). Notably, dogs from batch 1 in the KD cohort also had significantly higher urine concentrations of methionine (FDR <0.02, $p < 0.0002$) and 4-pyridoxic acid (FDR <0.04, $p < 0.002$) (**Supplementary Table 6**). There were no metabolites that significantly differed between diet cohorts of the healthy individuals (KD $n = 3$, RMBD $n = 3$), although several metabolite concentrations differed with $p < 0.05$ (FDR >0.05, $p < 0.05$) (**Supplementary Table 10**).

Two-Way ANOVA Between Sample Media and Diet at End of the Diet Intervention

A two-way ANOVA was used to see whether any significant difference in serum metabolite concentrations between the diet cohorts could be seen in urine metabolite concentrations (**Figure 2A**). Of the 63 metabolites that differed significantly between serum and urine, 10 also differed between diet cohorts with interaction detected in five of the metabolites (**Supplementary Table 10**). The significantly different metabolites between diet cohorts and sample type (serum and urine) from the two-way ANOVA were visualized with a heatmap (**Figure 2B**). To further explore how urine and serum samples differed between the diet cohorts of batch 1, a PLS-DA was performed. The parameters of the model, calculated with the LOOCV approach, are shown in **Supplementary Table 14A**. Components 1 and 2 were plotted against each other (**Figure 3**) with shaded circles representing the 95% confidence interval area for the respective diet cohorts. In the 2-D PLS-DA plot presented in **Figure 3**, the extent to how much within-cohort variation exists for diet cohorts and urine and serum samples was visualized. When the first two components of the PLS-DA were plotted against each other, the urine and serum samples were separable with the first component, and the RMBD and KD diet cohorts were separable with the second component. However, likely because of the low sample size, the predictability of the model calculated with R2 and its predictability when testing the model (Q2) were 0.108, and as such can be considered quite weak. However, although the Q2 is small, the model describes the extent to which the sample media accounts for most of the variance. There was a minor overlap of confidence intervals between diet cohorts observed in serum samples when separated with component 2.

Univariate Analysis of CADESI-4 Score, Weight, and Age With Diet

According to the evaluation of CAD severity at the end of the diet intervention, neither the KD nor the RMBD significantly changed the CADESI-4 score outcome of the CAD-diagnosed dogs. The difference between diet cohorts was insignificant, with a weak worsening trend in the KD cohort ($p = 0.104$). There was a general trend in worsening of CADESI-4 scores found in both diet cohorts (for the KD: $n = 9$, $\mu = 18.3$, $\sigma = 13.8$; for the RMBD: $n = 11$, $\mu = 6.9$, $\sigma = 6.5$). The change in

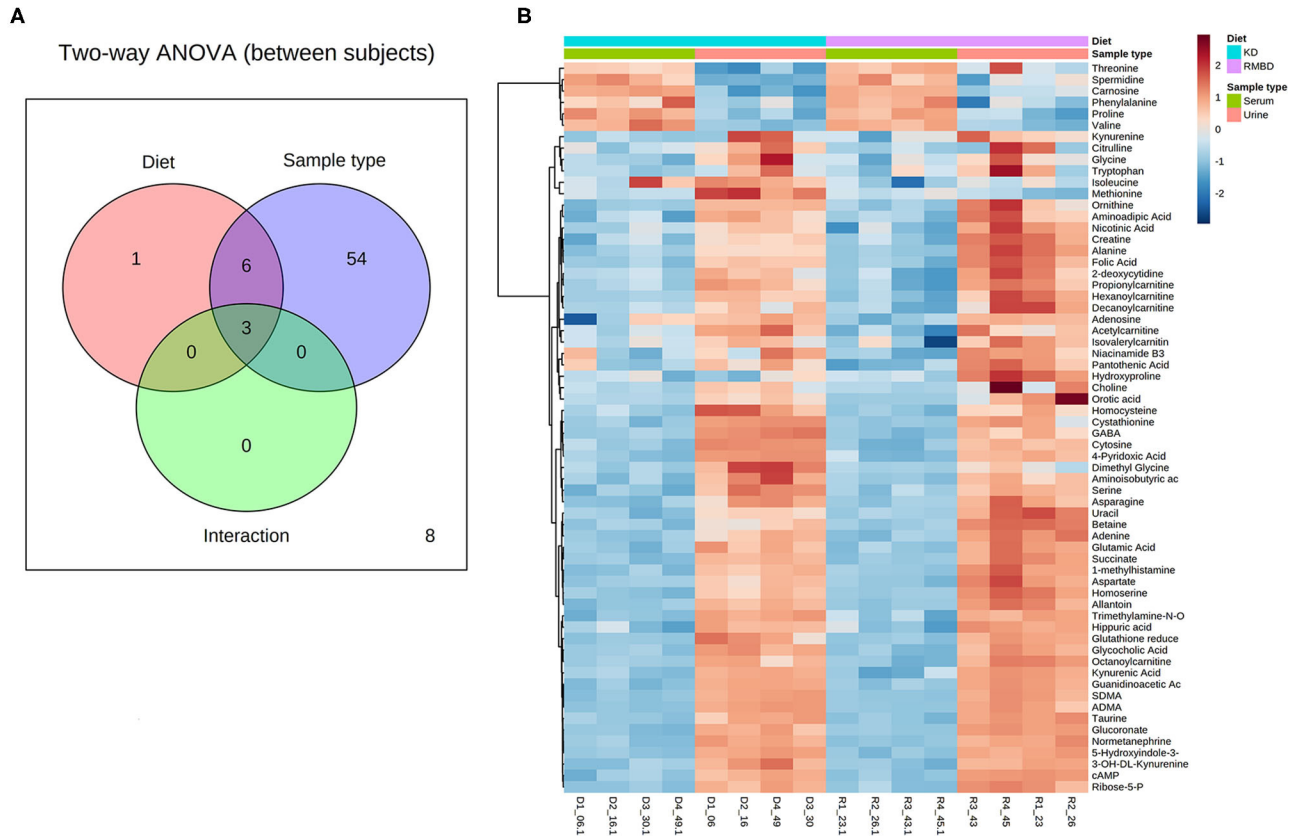

FIGURE 2 | Batch 1 ($n = 8$) comparison of serum and urine profiles between diet cohorts. **(A)** An overview of sample media and diet interaction at the end of diet intervention where metabolite values differ significantly (FDR < 0.05) between diet cohorts (red) and sample type (blue), as well as interaction between the two (dark green and purple). **(B)** A heatmap illustrating significant features from the two-way ANOVA. Values relative to the combined cohort average are represented as a color spectrum and have been scaled to −2 (blue) through 2 (red) (KD, kibble diet; RMBD, raw meat–based diet).

CADESI-4 scores did not result in a progression from mild to moderate CAD symptoms in any of the CAD-diagnosed canines however. In the serum samples of dogs from all dogs ($n = 20$), no significant weight and age differences between the KD and RMBD cohorts at the end of the diet intervention were detected. Results from the univariate analysis of CADESI-4, weight, and age across diet and disease cohorts are presented in **Supplementary Tables 3A–E**.

Analysis Between of Diet and Atopy

In all the atopic dogs, no significant differences in CADESI-4 scores between diet cohorts were found at the diet intervention baseline, where the dogs' diets were mixed, or at the end of the diet intervention. The outcome of serum concentrations of all dogs ($n = 20$) at the end of the diet intervention were visualized as a two-way ANOVA between diet and atopy and their interactions (**Figure 4A**). Here, the RMBD and KD cohorts were classified as either healthy (healthy-RMBD, $n = 3$, healthy-KD, $n = 3$) or atopic (RMBD, $n = 8$, KD, $n = 6$). Metabolite values that differed significantly between either diet or health status cohorts, or their interaction, are presented in **Supplementary Table 12**. The significantly different metabolites between diet cohorts from the two-way ANOVA of the atopic and healthy canines were visualized with a heatmap (**Figure 4B**). To further address the separation of cohorts based on diet and health status, PLS-DA analysis was performed to see how the metabolite profiles differed between diet and health status cohorts (**Figure 5A**). The parameters of the model were calculated using the LOOCV approach and are shown in **Supplementary Table 14B**. Likely due to the low sample size, as well as the similarity between the CAD-diagnosed and healthy individuals serum metabolite concentrations, the predictability of the model calculated with R2 and its predictability when testing the model (Q2) were 0.277, which is relatively weak. Nevertheless, the model gives an indication toward how the healthy individuals in both diet groups were more closely clustered among themselves than the atopic individuals of either diet cohort. The top 20 VIP scores were visualized as a heatmap that looks at the top 20 metabolites across all components (**Figure 5B**), with which the diet cohorts could be separated, but that the health status cohorts (CAD-diagnosed and healthy) could not. Many of the

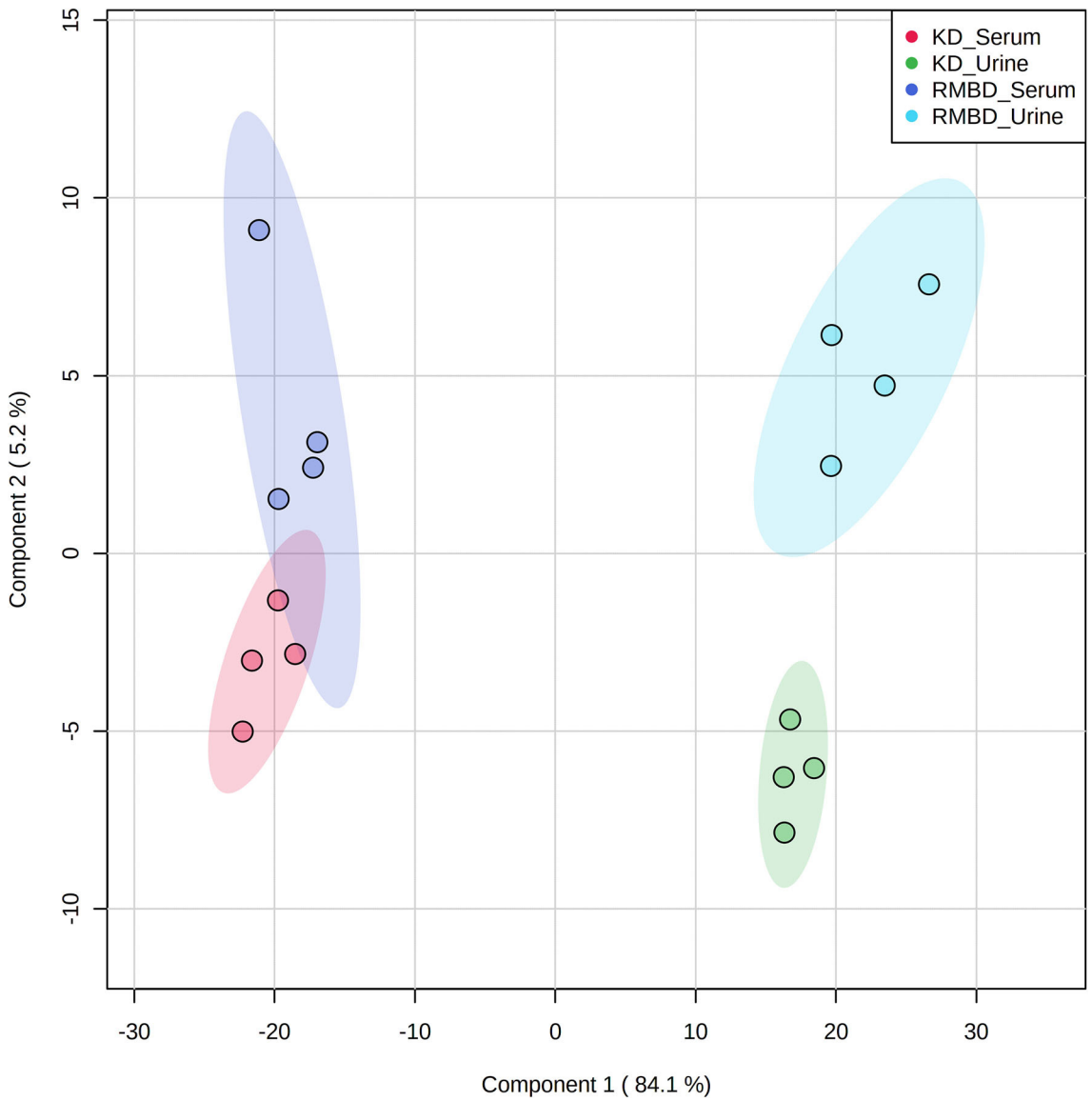

FIGURE 3 | PLS-DA shows how the serum and urine profiles of batch 1 ($n = 8$) can separate diet cohorts. PLS-DA of batch 1 dogs ($n = 8$) at the end of the diet intervention. Plot shows how serum, urine, and the KD ($n = 4$) and RMBD ($n = 4$) cohort metabolites differed at the end of diet intervention, shown with 95% confidence intervals (shaded regions) (KD, kibble diet; RMBD, raw meat–based diet).

metabolites found to be significantly different with the two-way ANOVA described above and the univariate analysis at end of the diet intervention (**Table 3**) were also found to have high VIP scores.

As a follow-up to the two-way ANOVA, an unprotected Fisher LSD test was used to compare how the metabolite concentrations at the end of the diet intervention differed between the four cohorts, i.e., the healthy and atopic dogs of both diet cohorts. The significant differences (FDR < 0.05) between these cohorts are presented in **Figure 6** as group averages. The tabulated results are included in **Supplementary Table 13**.

DISCUSSION

Diet Cohorts Readily Distinguished by Distinct Serum and Urine Metabolite Profiles

The two diets included in this study were remarkably different in terms of the types of raw ingredients used, their macronutrient and micronutrient composition, and their manufacturing methods. This suggests that the feeding of a particular diet could have a profound impact on metabolism, which in turn could have an effect on the dog's overall health and well-being. To

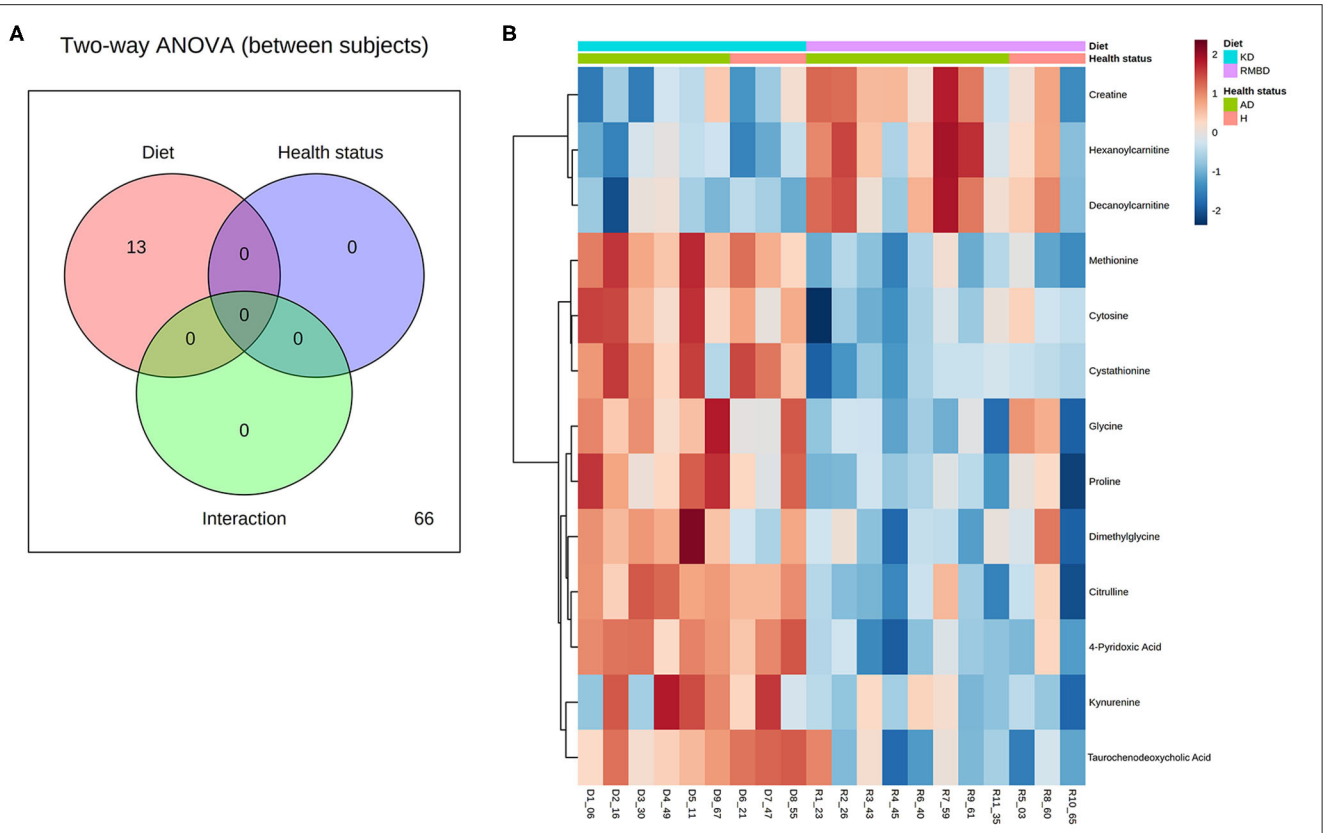

FIGURE 4 | ANOVA of serum from all dogs at the end of diet intervention shows significant metabolite differences between diet cohorts, but not between health status cohorts. **(A)** An overview of how metabolite values differ significantly (FDR < 0.05) between diet cohorts (red) and health status cohorts (blue), as well as any significant interaction between them (green) for all dogs ($n = 20$) at end of diet intervention. **(B)** A heatmap illustrating significant metabolite concentration differences in the two-way ANOVA for CAD-diagnosed ($n = 14$) and healthy individuals ($n = 6$) (green and orange) and between the kibble diet (KD) ($n = 9$) and raw meat–based diet (RMBD) ($n = 11$) cohorts at the end of diet intervention.

our best knowledge, no data are available about the comparative study of blood and urine metabolomics in response to raw meat–based and KDs. Most of the metabolomics-based studies performed before 2015 are referred to in a review paper by Allaway (59). To date, only the study by Schmidt et al. (60) compares the differences between an RMBD and a KD using metabolic profiling. However, that study considers the fecal metabolome. The first study to evaluate health outcomes as a result of feeding commercial RMBDs was published in 2012 (61). The authors concluded that no undesirable changes occurred to either blood biogenic amine concentrations or skin and coat conditions in dogs fed the RMBDs in their study. Here, the major differences in metabolite concentrations observed between the diet cohorts could indicate impact on blood biochemistry, overall health, and the CAD condition are discussed in light of literature found regarding these topics.

There were higher concentrations of several of the carnitines, creatine, and creatinine in the serum of the RMBD cohort than in the KD cohort (all dogs, $n = 20$) (**Table 3**). This finding is likely reflected in the markedly higher meat content of the RMBD diet. Meat is the main dietary source of carnitines (62) and creatine (63). It is likely that the elevated creatinine concentrations in the RMBD cohort because creatine is the direct precursor of creatinine (64). Furthermore, carnitines play crucial roles in long-chain fatty acid transport for mitochondrial oxidation, which is to be expected of canines eating a fat-rich diet. Higher serum carnitine concentrations have been associated with antiaging effects in canines (65). The authors note that higher carnitine concentrations are associated with younger dogs, but they make no claims as to age-related health benefits (65).

The urea-cycle metabolites citrulline and proline were found in significantly lower serum concentrations in the RMBD cohort than in the KD cohort (**Table 3**). These metabolites are involved in urea production and ammonia recycling (66, 67). Citrulline is the direct precursor for arginine synthesis (68). Meat protein contains high amounts of both arginine (69) and creatine (63), where arginine, and subsequently citrulline, is required for creatine synthesis (70). As citrulline is used to accept the amino groups of excess amino acids from dietary protein (71), the higher protein content in the RMBD may explain this observation; i.e., less citrulline would be required by the KD-fed dogs, which possibly explains the higher concentrations observed in the KD cohort. Proline is found in especially high concentrations in

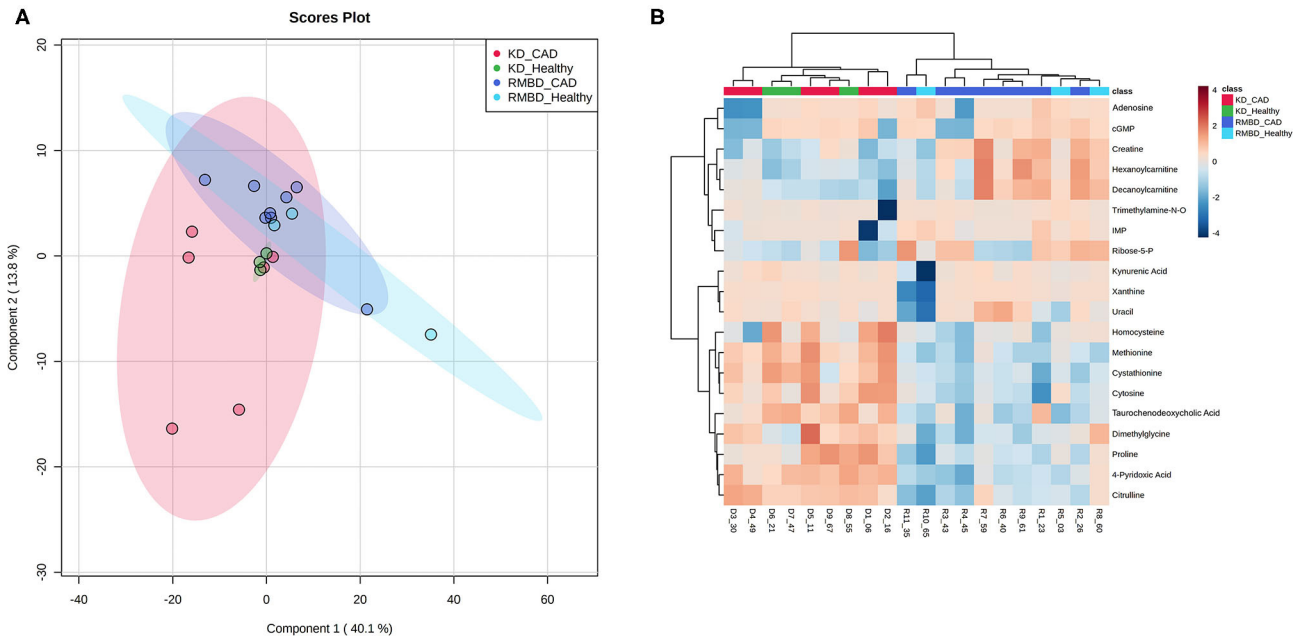

FIGURE 5 | PLS-DA analysis of the diet cohorts and health status cohorts. **(A)** PLS-DA (partial least squares–discriminant analysis) plot of the first two components, displayed with 95% confidence intervals for each diet group (shaded regions of same color). **(B)** A PLS-DA VIP score heatmap visualization of the most important features ($n = 20$) across components (KD, kibble diet; RMBD, raw meat–based diet; CAD, canine atopic dermatitis).

collagen (72), an unexpected finding considering the likely higher collagen content in the RMBD.

The significantly higher serum concentrations of the nucleobase cytosine observed in all of the dogs of the KD cohort (**Table 3**), as well as urine concentrations in the batch 1 KD cohort (**Supplementary Table 6**) at the end of the diet intervention, are notable. To the best of our knowledge, no studies have investigated the relationships of diet between cytosine concentrations in blood and urine.

In blood serum of all dogs in the KD cohort, higher concentrations of the primary bile acid taurochenodeoxycholic acid could be seen after the diet intervention than in the RMBD cohort. Elevated concentrations of the downstream product of taurochenodeoxycholic acid, deoxycholic acid, has been implicated in colon tumorigenesis in both mice and humans (73, 74). Colon cancer is exceptionally high in canines (75), although the links to bile acid concentrations remain poorly understood. Although insignificant, taurocholic acid was also found in higher serum concentrations in the KD cohort relative to the RMBD cohort. It has been established that the composition of the microbiota throughout the canine gut is largely defined by the nutritional profile of dietary intake (76, 77). The microbiota composition modulates the amount and composition of nutrients that are able to pass through the gut endothelium, hence affecting blood serum biochemistry (78). Most studies on this topic have focused solely on fecal samples (79). Bile acid concentrations have been suggested to be sensitive to changes in gut microbiota composition. It has been reported that fecal bile acid concentrations increase in canines when fed an animal-based, high-fat, low-fiber diet (80). Elevated primary bile acid concentrations in blood have been shown to be a sign of elevated inflammation (81), especially with regard to the liver (82). No reference values regarding what levels lead to increased inflammation have been reported for canines (80).

Because of their toxicity, bile acid concentrations are tightly regulated in mice (83) and furthermore are usually increased as a response to increased fat digestion (84) as they function essentially as emulsifiers to improve fat absorption through the endothelium. Given the far greater amounts of fat present in the RMBD, this finding comes as a surprise. However, it should be noted that there were also large amounts of carbohydrate present in the high-fat, low-fiber diet in the study performed by O'Keefe et al. (84). As the RMBD has little to no carbohydrate, the energy metabolism of the canines was likely markedly different from the humans participating in the diet interventions of the O'Keefe et al. (84) study. The RMBD-fed dogs were possibly even ketogenic, i.e., causing a switch over to increased β-oxidation of fatty acids as a primary means for ATP production (85). It has been shown that even in the presence of high fat content, glucose is the preferred energy substrate in mammals (86). Canines fed a high-fat diet, in particular, one rich in medium-chain triglycerides (MCTs), even in the presence of high carbohydrate, have been reported to be ketogenic (87, 88). However, results from both studies performed by Packer et al. (87) and Law et al. (88) are questionable, as the authors neglected to measure or report ketone body values in the dogs and thereby establish whether the diets were ketogenic (89). Furthermore, ketone body production has been shown to rely

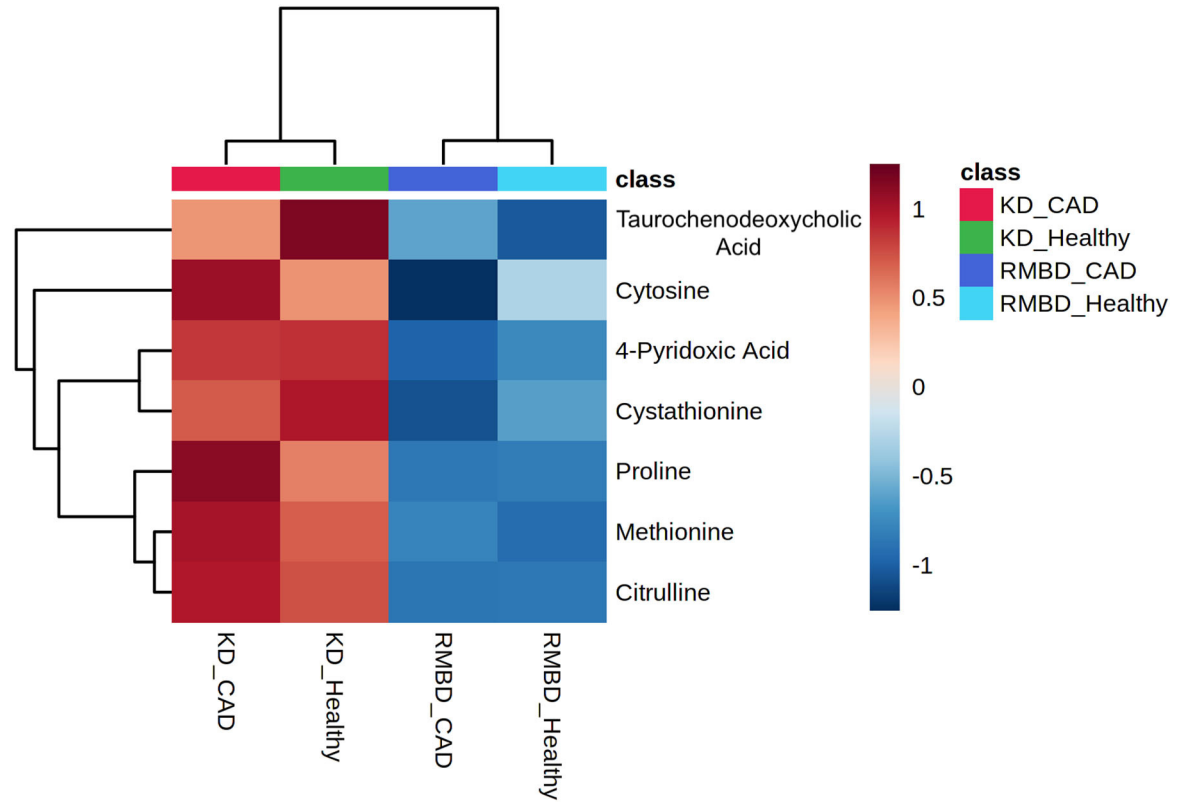

FIGURE 6 | Fisher least significant difference (LSD) test to determine the significant differences between diet cohorts of healthy and atopic individuals. Significant differences between metabolite concentrations calculated with Fisher LSD test (KD, kibble diet; RMBD, raw meat–based diet; CAD, canine atopic dermatitis).

on low levels of carbohydrate (90). Although MCTs are readily used for energy, even in the presence of carbohydrate (89), it does not necessarily switch the dog to a state of endogenous ketosis, i.e., where fat is the preferred metabolic energy source—the underlying assumption of a ketogenic diet (90). Ketogenic diets may affect serum bile acid concentrations in mice (91). In mammals, a switch over to ketogenic metabolism has major implications for altering glycolytic energy metabolism (92) and an increase in NADPH production, which is produced via the pentose phosphate pathway (93). In the RMBD cohort of batch 1 ($n = 4$) (**Supplementary Table 7**), a significantly higher level of ribose-5-phosphate was observed, indicating an upregulation of the pentose phosphate pathway (92) and subsequently a downregulation of glycolysis and upregulation of ketone body production. However, higher concentrations ribose-5-phosphate were not observed in the RMBD cohort of batch 2 ($n = 7$) or when observing all the dogs in the RMBD cohort ($n = 11$). The discrepancy between batches in itself merits further investigation. The finding in batch 1 may indicate that the RMBD was ketogenic, although to date no studies to our knowledge have considered the ketogenic properties of RMBDs, an area that merits further investigation.

At the end of the diet intervention, all canines in the KD cohort had higher serum concentrations of the sulfur-containing amino acid methionine than the RMBD cohort (**Table 3**). The batch 1 KD cohort also had significantly higher urine methionine concentrations than the RMBD cohort (**Supplementary Table 6**). The serum of all canines in the KD cohort had higher levels of cystathionine. Both play important roles in homocysteine metabolism via the remethylation pathway, via the transsulfuration pathway, and via one-carbon pathway (94). The amino acid homocysteine is remethylated to methionine in a process dependent on vitamin B_{12} (B_{12}) or is converted to cysteine via cystathionine in a vitamin B_6-dependent process (94). A schematic representation of the methionine and transsulfuration pathways is represented in **Figure 7**. Serum methionine concentrations have been implicated in the outcomes of many long-term health studies in a vast selection of organisms (95). It has been shown that lower consumption and subsequent blood concentrations of this essential amino acid are associated with longevity across species (95, 96), as well as improved blood glucose tolerance in rats, lower levels of oxidative stress in mice (97), and a lower risk of developing cancers in both species (98, 99). The amount of food that dogs are fed may also affect dog health; however, this consideration falls beyond the scope of the present study. Elevated serum methionine concentration serves as an indicator of overfeeding as has been shown in mice (100). As there was considerably more meat-based protein present in the RMBD, it could be expected to be reflected as higher blood serum and urine

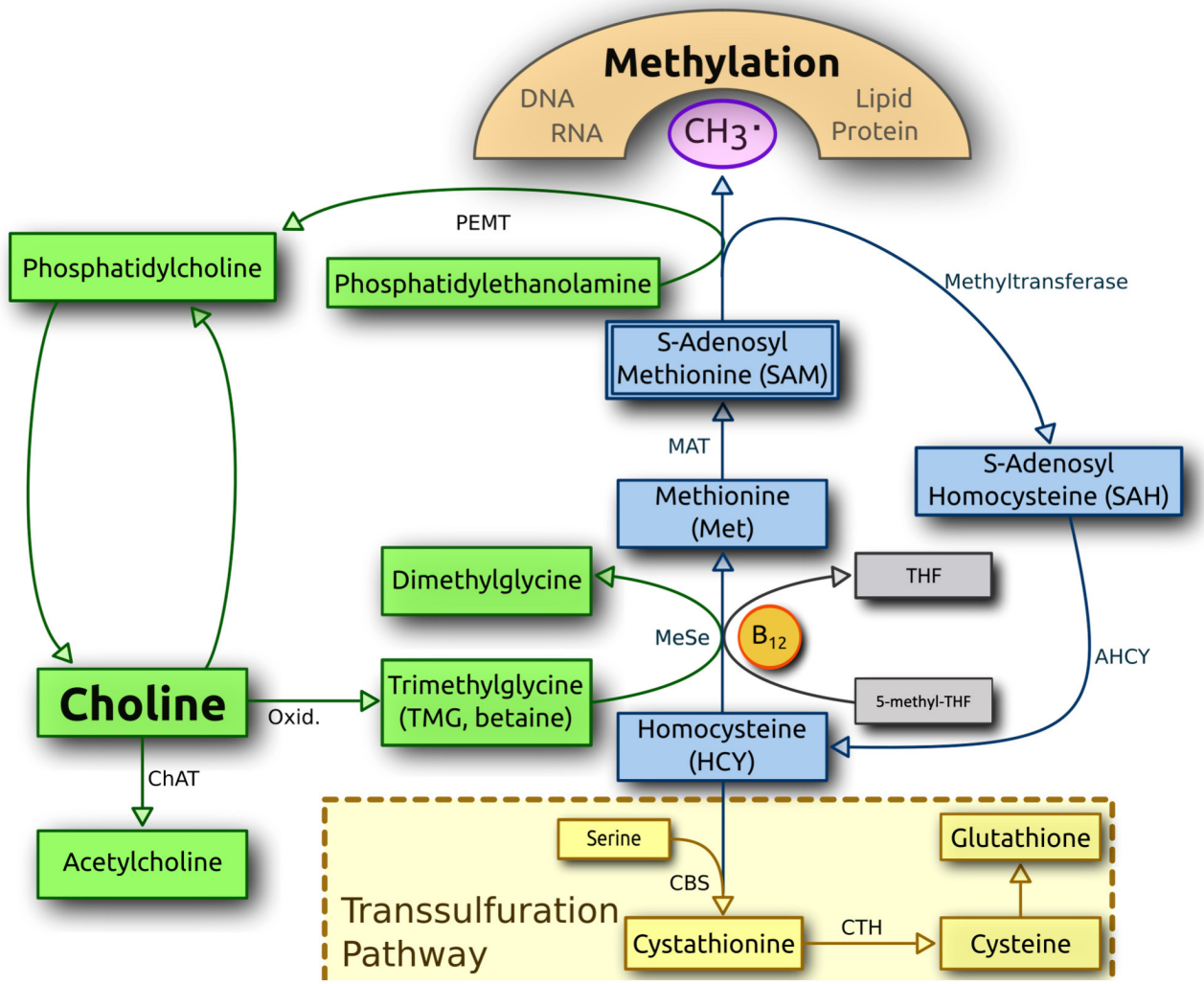

FIGURE 7 | An overview of homocysteine metabolism and the transsulfuration pathway. CBS, cystathione-beta synthase; MeSe, methionine synthase; THF, tetrahydrofolate (Figure uploaded by radio89 and labeled for reuse. https://commons.wikimedia.org/wiki/File:Choline_metabolism-en.svg. Image modified to present all terms in English).

concentrations of methionine in the RMBD cohort. However, the KD manufacturer apparently adds an unspecified amount of DL-methionine to the kibble (**Supplementary Table 1A**), which may in part explain this observation. Another explanation may be that canines in the KD cohort are actively eliminating or recycling greater concentrations of homocysteine than dogs fed the RMBD. In only the atopic dogs of the KD ($n = 6$) cohort, there is a trend of higher homocysteine concentrations vs. the atopic dogs of the RMBD ($n = 8$) cohort (**Supplementary File 21**, sheet 23). Concurrently, there is also a trend of higher urine homocysteine concentrations of CAD-diagnosed KD-fed dogs from batch 1 ($p = 0.05714$, FDR = 0.1934) (**Supplementary File 21**, sheet 24). Although insignificant, there is a trend of higher homocysteine concentrations in both urine and in the batch 1 KD cohort (**Supplementary File 21**, sheets 19 and 24). In a previously reported study regarding the hematology of the canines during the diet intervention (50), it was determined that the canines in the KD-fed cohort had elevated concentrations of blood serum B_{12} values. The significantly higher concentrations of methionine in the blood sera and urine of the batch 1 KD cohort (**Table 3**, **Supplementary Table 6**) and concurrently higher B_{12} serum concentrations (50) may be partially due to increased methionine synthase activity (94) as homocysteine is converted to methionine via this pathway (**Figure 7**). The higher concentrations of B_{12} comports with a higher methionine/homocysteine ratio as methylated B_{12} is converted into B_{12}, i.e., as its methyl group is donated to homocysteine, turning it into methionine. In the data reported by Anturaniemi et al. (50), serum folate concentrations were also significantly higher in KD-fed dogs, which also plays a role in homocysteine clearance (94). In the

present study, however, concentrations of folic acid, the acid form of folate, were not significantly different between diet cohorts for either urine or serum. There is a correlation between the amount of B_{12} in the food with serum B_{12} in dogs (101), indicating that B_{12} concentrations in dogs are tightly regulated, i.e., conserved in dogs fed a diet low B_{12}. Furthermore, 4-pyridoxic acid, a downstream product of pyridine (B_6), was also found in significantly higher concentrations in both the serum and urine of the KD cohorts. As B_6 is the cofactor for cystathione beta-synthase, which converts homocysteine to cystathionine via the transsulfuration pathway (102) (**Figure 7**), this may indicate that this pathway is significantly upregulated in the KD diet. Cystathionine, the first metabolite produced as a result of homocysteine clearance via the transsulfuration pathway (94), was found in far higher concentrations in all dogs in the KD cohort, with a high fold-change difference compared to the RMBD cohort (**Table 3**). Finally, higher serum concentrations of dimethylglycine were observed in all dogs in the KD cohort (**Table 3**), and a trend of higher serum concentration of betaine was found in all dogs in the RMBD cohort (**Supplementary Table 5**), as well as in the urine of the batch 1 RMBD cohort (**Supplementary Table 6**) compared to the KD cohort. Playing important roles in one-carbon metabolism, and subsequently often discussed in the context of DNA methylation, betaine is converted to dimethylglycine as its methyl group is added to homocysteine, producing methionine (103) (**Figure 7**).

Elevated homocysteine levels are often discussed as risk factors for various canine pathologies, including cardiovascular disease (104), increased inflammation (105), and certain renal pathologies (106). In humans, elevated levels of plasma homocysteine have been associated with irritable bowel syndrome and cancer (107). Elevated homocysteine levels and subsequent clearance have long been known to be a risk marker for MetS in humans (108). To our knowledge, no studies have observed any direct correlation between atopy and elevated homocysteine or methionine blood serum concentrations. However, a higher prevalence of AD in offspring was observed in the offspring of women with elevated circulating levels of vitamins B_{12} and folate, and hence upregulation of the homocysteine pathway may be related (109). Homocysteine is highly toxic for dogs (110), and blood homocysteine concentrations are kept low, lying within a narrow concentration range (111). Studies on mice have shown that homocysteine concentrations are kept low even in the case where serum concentrations of methionine (100), as well as cystathionine (106), are significantly increased. We find a similar phenomenon in the present study. It should be noted that the blood homocysteine concentrations in the canines of both diet cohorts were no higher than those reported for healthy canines elsewhere (112, 113). The significantly higher blood serum and urine concentrations may indicate that more methionine was added to the diet than biologically necessary (4, 114). This may also be true of other metabolites found in significantly higher concentrations in both the serum and urine samples of batch 1 (**Supplementary Table 11A**, **Supplementary Figure 2A**), including 4-pyridoxic acid, which as discussed above is likely related to the significantly higher cystathionine concentrations observed in the KD cohort.

The atopic complex is still not fully understood in canines (115), nor its relationship to MetS. Previous studies in mice (116) and humans (117) have provided contradictory evidence, indicating that AD both may (116) or may not (117) be linked to MetS in mammals (26). Whether underlying lifestyle choices predispose risk for both MetS and AD, or whether the development of MetS increases the risk of developing AD or vice versa, is not fully understood (26). According to the evaluation of CAD severity at the end of the diet intervention, neither the KD nor the RMBD significantly changed the CADESI-4 score outcome of the CAD-diagnosed canines, although there was a trend of greater CADESI-4 worsening in the KD cohort ($p = 0.219$) (**Supplementary Table 3C**). There was a general trend in worsening of CADESI-4 scores found in both diet cohorts (for the RMBD $= 6.9$, $\sigma = 6.5$, for the KD, $\mu = 18.3$, $\sigma = 13.8$) (**Supplementary Tables 3D,E**, respectively). In order to avoid interference from the seasonality of the disease, the diet trial was originally planned to take place during the late fall and winter months, when plant allergens known to exacerbate symptoms were not present. As discussed above, the trial had to be pushed forward, such that it ended when many plants had begun to bloom in Finland. It is likely that this delay caused the worsening of symptoms in both diet cohorts. There were disagreements between the owner-reported CAD diagnosis, which used the visual analog scale, and the dermatologist's diagnosis, which used the CADESI-4 scale. A metabolomics approach can potentially address and classify differing phenotypes of CAD, by combining "omics" with clinical and epidemiological data. However, in the present study, when considering the targeted metabolomic analysis that compared the atopic and healthy individuals, there were no significantly different metabolite concentrations at either the baseline or the end of the diet trial (**Figure 4A**). This suggests that diagnosing CAD by studying the blood serum with the targeted metabolites used in this study is also challenging.

A couple of studies looking at macronutrient preference among dogs served several food choices of varying macronutrient compositions *ad libitum* have indicated that several breeds of dogs are well-attuned to what they prefer and what their bodies require (17, 118). In the first study, the authors observed that several breeds of dogs adjusted to a preferred PFC macronutrient composition of 30%:63%:7% ME over a 7-day period (118), and another study observed that Harrier hound dogs adjusted to a PFC macronutrient ratio of 44%:52%:4% ME (17). The adequacy of diets for domesticated dogs, especially with regard to macronutrient composition, has been studied by comparing their diet with the diet of wolf (*Canis lupus*) populations (119). A meta-analysis of 41 studies that observed the wolf diet in Europe and North America concluded that the average wolf diet has a PFC of 54%:45%:1% ME (119). With the lack of carbohydrate and relatively high protein content, it resembles the RMBD used in our study (**Table 1**). This macronutrient ratio also resembles the ratio that the dogs in the two *ad libitum* studies mentioned above preferred (17). The ratio these breeds tend toward comports with current nutritional guidelines for dogs (120), which classify

proteins and fats as essential and carbohydrates as non-essential. It remains unclear whether increased starch digestibility offers any advantage to dogs with regard to their health span or whether the artificial selection for improved tolerance toward a starch-rich diet may outweigh the predisposition for other illnesses. Both of these topics deserve further study.

Strengths and Limitations of the Study

To our best knowledge this pilot study was the first ever to apply a serum and urine metabolomics-based approach to study how feeding canines a high-fat, moderate-protein, very low-carbohydrate RMBD affects serum and urine metabolite concentrations, as well as compare the outcome with the serum and urine metabolite profiles of dogs fed a moderate-fat, moderate-protein, high-carbohydrate KD. This targeted metabolomics approach offers quantitative and reliable data of blood serum and urine metabolite concentrations. Both urine and serum were analyzed simultaneously, giving insight into the relationships between the serum and urine media and diet. All dogs were pedigreed Staffordshire bull terriers. Their health status was diagnosed by a dermatologist using Favrot's criteria and the CADESI-4 scale to produce validated clinical scores.

As the present study focuses specifically on nutrition, there were no controls for quantitative markers for sleep, physical activity, or overall stress. Because of the high cost of analysis, the number of dogs that were used for the study was kept to a minimum of three dogs per cohort (KD-healthy, RMBD-healthy). Targeted metabolomic analysis of the serum samples collected from the dogs was performed in two batches. The ACQUITY UPLC/MS-MS instrument used for metabolomic analysis was serviced in between the analysis of the two batches, resulting in significantly different metabolite values between batches. Of the 102 metabolites targeted, a considerable amount had to be removed from the first batch analysis. Targeted analysis of the serum samples of the second batch went considerably better. Even so, many of the metabolites were unable to be used in the combined-batch analysis. Given the vast variety of metabolites circulating in both serum and urine media, it is clear in retrospect that numerous metabolites not studied were worthy of analysis. As discussed in *Design and Animals*, the postponed end of the diet intervention possibly allowed the introduction of undesired seasonal effects on CAD severity due to plant allergens. The study used more CAD-diagnosed than healthy dogs. Several dogs considered healthy prior to their official diagnosis by the dermatologist had to be reclassified as CAD sufferers. There were no metabolites that significantly differed between diet cohorts of the healthy individuals at the end of the diet intervention (KD-healthy $n = 3$, RMBD-healthy $n = 3$). This is likely due to the small sample size. The far fewer significant differences in metabolites between diet cohorts of batch 2 (**Supplementary Table 8**) may indicate that the underlying health status (CAD or healthy) had an impact on the results and may explain why the response to diet in the fully atopic cohort (batch 1) showed starker differences than for batch 2. Alternatively, this result may be an artifact due solely to the smaller sample size of batch 1.

CONCLUSIONS

Three key differences were observed with regard to the effects of diet on the canine metabolite profiles studied. First, there were markedly higher levels of carnitines and related compounds in canines fed the RMBD. Additionally higher levels of nitrogen excretion were indicated, also a result of the diet's high meat content. Second, the KD-fed cohort showed elevated bile acid concentrations that have a condition implicated, for example, in colon tumorigenesis in mice and humans. In addition to reflecting the macronutrient profile, it may also implicate a change in the gut microbiota composition. Further study is needed to confirm this. Third, there were higher concentrations of sulfur-containing compounds such as methionine and cystathionine, as well as compounds related to their metabolism, in the serum and urine of KD-fed dogs. Higher serum concentrations of these compounds are associated with increased inflammation in mammals. Furthermore, lower serum methionine concentrations as seen in the RMBD cohort have long been established as a marker associated with long life span and are generally considered beneficial for metabolic health. The latter two differences suggest that the KD may be less beneficial to the metabolic health of canines as metabolite concentrations that have been previously implicated in various pathologies were found in higher concentrations than in the RMBD-fed dogs. Given the limitations of the present study, however, such speculation requires further study to establish causality. Given the challenge of identifying CAD at the serum metabolite level, addressing and classifying differing phenotypes of CAD may be beyond the scope of a targeted metabolomics approach. Future studies will likely require both a larger set of metabolites to be targeted and larger sample cohorts. In summary, this experiment sought to clarify how nutrition may relate to CAD, as well as determine whether the impact of different diets could be seen on the metabolite level. While these topics are still novel for canine studies, the use of diet as a form of health maintenance, a notion that has gained popularity in recent years, will eventually be substantiated or rejected with quantitative clinical data.

EPILOGUE

A growing movement based on lay publications and anecdotal evidence regarding canine nutrition asserts that a diet consisting of raw meats lowers the risk of disease in canines (13, 15, 16). Diet as a form of disease prevention has become popular in human research but has yet to be adopted by the mainstream veterinary science community. The health maintenance chapter in the authoritative compendium *Small Animal Clinical Nutrition* (121) begins with the quote: "To ward off disease or recover health, people as a rule find it easier to depend on healers than to attempt the more difficult task of living wisely." Many dog owners want to take preventive measures to ward off disease in their pets, yet must place trust in veterinarians who may be unfamiliar with preventive measures to improve health span. To overcome the anecdotal nature of the discussion, scientific evidence can help in understanding the role of diet in promoting dog health.

DATA AVAILABILITY STATEMENT

Being funded by commercial sources has not altered our adherence to Frontiers policies on sharing data and materials. While the data is still used for graduation work, it will be disclosed later. However, for research purposes the data can be obtained upon request from the authors: anna.hielm-bjorkman@helsinki.fi.

ETHICS STATEMENT

Owners provided informed written consent for inclusion of their dogs in the study. The protocol was also approved by the Animal Experiment Board in Finland (ELLA) (permit number: ESAVI/3244/04.10.07/2013).

AUTHOR CONTRIBUTIONS

AH-B and JA contributed conception and design of the study. RM and AH-B organized the database. VV and JN performed the metabolomic analysis. RM did the statistical analysis and wrote the first draft of the manuscript. RM, AH-B, JA, VV, and SB-M wrote sections of the manuscript. All authors contributed to manuscript revision and approval of the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2020.554296/full#supplementary-material

REFERENCES

1. Warth B, Spangler S, Fang M, Johnson CH, Forsberg EM, Granados A, et al. Exposome-scale investigations guided by global metabolomics, pathway analysis, and cognitive computing. *Anal Chem.* (2017) 89:11505–13. doi: 10.1021/acs.analchem.7b02759
2. Kaddurah-Daouk R, Kristal BS, Weinshilboum RM. Metabolomics: a global biochemical approach to drug response and disease. *Annu Rev Pharmacol Toxicol.* (2008) 48:653–83. doi: 10.1146/annurev.pharmtox.48.113006.094715
3. Allaway D, Kamlage B, Gilham MS, Hewson-Hughes AK, Wiemer JC, Colyer A, et al. Effects of dietary glucose supplementation on the fasted plasma metabolome in cats and dogs. *Metabolomics.* (2013) 9:1096–108. doi: 10.1007/s11306-013-0527-8
4. Gibney MJ, Walsh M, Brennan L, Roche HM, German B, Van Ommen B. Metabolomics in human nutrition: opportunities and challenges. *Am J Clin Nutr.* (2005) 82:497–503. doi: 10.1093/ajcn/82.3.497
5. Gao X, Chen W, Li R, Wang M, Chen C, Zeng R, et al. Systematic variations associated with renal disease uncovered by parallel metabolomics of urine and serum. *BMC Syst Biol.* (2012) 6:S14. doi: 10.1186/1752-0509-6-S1-S14
6. Gibney MJ. Ultra-processed foods: definitions and policy issues. *Curr Dev Nutr.* (2019) 3:nzy077. doi: 10.1093/cdn/nzy077
7. Martinez Steele E, Baraldi LG, Louzada ML, Moubarac JC, Mozaffarian D, Monteiro CA. Ultra-processed foods and added sugars in the US diet: evidence from a nationally representative cross-sectional study. *BMJ Open.* (2016) 6:e009892. doi: 10.1136/bmjopen-2015-009892
8. Monteiro CA, Cannon G, Levy R, Moubarac J, Jaime P, Martins AP, et al. NOVA. The star shines bright. *World Nutr.* (2016) 7:28–38.
9. Gibson MW, Sajid A. Pet food processing: understanding transformations in starch during extrusion and baking. *Cereal Foods World.* (2013) 58:232–6. doi: 10.1094/CFW-58-5-0232
10. Tobie C, Péron F, Larose C. Assessing food preferences in dogs and cats: a review of the current methods. *Animals.* (2015) 5:126–37. doi: 10.3390/ani5010126
11. Suomen Ruokavirasto. *Koirille ja Kissoille Tarkoitettujen Ruokien Valmistus.* (2019). Available online at: https://www.ruokavirasto.fi/globalassets/yritykset/rehuala/tilastot/lemmikkielainruuat_2011-.pdf (accessed August 19, 2020).
12. Euromonitor. *Passport- The World Market for Pet Care* (behind paywall) (2018). Available online at: https://www.researchandmarkets.com/reports/4602351/the-world-market-for-pet-care#pos-0 (accessed August 19, 2020).
13. Freeman LM, Chandler ML, Hamper BA, Weeth LP. Current knowledge about the risks and benefits of raw meat-based diets for dogs and

cats. *J Am Vet Med Assoc.* (2013) 243:1549–58. doi: 10.2460/javma.243.11.1549
14. Schlesinger DP, Joffe DJ. Raw food diets in companion animals: a critical review. *Can Vet J.* (2011) 52:50–54.
15. Dijcker JC, Hagen-Plantinga EA, Everts H, Bosch G, Kema IP, Hendriks WH. Dietary and animal-related factors associated with the rate of urinary oxalate and calcium excretion in dogs and cats. *Vet Rec.* (2012) 171:46. doi: 10.1136/vr.100293
16. Lefebvre S, Reid-Smith R, Boerlin P, Weese J. Evaluation of the risks of shedding Salmonellae and other potential pathogens by therapy dogs fed raw diets in Ontario and Alberta. *Zoo Public Health.* (2008) 55:470–80. doi: 10.1111/j.1863-2378.2008.01145.x
17. Roberts M, Bermingham E, Cave N, Young W, McKenzie C, Thomas D. Macronutrient intake of dogs, self-selecting diets varying in composition offered ad libitum. *J Anim Physiol Anim Nutr.* (2018) 102:568–75. doi: 10.1111/jpn.12794
18. Darlenski R, Kazandjieva J, Hristakieva E, Fluhr JW. Atopic dermatitis as a systemic disease. *Clin Dermatol.* (2014) 32:409–13. doi: 10.1016/j.clindermatol.2013.11.007
19. Favrot C, Steffan J, Seewald W, Picco F. A prospective study on the clinical features of chronic canine atopic dermatitis and its diagnosis. *Vet Dermatol.* (2010) 21:23–31. doi: 10.1111/j.1365-3164.2009.00758.x
20. Olivry T, Saridomichelakis M, Nuttall T, Bensignor E, Griffin CE, Hill PB. Validation of the canine atopic dermatitis extent and severity index (CADESI)-4, a simplified severity scale for assessing skin lesions of atopic dermatitis in dogs. *Vet Dermatol.* (2014) 25:77–e25. doi: 10.1111/vde.12107
21. Huang PL. A comprehensive definition for metabolic syndrome. *Dis Model Mech.* (2009) 2:231–7. doi: 10.1242/dmm.001180
22. Cohen JB, Janniger CK, Piela Z, Szepietowski JC, Samady JA, Schwartz RA. Dermatologic correlates of selected metabolic events. *J Med.* (1999) 30:149–56.
23. Almela RM, Rubio CP, Cerón JJ, Ansón A, Tichy A, Mayer U. Selected serum oxidative stress biomarkers in dogs with non-food-induced and food-induced atopic dermatitis. *Vet Dermatol.* (2018) 29:229–e82. doi: 10.1111/vde.12525
24. Silverberg J, Garg N, Silverberg NB. New developments in comorbidities of atopic dermatitis. *Cutis.* (2014) 93:222–4.
25. Peikes H, Morris DO, Hess RS. Dermatologic disorders in dogs with diabetes mellitus: 45 cases (1986–2000). *J Am Vet Med Assoc.* (2001) 219:203–8. doi: 10.2460/javma.2001.219.203
26. Wollina U. Atopic dermatitis and the metabolic syndrome. *Clin Dermatol.* (2018) 36:62–6. doi: 10.1016/j.clindermatol.2017.09.010
27. Stefanadi EC, Dimitrakakis G, Antoniou C, Challoumas D, Punjabi N, Dimitrakaki IA, et al. Metabolic syndrome and the skin: a more than superficial association. Reviewing the association between skin diseases and metabolic syndrome and a clinical decision algorithm for high risk patients. *Diabetol Metab Syndr.* (2018) 10:9. doi: 10.1186/s13098-018-0311-z
28. Van Hecke E. Cutaneous manifestations of internal diseases. *Acta Clin Belg.* (2003) 58:302–7. doi: 10.1179/acb.2003.58.5.007
29. Pucheu-Haston CM, Santoro D, Bizikova P, Eisenschenk MNC, Marsella R, Nuttall T. Review: Innate immunity, lipid metabolism and nutrition in canine atopic dermatitis. *Vet Dermatol.* (2015) 26:104–e28. doi: 10.1111/vde.12199
30. Reiter LV, Torres SMF, Wertz PW. Characterization and quantification of ceramides in the nonlesional skin of canine patients with atopic dermatitis compared with controls. *Vet Dermatol.* (2009) 20:260–6. doi: 10.1111/j.1365-3164.2009.00759.x
31. Shimada K, Yoon J, Yoshihara T, Iwasaki T, Nishifuji K. Increased transepidermal water loss and decreased ceramide content in lesional and non-lesional skin of dogs with atopic dermatitis. *Vet Dermatol.* (2009) 20:541–6. doi: 10.1111/j.1365-3164.2009.00847.x
32. Franco J. *Lipid biomarkers for atopic dermatitis* [Ph. D. thesis]. Purdue University Graduate School, West Lafayette, IN, United States (2019).
33. Chandra RK. Nutrition and the immune system: an introduction. *Am J Clin Nutr.* (1997) 66:460–3S. doi: 10.1093/ajcn/66.2.460S
34. Benson A, Toh J, Vernon N, Jariwala SP. The role of vitamin D in the immunopathogenesis of allergic skin diseases. *Allergy.* (2012) 67:296–301. doi: 10.1111/j.1398-9995.2011.02755.x
35. Nesbitt GH, Freeman LM, Hannah SS. Effect of n-3 fatty acid ratio and dose on clinical manifestations, plasma fatty acids and inflammatory mediators in dogs with pruritus. *Vet Dermatol.* (2003) 14:67–74. doi: 10.1046/j.1365-3164.2003.00328.x
36. Ahlstrøm Ø, Krogdahl A, Vhile SG, Skrede A. Fatty acid composition in commercial dog foods. *J Nutr.* (2004) 134:2145S–7S. doi: 10.1093/jn/134.8.2145S
37. Popa I, Pin D, Remoué N, Osta B, Callejon S, Videmont E, et al. Analysis of epidermal lipids in normal and atopic dogs, before and after administration of an oral omega-6/omega-3 fatty acid feed supplement. A pilot study. *Vet Res Commun.* (2011) 35:501–9. doi: 10.1007/s11259-011-9493-7
38. Kaeberlein M, Rabinovitch PS, Martin GM. Healthy aging: the ultimate preventative medicine. *Science.* (2015) 350:1191–3. doi: 10.1126/science.aad3267
39. Chen M, Chen X, Nsor-Atindana J, Masamba KG, Ma J, Zhong F. Optimization of key aroma compounds for dog food attractant. *Anim Feed Sci Technol.* (2017) 225:173–81. doi: 10.1016/j.anifeedsci.2016.12.005
40. Hall NJ, Péron F, Cambou S, Callejon L, Wynne CD. Food and food-odor preferences in dogs: a pilot study. *Chem Senses.* (2017) 42:361–70. doi: 10.1093/chemse/bjx016
41. Hall JA, Melendez LD, Jewell DE. Using gross energy improves metabolizable energy predictive equations for pet foods whereas undigested protein and fiber content predict stool quality. *PLoS ONE.* (2013) 8:e54405. doi: 10.1371/journal.pone.0054405
42. Laflamme D, Izquierdo O, Eirmann L, Binder S. Myths and misperceptions about ingredients used in commercial pet foods. *Vet Clin North Am Small Anim Pract.* (2014) 44:689–98. doi: 10.1016/j.cvsm.2014.03.002
43. Rauber F, da Costa Louzada, Maria Laura, Steele EM, Millett C, Monteiro CA, et al. Ultra-processed food consumption and chronic non-communicable diseases-related dietary nutrient profile in the UK (2008–2014). *Nutrients.* (2018) 10:587. doi: 10.3390/nu10050587
44. Fiolet T, Srour B, Sellem L, Kesse-Guyot E, Alles B, Mejean C, et al. Consumption of ultra-processed foods and cancer risk: results from NutriNet-sante prospective cohort. *BMJ.* (2018) 360:k322. doi: 10.1136/bmj.k322
45. Kim H, Hu EA, Rebholz CM. Ultra-processed food intake and mortality in the USA: results from the third national health and nutrition examination survey (NHANES III, 1988–1994). *Public Health Nutr.* (2019) 22:1777–85. doi: 10.1017/S1368980018003890
46. Buddhachat K, Siengdee P, Chomdej S, Soontornvipart K, Nganvongpanit K. Effects of different omega-3 sources, fish oil, krill oil, and green-lipped mussel against cytokine-mediated canine cartilage degradation. *In Vitro Cell Dev Biol Anim.* (2017) 53:448–57. doi: 10.1007/s11626-016-0125-y
47. Barrouin-Melo SM, Terán YAM, Anturaniemi J, Hielm-Björkman AK. Interaction between nutrition and metabolism. In: Silvestre R, Torrado E, editors. *Metabolic Interaction in Infection.* Cham: Springer (2018). p. 29–114.
48. Barrouin-Melo SM, Anturaniemi J, Sankari S, Griinari M, Atroshi F, Ounjaijean S, et al. Evaluating oxidative stress, serological-and haematological status of dogs suffering from osteoarthritis, after supplementing their diet with fish or corn oil. *Lipids Health Dis.* (2016) 15:139. doi: 10.1186/s12944-016-0304-6
49. Anturaniemi J. *The relationships between environment, diet, transcriptome and atopic dermatitis in dogs* [Ph. D. thesis]. University of Helsinki, Helsinki, Finland (2018).
50. Anturaniemi J, Zaldívar-López S, Moore R, Kosola M, Sankari S, Barrouin-Melo SM. The effect of a raw vs dry diet on serum biochemical, hematologic, blood iron, B12, and folate levels in Staffordshire Bull Terriers. *Vet Clin Pathol.* (2020) 49:258–69. doi: 10.1111/vcp.12852
51. Picco F, Zini E, Nett C, Naegeli C, Bigler B, Rüfenacht S, et al. A prospective study on canine atopic dermatitis and food-induced allergic dermatitis in Switzerland. *Vet Dermatol.* (2008) 19:150–5. doi: 10.1111/j.1365-3164.2008.00669.x
52. Favrot C. Clinical signs of canine atopic dermatitis. *Vet Allergy.* (2013) 2013:65–9. doi: 10.1002/9781118738818.ch9
53. National Research Council. *Nutrient Requirements of Dogs and Cats.* Washington, DC: National Academies Press (2006).

54. Roman-Garcia P, Quiros-Gonzalez I, Mottram L, Lieben L, Sharan K, Wangwiwatsin A, et al. Vitamin B 12–dependent taurine synthesis regulates growth and bone mass. *J Clin Invest.* (2014) 124:2988–3002. doi: 10.1172/JCI72606
55. Chong J, Xia J. MetaboAnalystR: an R package for flexible and reproducible analysis of metabolomics data. *Bioinformatics.* (2018) 34:4313–14. doi: 10.1093/bioinformatics/bty528
56. Johnson WE. *Statistical Models for Removing Microarray Batch Effects and Analyzing Genome Tiling Microarrays*. Ann Arbor, MI: Harvard University (2007).
57. Chong J, Soufan O, Li C, Caraus I, Li S, Bourque G, et al. MetaboAnalyst 4.0: towards more transparent and integrative metabolomics analysis. *Nucleic Acids Res.* (2018) 46:W486–94. doi: 10.1093/nar/gky310
58. Benjamini Y, Hochberg Y. Controlling the false discovery rate: a practical and powerful approach to multiple testing. *J R Statist Soc.* (1995) 57:289–300. doi: 10.1111/j.2517-6161.1995.tb02031.x
59. Allaway D. Nutritional metabolomics: lessons from companion animals. *Curr Metabol.* (2015) 3:80–9. doi: 10.2174/2213235X03666141216203315
60. Schmidt M, Unterer S, Suchodolski JS, Honneffer JB, Guard BC, Lidbury JA, et al. The fecal microbiome and metabolome differs between dogs fed bones and raw food (BARF) diets and dogs fed commercial diets. *PLoS ONE.* (2018) 3:e0201279. doi: 10.1371/journal.pone.0201279
61. Beloshapka AN, Duclos LM, Vester Boler BM, Swanson KS. Effects of inulin or yeast cell-wall extract on nutrient digestibility, fecal fermentative end-product concentrations, and blood metabolite concentrations in adult dogs fed raw meat–based diets. *Am J Vet Res.* (2012) 73:1016–23. doi: 10.2460/ajvr.73.7.1016
62. Steiber A, Kerner J, Hoppel CL. Carnitine: a nutritional, biosynthetic, and functional perspective. *Mol Aspects Med.* (2004) 25:455–73. doi: 10.1016/j.mam.2004.06.006
63. Brosnan ME, Brosnan JT. The role of dietary creatine. *Amino Acids.* (2016) 48:1785–91. doi: 10.1007/s00726-016-2188-1
64. Wyss M, Kaddurah-Daouk R. Creatine and creatinine metabolism. *Physiol Rev.* (2000) 80:1107–213. doi: 10.1152/physrev.2000.80.3.1107
65. Hall JA, Jewell DE. Feeding healthy beagles medium-chain triglycerides, fish oil, and carnitine offsets age-related changes in serum fatty acids and carnitine metabolites. *PLoS ONE.* (2012) 7:e49510. doi: 10.1371/journal.pone.0049510
66. Rabier D, Kamoun P. Metabolism of citrulline in man. *Amino Acids.* (1995) 9:299–316. doi: 10.1007/BF00807268
67. Barbul A. Proline precursors to sustain mammalian collagen synthesis. *J Nutr.* (2008) 138:2021–24S. doi: 10.1093/jn/138.10.2021S
68. Haines RJ, Pendleton LC, Eichler DC. Argininosuccinate synthase: at the center of arginine metabolism. *Int J Biochem Mol Biol.* (2011) 2:8–23.
69. Hill D, editor. Alternative proteins in companion animal nutrition. In: *Pet Food Association of Canada Fall Conference 2004*. Toronto, ON; Quincy, IL: ADM Animal Health & Nutrition (2004).
70. Brosnan JT, Da Silva RP, Brosnan ME. The metabolic burden of creatine synthesis. *Amino Acids.* (2011) 40:1325–31. doi: 10.1007/s00726-011-0853-y
71. Weiner ID, Mitch WE, Sands JM. Urea and ammonia metabolism and the control of renal nitrogen excretion. *Clin J Am Soc Nephrol.* (2015) 10:1444–58. doi: 10.2215/CJN.10311013
72. Wu G, Bazer FW, Burghardt RC, Johnson GA, Kim SW, Knabe DA, et al. Proline and hydroxyproline metabolism: implications for animal and human nutrition. *Amino Acids.* (2011) 40:1053–63. doi: 10.1007/s00726-010-0715-z
73. Cao H, Luo S, Xu M, Zhang Y, Song S, Wang S, et al. The secondary bile acid, deoxycholate accelerates intestinal adenoma–adenocarcinoma sequence in Apc min/ mice through enhancing Wnt signaling. *Fam Cancer.* (2014) 13:563–71. doi: 10.1007/s10689-014-9742-3
74. Bernstein C, Bernstein H, Garewal H, Dinning P, Jabi R, Sampliner RE, et al. A bile acid-induced apoptosis assay for colon cancer risk and associated quality control studies. *Cancer Res.* (1999) 59:2353–7.
75. Lingeman CH, Garner F. Comparative study of intestinal adenocarcinomas of animals and man. *J Natl Cancer Inst.* (1972) 48:325–46.
76. Hang I, Rinttila T, Zentek J, Kettunen A, Alaja S, Apajalahti J, et al. Effect of high contents of dietary animal-derived protein or carbohydrates on canine faecal microbiota. *BMC Vet Res.* (2012) 8:90. doi: 10.1186/1746-6148-8-90
77. Beloshapka AN, Dowd SE, Suchodolski JS, Steiner JM, Duclos L, Swanson KS. Fecal microbial communities of healthy adult dogs fed raw meat-based diets with or without inulin or yeast cell wall extracts as assessed by 454 pyrosequencing. *FEMS Microbiol Ecol.* (2013) 84:532–41. doi: 10.1111/1574-6941.12081
78. Forster GM, Stockman J, Noyes N, Heuberger AL, Broeckling CD, Bantle CM, et al. A comparative study of serum biochemistry, metabolome and microbiome parameters of clinically healthy, normal weight, overweight, and obese companion dogs. *Top Comp Anim Med.* (2018) 33:126–35. doi: 10.1053/j.tcam.2018.08.003
79. Deng P, Swanson KS. Gut microbiota of humans, dogs and cats: current knowledge and future opportunities and challenges. *Br J Nutr.* (2015) 113:S6–S17. doi: 10.1017/S0007114514002943
80. Herstad KMV, Rønning HT, Bakke AM, Moe L, Skancke E. Changes in the faecal bile acid profile in dogs fed dry food vs high content of beef: a pilot study. *Acta Vet Scand.* (2018) 60:29. doi: 10.1186/s13028-018-0383-7
81. Janssen AWF, Houben T, Katiraei S, Dijk W, Boutens L, van der Bolt N, et al. Modulation of the gut microbiota impacts nonalcoholic fatty liver disease: a potential role for bile acids. *J Lipid Res.* (2017) 58:1399–416. doi: 10.1194/jlr.M075713
82. Masubuchi N, Sugihara M, Sugita T, Amano K, Nakano M, Matsuura T. Oxidative stress markers, secondary bile acids and sulfated bile acids classify the clinical liver injury type: promising diagnostic biomarkers for cholestasis. *Chem Biol Interact.* (2016) 255:83–91. doi: 10.1016/j.cbi.2015.08.016
83. Kim I, Ahn SH, Inagaki T, Choi M, Ito S, Guo GL, et al. Differential regulation of bile acid homeostasis by the farnesoid X receptor in liver and intestine. *J Lipid Res.* (2007) 48:2664–72. doi: 10.1194/jlr.M700330-JLR200
84. O'Keefe SJ, Li JV, Lahti L, Ou J, Carbonero F, Mohammed K, et al. Fat, fibre and cancer risk in African Americans and rural Africans. *Nat Commun.* (2015) 6:6342. doi: 10.1038/ncomms7342
85. Fukao T, Lopaschuk GD, Mitchell GA. Pathways and control of ketone body metabolism: on the fringe of lipid biochemistry. *Prostaglandins Leukot Essent Fatty Acids.* (2004) 70:243–51. doi: 10.1016/j.plefa.2003.11.001
86. Mitchell GA, Kassovska-Bratinova S, Boukaftane Y, Robert MF, Wang SP, Ashmarina L, et al. Medical aspects of ketone body metabolism. *Clin Invest Med.* (1995) 18:193–216.
87. Packer RM, Law TH, Davies E, Zanghi B, Pan Y, Volk HA. Effects of a ketogenic diet on ADHD-like behavior in dogs with idiopathic epilepsy. *Epilepsy Behav.* (2016) 55:62–8. doi: 10.1016/j.yebeh.2015.11.014
88. Law TH, Volk HA, Pan Y, Zanghi B, Want EJ. Metabolic perturbations associated with the consumption of a ketogenic medium-chain TAG diet in dogs with idiopathic epilepsy. *Br J Nutr.* (2018) 120:484–90. doi: 10.1017/S0007114518001617
89. Studzinski CM, MacKay WA, Beckett TL, Henderson ST, Murphy MP, Sullivan PG, et al. Induction of ketosis may improve mitochondrial function and decrease steady-state amyloid-β precursor protein (APP) levels in the aged dog. *Brain Res.* (2008) 1226:209–17. doi: 10.1016/j.brainres.2008.06.005
90. Paoli A, Rubini A, Volek J, Grimaldi K. Beyond weight loss: a review of the therapeutic uses of very-low-carbohydrate (ketogenic) diets. *Eur J Clin Nutr.* (2013) 67:789–96. doi: 10.1038/ejcn.2013.116
91. Kesl S, Poff A, Ward N, Fiorelli T, Ari C, Van Putten A, et al. Effect of sustaining dietary ketosis on the hippocampal and serum metabolome of Sprague-Dawley rats. *FASEB J.* (2015) 29(Suppl. 1):745.4. doi: 10.1096/fasebj.29.1_supplement.745.4
92. Veech RL, Chance B, Kashiwaya Y, Lardy HA, Cahill GF Jr. Ketone bodies, potential therapeutic uses. *IUBMB Life.* (2001) 51:241–7. doi: 10.1080/152165401753311780
93. Maalouf M, Sullivan PG, Davis L, Kim DY, Rho JM. Ketones inhibit mitochondrial production of reactive oxygen species production following glutamate excitotoxicity by increasing NADH oxidation. *Neuroscience.* (2007) 145:256–64. doi: 10.1016/j.neuroscience.2006.11.065
94. Finkelstein D, editor. Pathways and regulation of homocysteine metabolism in mammals. In: *Seminars in Thrombosis and Hemostasis.* New York, NY: Copyright© 2000 by Thieme Medical Publishers, Inc. (2000).
95. McIsaac RS, Lewis KN, Gibney PA, Buffenstein R. From yeast to human: exploring the comparative biology of methionine restriction in extending eukaryotic life span. *Ann N Y Acad Sci.* (2016) 1363:155–70. doi: 10.1111/nyas.13032

96. Fontana L, Partridge L. Promoting health and longevity through diet: from model organisms to humans. *Cell.* (2015) 161:106–18. doi: 10.1016/j.cell.2015.02.020
97. Hulbert A, Faulks SC, Harper JM, Miller RA, Buffenstein R. Extended longevity of wild-derived mice is associated with peroxidation-resistant membranes. *Mech Ageing Dev.* (2006) 127:653–7. doi: 10.1016/j.mad.2006.03.002
98. Komninou D, Leutzinger Y, Reddy BS, Richie JP Jr. Methionine restriction inhibits colon carcinogenesis. *Nutr Cancer.* (2006) 54:202–8. doi: 10.1207/s15327914nc5402_6
99. Sinha R, Cooper TK, Rogers CJ, Sinha I, Turbitt WJ, Calcagnotto A, et al. Dietary methionine restriction inhibits prostatic intraepithelial neoplasia in TRAMP mice. *Prostate.* (2014) 74:1663–73. doi: 10.1002/pros.22884
100. Mentch S, Mehrmohamadi M, Huang L, Liu X, Gupta D, Mattocks D, et al. Histone methylation dynamics and gene regulation occur through the sensing of one-carbon metabolism. *Cell Metab.* (2015) 22:861–73. doi: 10.1016/j.cmet.2015.08.024
101. Davenport DJ, Ching RJ, Hunt JH, Bruyette DS, Gross KL. The effect of dietary levels of folate and cobalamin on the serum concentration of folate and cobalamin in the dog. *J Nutr.* (1994) 124(Suppl. 12):2559–62S. doi: 10.1093/jn/124.suppl_12.2559S
102. Finkelstein JD. Methionine metabolism in mammals. *J Nutr Biochem.* (1990) 1:228–37. doi: 10.1016/0955-2863(90)90070-2
103. Dominguez-Salas P, Cox SE, Prentice AM, Hennig BJ, Moore SE. Maternal nutritional status, C 1 metabolism and offspring DNA methylation: a review of current evidence in human subjects. *Proc Nutr Soc.* (2012) 71:154–65. doi: 10.1017/S0029665111003338
104. Heilmann RM, Grützner N, Iazbik M, Lopes R, Bridges C, Suchodolski JS, et al. Hyperhomocysteinemia in greyhounds and its association with hypofolatemia and other clinicopathologic variables. *J Vet Intern Med.* (2017) 31:109–16. doi: 10.1111/jvim.14597
105. Undas A, Brozek J, Szczeklik A. Homocysteine and thrombosis: from basic science to clinical evidence. *Thromb Haemost.* (2005) 94:907–15. doi: 10.1160/TH05-05-0313
106. Friedman AN, Bostom AG, Selhub J, Levey AS, Rosenberg IH. The kidney and homocysteine metabolism. *J Am Soc Nephrol.* (2001) 12:2181–9.
107. Keshteli AH, Baracos VE, Madsen KL. Hyperhomocysteinemia as a potential contributor of colorectal cancer development in inflammatory bowel diseases: a review. *World J Gastroenterol.* (2015) 21:1081–90. doi: 10.3748/wjg.v21.i4.1081
108. Sreckovic B, Sreckovic VD, Soldatovic I, Colak E, Sumarac-Dumanovic M, Janeski H, et al. Homocysteine is a marker for metabolic syndrome and atherosclerosis. *Diabetes Metab Syndr.* (2017) 11:179–82. doi: 10.1016/j.dsx.2016.08.026
109. Kiefte-de Jong JC, Timmermans S, Jaddoe VW, Hofman A, Tiemeier H, Steegers EA, et al. High circulating folate and vitamin B-12 concentrations in women during pregnancy are associated with increased prevalence of atopic dermatitis in their offspring. *J Nutr.* (2012) 142:731–8. doi: 10.3945/jn.111.154948
110. Patterson B, Barr J, Fosgate GT, Berghoff N, Steiner JM, Suchodolski J, et al. Homocysteine in dogs with systemic inflammatory response syndrome. *J Small Anim Pract.* (2013) 54:620–4. doi: 10.1111/jsap.12144
111. Kakimoto T, Iwanaga T, Kanouchi H. Plasma homocysteine concentrations in dogs. *Int J Vet Med Res Rep*. (2014) 2014:141449. doi: 10.5171/2014.141449
112. Trisolini C, Minoia G, Manca R, Rizzo A, Robbe D, Valentini L, et al. Plasma homocysteine levels in cycling, pregnant, and spayed bitches. *Anim Reprod Sci.* (2008) 108:29–36. doi: 10.1016/j.anireprosci.2007.06.027
113. Çayir C, Kozat S. Investigation of homocysteine levels in healthy dogs. *J Vet Sci Anim Husb.* (2016) 4:305. doi: 10.15744/2348-9790.4.305
114. Shih VE. Amino acid analysis. In: Blau N, Duran M, Blaskovics ME, Gibson KM, editors. *Physician's Guide to the Laboratory Diagnosis of Metabolic Diseases*. Berlin; Heidelberg: Springer (2003). p. 11–26.
115. Santoro D, Marsella R, Pucheu-Haston CM, Eisenschenk MN, Nuttall T, Bizikova P. Pathogenesis of canine atopic dermatitis: skin barrier and host–micro-organism interaction. *Vet Dermatol.* (2015) 26:84–e25. doi: 10.1111/vde.12197
116. Seino S, Tanaka Y, Honma T, Yanaka M, Sato K, Shinohara N, et al. Atopic dermatitis causes lipid accumulation in the liver of NC/Nga mouse. *J Clin Biochem Nutr*. (2011) 2011:1111160123. doi: 10.3164/jcbn.11-29
117. Wakkee M, Nijsten T. Comorbidities in dermatology. *Dermatol Clin.* (2009) 27:137–47. doi: 10.1016/j.det.2008.11.013
118. Hewson-Hughes AK, Hewson-Hughes VL, Colyer A, Miller AT, McGrane SJ, Hall SR, et al. Geometric analysis of macronutrient selection in breeds of the domestic dog, Canis lupus familiaris. *Behav Ecol.* (2013) 24:293–304. doi: 10.1093/beheco/ars168
119. Bosch G, Hagen-Plantinga EA, Hendriks WH. Dietary nutrient profiles of wild wolves: insights for optimal dog nutrition? *Br J Nutr.* (2015) 113:S40–54. doi: 10.1017/S0007114514002311
120. Association of American Feed Control Officials. *AAFCO Dog Food Nutrient Profiles*. 2017 Official Publication. (2016). p. 154–56.
121. Hand MS, Lewis LD. *Small Animal Clinical Nutrition*. 4th Edn. Topeka, KS: Mark Morris Associates (2000).

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Supplementation of Yeast Cell Wall Fraction Tends to Improve Intestinal Health in Adult Dogs Undergoing an Abrupt Diet Transition

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When owners decide to change their pet's food, a rapid transition may cause gastrointestinal distress. Yeast products may help with digestive upset during diet transition due to the bioactive compounds they possess, which may lead to improved intestinal morphology and integrity, modified gut microbiota, and modulated immune responses. The objective of this study was to determine the effects of a yeast cell wall fraction supplement on measures of gut integrity and fecal characteristics of adult dogs undergoing an abrupt diet transition. Twelve adult female beagles (mean age: 5.16 ± 0.87 years; mean body weight: 13.37 ± 0.68 kg) were used in a replicated 4 × 4 Latin square design with four 28-day experimental periods. During days 1–14, dogs were fed a dry kibble diet and supplemented with a placebo (cellulose; 125 mg/d) or yeast product (365 mg/d; equivalent to 0.2% of diet). During days 15–28, dogs remained on their placebo or yeast treatments but were rapidly transitioned to a canned diet or high-fiber diet. Fresh fecal samples were collected on days 13, 16, 20, 24, and 27 for measurement of pH, dry matter, calprotectin, immunoglobulin A (IgA), *Escherichia coli*, and *Clostridium perfringens.* Blood samples were collected on days 14, 17, and 28 to measure serum lipopolysaccharide-binding protein concentrations. All data were analyzed using the Mixed Models procedure of SAS 9.4. Fecal pH, dry matter, calprotectin, IgA, and *E. coli* were not affected ($P > 0.05$) by treatment before diet transition. Dogs supplemented with yeast cell wall fraction tended to have higher ($P = 0.06$) fecal *C. perfringens* counts than the controls. After diet transition, most parameters were not altered ($P > 0.05$) by treatment except that yeast-supplemented dogs tended to have higher ($P = 0.06$) fecal IgA than controls. Our results suggest that the yeast product may modestly improve intestinal health after an abrupt diet transition in adult dogs by enhancing intestinal immunity.

Keywords: canine nutrition, fecal characteristics, gut stability, intestinal immunity, stool quality

INTRODUCTION

Diet transition commonly occurs in dogs when owners purchase new products. If not transitioned slowly, digestive upset may occur, which results in poor stool quality and gastrointestinal (GI) upset. Based on past research, the supplementation of functional ingredients such as yeast products may serve as a potential approach to minimizing digestive upset in such situations.

Yeast products are ingredients containing yeast cells or yeast derivatives and are commonly used in livestock feeds to improve animal performance (1, 2). Yeast products have also been shown to improve GI health in various ways. For example, dogs (3–6) and cats (7) supplemented with yeast products had reduced potential pathogenic bacteria (e.g., *Escherichia coli*; *Clostridium perfringens*) and increased beneficial bacteria (e.g., *Lactobacillus*; *Bifidobacterium*) in feces. In pigs (2, 8) and chickens (1), supplementation of yeast products has led to increased small intestinal villus height and/or villus height: crypt depth ratio. Yeast supplementation enhanced colonic barrier function and decreased intestinal permeability in murine intestinal obstruction and colitis models (9, 10). Lastly, yeast-supplemented dogs (4, 11) and pigs (12) had greater small intestinal immunoglobulin A (IgA) concentrations vs. controls.

Results from those studies indicate that supplementation of yeast products may improve GI health by modifying gut microbiota, improving intestinal morphology and integrity, and/or modulating immune responses. In this study, we aimed to determine the effects of a yeast cell wall fraction product on measures of GI tract stability and fecal characteristics of adult dogs undergoing an abrupt diet transition. We hypothesized that yeast cell wall fraction-supplemented dogs would have greater gastrointestinal stability and protection from a disrupted gut barrier function due to abrupt diet change, as well as reduced fecal pathogens compared to control dogs receiving a placebo treatment.

MATERIALS AND METHODS

Animals, Diets, and Treatments

All animal care and experimental procedures were approved by the University of Illinois Institutional Animal Care and Use Committee (Protocol No. 17276) prior to experimentation. All methods were performed in accordance with the United States Public Health Service Policy on Humane Care and Use of Laboratory Animals. Twelve adult female beagles (mean age: 5.16 ± 0.87 years; mean BW: 13.37 ± 0.68 kg) were used and housed individually in pens (1.0 m wide × 1.8 m long) in a humidity- and temperature-controlled room on a 14 h light: 10 h dark cycle. Dogs had access to fresh water *ad libitum* at all times. All diets were offered to dogs once a day in the morning to maintain body weight throughout the study. Dogs were weighed and body condition scores were assessed using a 9-point scale (13) weekly before feeding.

A replicated 4 × 4 Latin square design was conducted. Each 28-d experimental period consisted of an adaptation phase (d 1–14) and a diet transition phase (d 15–28). Three diets that met all Association of American Feed Control Officials (AAFCO) (14) nutrient recommendations for adult dogs at maintenance were fed, which included (1) a baseline diet (a dry kibble experimental diet; **Tables 1**, **2**); (2) a commercial canned diet (CD; Ol' Roy Cuts in Gravy with Savory Beef; Walmart, Bentonville, AR; **Table 2**); and (3) a high-fiber diet (HFD) composed of the experimental diet plus 22.5 g/d of soluble corn fiber (Nutriose® Soluble Digestion-Resistant Prebiotic Corn Fiber; Roquette America Inc., Geneva, IL) that was top-dressed on the diet just prior to feeding. Treatments were given to dogs via gelatin capsules (d 1–28) prior to the diet each day, which included a placebo (125 mg cellulose/d) or yeast cell wall fraction (Safmannan®; Phileo by Lesaffre, Marcq-en-Barœul, France; 365 mg/d; equivalent to 0.2% of diet). The yeast cell wall fraction tested was from *Saccharomyces cerevisiae* and the analyzed chemical composition is presented in **Table 3**. During the adaptation phase, all dogs were fed the experimental diet and supplemented with placebo or yeast product. During the diet transition phase, dogs remained on their placebo or yeast treatments, but were fed their new diet (CD or HFD). Therefore, dogs were allotted to one of four groups each experimental period: (1) Yeast CD; (2) Yeast HFD; (3) Placebo CD; and (4) Placebo HFD.

TABLE 1 | Ingredient composition of the experimental diet fed to yeast- or cellulose-supplemented dogs.

Ingredient	Amount (%, as-is)
Brewer's rice	45.26
Chicken by-product meal	32.00
Chicken fat	9.00
Corn	6.00
Cellulose	6.00
Salt	0.50
Potassium chloride	0.45
Taurine	0.30
Mineral premix[a]	0.18
Vitamin premix[b]	0.18
Choline chloride	0.13

[a] *Provided per kg diet: Mn (as $MnSO_4$), 66.0 mg; Fe (as $FeSO_4$), 120.0 mg; Cu (as $CuSO_4$), 18.0 mg; Co (as $CuSO_4$), 1.20 mg; Zn (as $ZnSO_4$), 240.0 mg; I (as KI), 1.80 mg; Se (as Na_2SeO_3), 0.24 mg.*

[b] *Provided per kg diet: vitamin A, 5.28 mg; vitamin D_3, 0.04 mg; vitamin E, 120.0 mg; vitamin K, 0.88 mg; thiamine, 4.40 mg; riboflavin, 5.72 mg; pantothenic acid, 22.0 mg; niacin, 39.6 mg; pyridoxine, 3.52 mg; biotin, 0.13 mg; folic acid, 0.44 mg; vitamin B_{12}, 0.11 mg.*

Fecal Sample Collection

On d 13, 16, 20, 24, and 27 of each experimental period, one fresh fecal sample from each dog was collected within 15 min of defecation. Fecal samples were scored according to a 5-point scale: 1 = hard, dry pellets, small hard mass; 2 = hard, formed, dry stool; remains firm and soft; 3 = soft, formed, and moist stool, retains shape; 4 = soft, unformed stool, assumes shape of container; and 5 = watery, liquid that can be poured. Fecal pH was measured immediately using an AP10 pH meter (Denver Instrument, Bohemia, NY) equipped with a Beckman Electrode (Beckman Instruments Inc., Fullerton, CA). An aliquot

of fresh feces was dried at 105°C for 2 d for dry matter (DM) determination.

On d 13, 20, and 27 of each experimental period, two more aliquots of fresh fecal samples were collected for determination of fecal IgA, calprotectin and potential pathogenic bacteria (*E. coli* and *C. perfringens*). For fecal IgA and calprotectin measurements, the aliquot was stored at −80°C until analyses. For fecal bacteria analyses, the aliquot was processed for bacterial culture immediately after collection.

Fecal IgA and Calprotectin

Fecal proteins were extracted according to Vilson et al. (15). Fecal samples (250 mg) were vortexed with 750 µl extraction buffer containing 50 mM-EDTA (ThermoFisher, Waltham, MA, United States) and 100 µg/l soybean trypsin inhibitor (Sigma, St. Louis, MO, United States) in phosphate-buffered saline/1 per cent bovine serum albumin (Tocris Bioscience, Bristol, UK). Phenylmethanesulphonyl fluoride (12.5 µl, 350 mg/l; Sigma, St. Louis, MO) was added to each tube, followed by centrifugation at 10,000 × g at 4°C for 10 min. The supernatants were collected for measurements of IgA and calprotectin using canine-specific commercial ELISA kits (IgA: # E-40A, Immunology Consultants Laboratory, Portland, OR; calprotectin: #MBS030023, MyBioSource, San Diego, CA, United States).

TABLE 2 | Analyzed chemical composition of the experimental diet and canned diet (CD) fed to yeast- or cellulose-supplemented dogs.

Item	Experimental diet	Canned diet
Dry matter (DM; %)	92.82	23.90
	%, of DM	
Crude protein	28.30	41.27
Acid-hydrolyzed fat	14.36	24.35
Total dietary fiber	15.98	11.72
Ash	5.95	12.96
Nitrogen-free extract[a]	35.41	9.72
Metabolizable energy[b] (kcal/g)	3.51	3.91

[a] *Nitrogen-free extract (%) = 100% − (crude protein% + acid-hydrolyzed fat% + total dietary fiber% + ash%).*
[b] *Metabolizable energy = 3.5 kcal/g × crude protein (%) + 8.5 kcal/g × acid-hydrolyzed fat (%) + 3.5 kcal/g × nitrogen-free extract (%).*

TABLE 3 | Analyzed chemical composition of the yeast cell wall fraction tested.

Item	%	Free sugars (mg/g)	Hydrolyzed monosaccharides corrected for free sugars (mg/g)
Dry matter (DM; %)	95.36		
	%, of DM		
Ash	4.78		
Crude protein	14.65		
Acid-hydrolyzed fat	9.28		
Total dietary fiber	57.47		
Soluble fiber	53.38		
Insoluble fiber	4.10		
Arabinose		0.00	0.00
Fructose		0.00	0.00
Galactose		0.00	0.00
Glucose		0.13	227.35
Inositol		32.78	0.00
Mannose		2.44	242.46
Sorbitol		0.15	11.60
Xylose		0.00	0.00

Fecal *E. coli* and *C. perfringens*

Fecal *E. coli* and *C. perfringens* were measured using standard culture methods. Briefly, 50 mg feces were homogenized in 1 mL PBS followed by 10^{-1} to 10^{-7} serial dilutions. Diluted fecal samples were inoculated onto petri dishes with bacterial selective agar. The selective agar media used in this study were M-TEC agar (HiMedia Laboratories, Kelton, PA, United States) for *E. coli* and Perfringens OPSP agar (ThermoFisher, Waltham, MA, United States) for *C. perfringens*. *E. coli* was incubated aerobically at 37°C for 12–48 h, while *C. perfringens* was incubated anaerobically at 37°C for 12–48 h. Colony forming units were enumerated after incubation.

Blood Sample Collection and Serum Lipopolysaccharide (LPS)-Binding Protein

On d 14, 17, and 28 of each experimental period, a fasted blood sample was collected via jugular puncture and transferred to serum tubes containing a clot activator and gel for serum separation (no. 367986, Becton Dickinson, Franklin Lakes, NJ, United States). Serum was separated and collected by centrifuging blood tubes at 1,300 × g at 4°C for 10 min (Beckman CS-6R centrifuge; Beckman Coulter Inc., Brea, CA). Serum lipopolysaccharide-binding protein was measured

TABLE 4 | Daily food, nutrient, and energy intakes, body weight, and body condition score of yeast- or cellulose-supplemented dogs before diet transition[a].

	Treatment			
Item	Cellulose	Yeast	Pooled SEM	*P*-value
Daily intake (g/d)				
Food, as-is	177.3	180.2	3.98	0.22
Food, DMB[b]	164.6	167.3	3.69	0.22
Protein, DMB	46.6	47.3	1.04	0.23
Fat, DMB	25.5	25.9	0.57	0.22
Total dietary fiber, DMB	26.3	26.7	0.59	0.22
Nitrogen-free extract, DMB	56.5	57.4	1.27	0.22
Energy (kcal/d)	577.1	586.5	12.95	0.22
Body weight (kg)	13.51	13.53	0.06	0.78
Body condition score[c]	7.17	7.13	0.06	0.38

[a] *Data are expressed as means ± pooled standard error of the mean, n = 24.*
[b] *DMB, dry matter basis.*
[c] *A 9-point scale body condition scoring system was used (13).*

TABLE 5 | Daily food, nutrient, and energy intakes of yeast- or cellulose-supplemented dogs transitioned to a high-fiber diet (HFD) or canned diet (CD)[a].

Item	Cellulose		Yeast (375 mg/d)		*P*-value		
	HFD	CD	HFD	CD	Treatment	Diet	Treatment *Diet
Daily intake (g/d)							
Food, as-is	177.0 ± 4.62	695.2 ± 12.91	175.9 ± 4.44	678.7 ± 27.08	0.48	< 0.01	0.53
Food, DMB[b]	164.2 ± 4.28	160.8 ± 2.99	163.3 ± 4.13	157.0 ± 6.26	0.43	0.11	0.64
Protein, DMB	46.5 ± 1.21	66.4 ± 1.23	46.2 ± 1.17	64.8 ± 2.58	0.43	< 0.01	0.57
Fat, DMB	25.4 ± 0.66	40.8 ± 0.76	25.3 ± 0.64	39.9 ± 1.59	0.43	< 0.01	0.56
Total dietary fiber, DMB	44.2 ± 0.68	18.8 ± 0.35	44.1 ± 0.66	18.4 ± 0.73	0.47	< 0.01	0.73
Nitrogen-free extract, DMB	56.4 ± 1.47	13.9 ± 0.26	56.0 ± 1.41	13.6 ± 0.54	0.68	< 0.01	1.00
Energy (kcal/d)	575.8 ± 15.02	628.1 ± 11.66	572.4 ± 14.46	613.1 ± 24.47	0.43	< 0.01	0.62

[a]*Data are expressed as means ± standard error of the mean, n = 12 for HFD and CD.*
[b]*DMB, dry matter basis.*

using a canine-specific commercial ELISA kit (#MBS093112, MyBioSource, San Diego, CA, United States).

Chemical Analysis of Diets and Yeast Cell Wall Fraction

The CD was dried at 55°C in a forced-air oven for approximately 1 wk. Both diets were then subsampled and ground through a Wiley mill (model 4, Thomas Scientific, Swedesboro, NJ, United States) through a 2 mm screen. Diets and yeast cell wall fractions were analyzed for DM, organic matter (OM), crude protein, acid-hydrolyzed fat, and total dietary fiber (TDF). Dry matter and OM were analyzed according to Association of Official Analytical Chemists (AOAC, 2006; DM: methods 934.01; OM: 942.05) (16). Crude protein content was calculated from Leco (FP2000 and TruMac) total nitrogen values according to AOAC (2006, method 992.15) (16). Fat content was determined using acid hydrolysis methods of the American Association of Cereal Chemists (17) and lipid extraction by Budde (18). Total dietary fiber content was determined according to Prosky et al. (19). Nitrogen free extract (NFE) was calculated using the following equation: 100% – (crude protein% + acid-hydrolyzed fat% + TDF% + ash%). Monosaccharide composition of the yeast cell wall fraction was analyzed according to Bourquin et al. (20).

Statistical Analysis

For the baseline samples, data were analyzed using the Mixed Models procedure of SAS (version 9.4; SAS Institute, Cary, NC, United States) to test the main effect of treatment, with treatment as a fixed effect and dog as a random effect. For post-diet transition samples, data were expressed as change from baseline and analyzed using the Mixed Models procedure of SAS (version 9.4; SAS Institute, Cary, NC, United States) as repeated measures. The main effects of treatment, diet and time as well as interactions of diet and treatment, diet and time, and treatment and time were tested, with dog as a random effect. Data are reported as means ± standard error of the mean (SEM) with statistical significance set as $P \leq 0.05$ and trends set as $0.05 < P \leq 0.10$.

RESULTS

Food Intake and Body Weight

Average daily food, energy, and nutrient intakes, including protein, fat, TDF, and NFE were not changed by yeast treatment prior to diet transition (**Table 4**). Post-transition diets affected nutrient intakes. Daily intakes of protein, fat, and energy were higher ($P < 0.01$), while daily intakes of TDF and NFE were lower ($P < 0.01$) in dogs fed the CD than those fed the HFD (**Table 5**). Average BW (13.52 kg) and body condition score (7.15/9) were not altered by yeast treatment prior to and after diet transition (**Table 4**; **Figure 1A**). Body weight was affected by diet after diet transition. Dogs consuming CD had greater decreases ($P < 0.01$) in BW than dogs consuming HFD (**Figure 1A**).

Fecal Characteristics

Fecal characteristics, including pH, score, and DM were not altered by yeast treatment prior to diet transition (**Table 6**). After diet transition, the change from baseline values for fecal characteristics were affected by diet and time, but not yeast treatment (**Figure 1**). Fecal pH decreased after diet transition. The change from baseline values for fecal pH were greater ($P < 0.01$) in dogs fed HFD than in those consuming CD. The decrease in pH on d 16 was larger ($P < 0.01$) than other days (**Figure 1B**). Fecal scores increased (wetter feces) after diet transition. The increases in fecal scores were greater ($P < 0.01$) in dogs fed HFD vs. dogs fed CD, with the largest change from baseline appearing on d 20 ($P < 0.01$) (**Figure 1C**). Fecal DM decreased after diet transition (**Figure 1D**). Dogs consuming CD had larger decreases ($P = 0.03$) in fecal DM than dogs consuming HFD.

Fecal Bacteria, IgA, and Calprotectin

Fecal *E. coli*, IgA, and calprotectin were not affected by yeast treatment prior to diet transition (**Table 6**). Dogs supplemented with yeast product tended to have higher ($P = 0.06$) fecal *C. perfringens* counts than control dogs. After diet transition, fecal bacteria were changed by diet, but not yeast treatment. Fecal *E. coli* decreased in HFD-fed dogs but increased in CD-fed dogs ($P < 0.05$; **Figure 1E**). Fecal *C. perfringens*

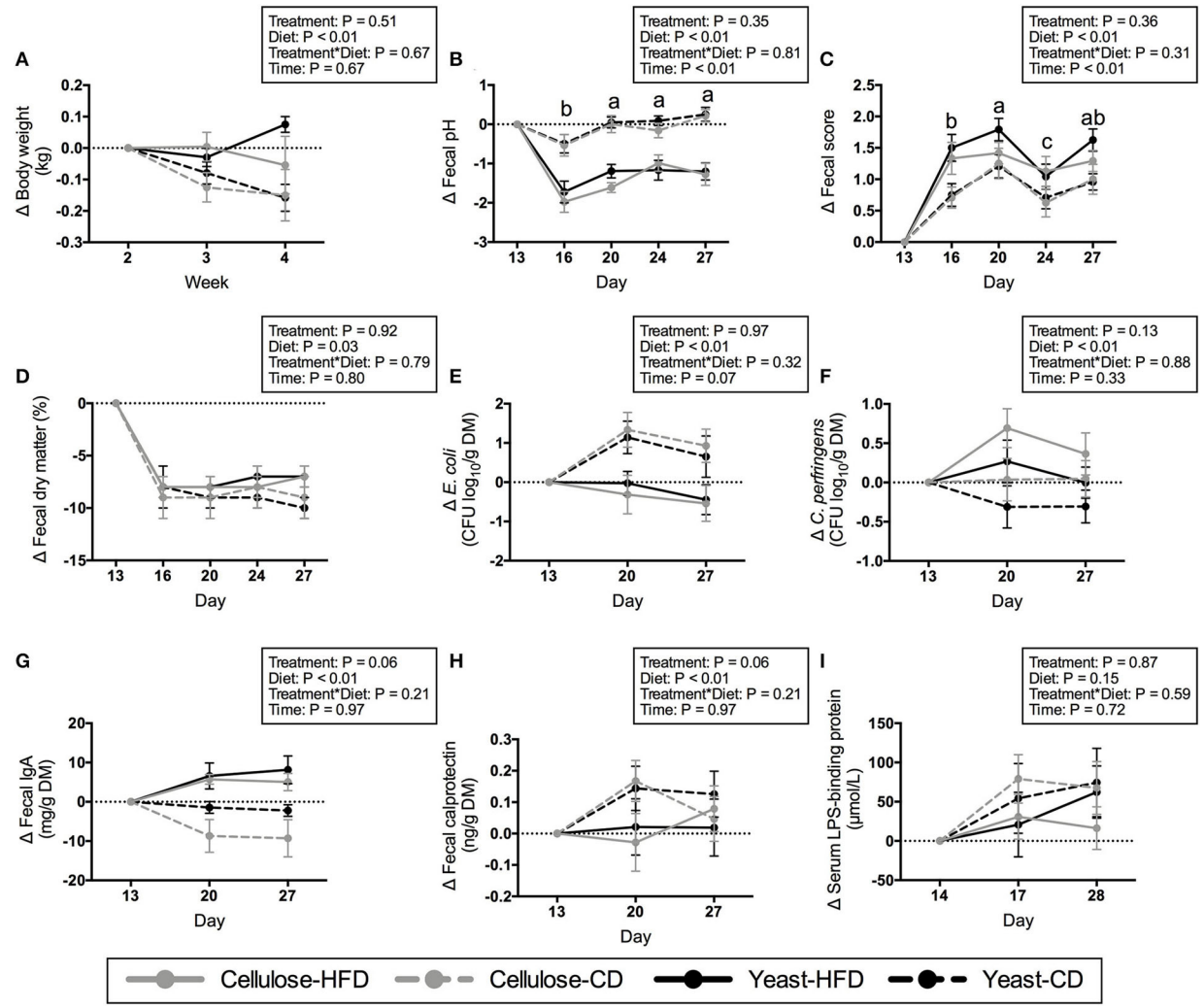

FIGURE 1 | Changes in body weight **(A)**, fecal pH **(B)**, fecal score **(C)**, fecal dry matter **(D)**, fecal *E. coli* **(E)** and *C. perfringens* **(F)** populations, fecal IgA concentration **(G)**, fecal calprotectin concentration **(H)**, and serum lipopolysaccharide-binding protein concentration **(I)** of yeast- or cellulose-supplemented dogs transitioned to a high-fiber diet (HFD) or canned diet (CD). Values represent mean ± SEM changes from baseline (day 13 or 14). [a,b,c]Mean values within a day with unlike letters were different ($P < 0.05$).

increased in dogs fed HFD, but decreased in dogs fed CD ($P < 0.01$; **Figure 1F**). The change in fecal IgA after diet transition was affected by treatment and diet. Yeast-supplemented dogs tended to have higher ($P = 0.06$) fecal IgA than the control dogs. In addition, fecal IgA increased ($P < 0.01$) in HFD-fed dogs, but decreased ($P < 0.01$) in CD-fed dogs (**Figure 1G**). Finally, fecal calprotectin increased after diet transition (**Figure 1H**). Dogs consuming CD tended to produce more ($P = 0.06$) calprotectin than dogs consuming HFD.

Serum LPS-Binding Protein

Serum LPS-binding protein concentration was not affected by yeast treatment prior to diet transition (**Table 6**). After diet transition, serum LPS-binding protein increased, but the changes from baseline were not affected by treatment or diet (**Figure 1I**).

DISCUSSION

Diet transition occurs when pet owners purchase different products, which might result in GI discomfort. Yeast products may serve as a functional ingredient in dog foods and may protect animals from digestive upset due to its benefits on GI health. Here, we investigated the effects of yeast cell wall fraction on fecal characteristics and GI tract stability in adult dogs undergoing diet transition from a dry kibble diet to a high-fiber diet or canned diet. Our findings revealed that before diet transition, all indices except for fecal *C. perfringens* counts, were not altered by yeast supplementation. After diet transition, all measurements, except for fecal IgA concentration were not changed by yeast treatment. Fecal IgA concentrations tended to increase in yeast-supplemented dogs.

TABLE 6 | Fecal characteristics, fecal bacteria counts, fecal IgA, fecal calprotectin, and serum lipopolysaccharide (LPS)-binding protein of yeast- or cellulose-supplemented dogs before diet transition (day 13 or 14)[a].

Item	Treatment		Pooled SEM	*P*-value
	Cellulose	Yeast (375 mg/d)		
Fecal characteristics				
Fecal pH	7.43	7.30	0.02	0.21
Fecal score[b]	2.15	2.08	0.03	0.69
Fecal dry matter (DM; %)	39.77	40.15	0.22	0.66
Fecal bacteria (CFU)[c]				
E. coli (log_{10}/g DM)	6.76	6.71	0.06	0.82
C. perfringens (log_{10}/g DM)	7.99	8.40	0.04	0.06
Fecal IgA (mg/g DM)	7.94	4.87	0.50	0.55
Fecal calprotectin (ng/g DM)	0.47	0.46	0.01	0.82
Serum LPS-binding protein (μmol/L)	186.58	200.74	6.52	0.44

[a] *Data are expressed as means ± pooled standard error of the mean, n = 24.*
[b] *Fecal scoring system: 1 = hard, dry pellets; small, hard mass; 2 = hard, formed, dry stool; remains firm and soft; 3 = soft, formed, and moist stool; 4 = soft, unformed stool; assumes shape of container; 5 = watery; liquid that can be poured.*
[c] *CFU, colony-forming units.*

Diet transition led to significant changes to fecal characteristics and inflammatory biomarkers. Fecal pH decreased after dogs were fed HFD, which was expected. Lower fecal pH is due primarily to greater short-chain fatty acid concentrations as a result of fiber fermentation by microbiota in the hindgut (21, 22). Fecal scores increased and fecal DM decreased when dogs were fed both HFD and CD, indicating wetter and/or looser stools. Fecal calprotectin, a protein complex present in neutrophils, monocytes and reactive macrophages, has been reported to be increased in dogs with chronic diarrhea and chronic inflammatory enteropathies (23, 24). Here, we observed increased fecal calprotectin concentrations after diet transition, which reveals a slight elevation in GI inflammation. Finally, serum LPS-binding protein concentrations were elevated after diet transition. LPS binding-protein binds to endotoxins, including LPS from Gram-negative bacteria, with their concentration being increased after endotoxemia or bacteremia (25, 26). The increased serum LPS-binding protein concentrations in this study suggested greater GI permeability (leaky gut) after diet transition.

Fecal IgA was the only parameter that tended to be affected by yeast supplementation after diet transition. Elevated secretory IgA concentrations indicate enhanced mucosal immunity. Secretory IgA in the intestine plays a role in protecting mucosal surface against enteric antigens by inhibiting colonization and invasion of pathogens as well as food antigen uptake (27, 28). This response was in agreement with previous studies that reported elevated IgA concentrations in dogs consuming yeast products, indicating enhanced mucosal immunity. Middelbos et al. (4) noted that ileal IgA concentrations tended to respond quadratically ($P = 0.09$) to yeast cell wall supplementation in adult healthy dogs, with the highest concentration coming from the 0.25% supplementation level. Swanson et al. (11) supplemented 0.25% mannanoligosaccharides (MOS), the main component of the yeast cell walls, to adult healthy dogs and observed a tendency ($P = 0.06$) of increased ileal IgA concentrations. Increased IgA concentration may possibly be explained by two major components of the yeast cell walls, beta-glucans and MOS. Beta-glucans have been shown to be an immunostimulant that modulates innate and adaptive immune responses in various animal species, including dogs (29–31). As an immunostimulant, beta-glucans induce a cascade of immune responses by binding to dectin-1 receptors that are expressed on immune cells such as macrophages, monocytes, dendritic cells and neutrophils (32). The possible mechanisms by which MOS induce immune responses include the binding of mannose receptors that are expressed on antigen-presenting cells, including macrophage and dendritic cells (33). This response is supported by findings from previous studies in dogs, broilers, and pigs whereby oral administration of MOS led to enhanced immunity (34–37).

Supplementation of yeast products have been shown to decrease potential pathogenic bacteria such as *E. coli* in previous studies where dogs were supplemented with yeast cell wall, MOS or live *S. cerevisiae* (4, 5, 37). Decreased pathogenic bacteria are believed to be due to the ability of yeast cell wall to bind and inactivate bacteria. Mannose in yeast cell walls binds to type-1 fimbriae of pathogenic bacteria and thus prevent their adhesion and colonization to the host mucosa (38). We did not observe changes to fecal *E. coli* in this study, however, which is in line with findings from Swanson et al. (11) where dogs were supplemented with MOS. Although decreased fecal *C. perfringens* was noted in cats consuming yeast cell walls (7), lower fecal *C. perfringens* was not observed in dogs supplemented with MOS or live *S. cerevisiae* (5, 11, 37). Middelbos et al. (4) reported a cubic response of fecal *C. perfringens* counts to yeast cell wall supplementation in dogs, with greatest counts being observed at the 0.25% supplementation level. This is similar to our findings in dogs supplemented with 0.2% yeast cell wall fractions, who tended to have greater fecal *C. perfringens* counts compared to the control dogs before diet transition. Fecal *C. perfringens* counts in our study were 8.0–8.4 colony-forming units (CFU) log_{10}/g DM, which is lower than those (9.5–10.0 CFU log_{10}/g DM) reported by Middelbos et al. (4). The difference could be contributed by several factors, including diet composition, dog breed studied, and plating methods used to quantify microbes. The increased fecal *C. perfringens*, however, did not negatively affect measures of GI distress. *C. perfringens* are parts of commensal microbiota in dogs and can be cultured from more than 80% of healthy dogs and diarrheic dogs (39, 40). Therefore, greater fecal *C. perfringens* counts usually do not lead to diarrhea. Canine *C. perfringens*-associated diarrhea is more likely the result of intestinal microbiota disruption, which enables sporulation of commensal *C. perfringens* and production of enterotoxin (41). It also has been shown that *C. perfringens* enterotoxin is only detected in 5–15% of healthy dogs but in 34% of diarrheic dogs (39, 40). Fecal enterotoxin concentrations were not evaluated in the current study, but

was unlikely because fecal scores were not affected by yeast cell walls.

The lack of significant effects on most intestinal health indices by yeast supplementation in this study could be due to the short treatment period used or the low supplement dosage tested. Grieshop et al. (37) observed decreased fecal *E. coli* counts in dogs supplemented with 1% MOS for 28 d. In dogs with enteropathogenic *E. coli*-induced diarrhea, supplementation of 2 g/kg BW MOS for 20 d resulted in faster recovery (42). Therefore, testing the yeast cell wall fractions with longer supplementation periods and/or higher dosages may be suggested in the future.

In conclusion, supplementation of 0.2% yeast cell wall fractions to dogs tended to increase fecal IgA concentrations, but did not affect fecal characteristics, fecal bacterial populations, or serum LPS-binding protein concentrations after an abrupt diet transition. Inclusion of yeast cell wall fractions in diets may modestly improve intestinal health for dogs undergoing diet transition.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by the University of Illinois Institutional Animal Care and Use Committee.

AUTHOR CONTRIBUTIONS

KS and RR designed the experiment. C-YL and MM performed all laboratory analyses. C-YL and MC performed the animal trial and sample collection. C-YL conducted statistical analysis and wrote the manuscript. All authors have read and approved the manuscript.

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REFERENCES

1. Gao J, Zhang HJ, Yu SH, Wu SG, Yoon I, Quigley J, et al. Effects of yeast culture in broiler diets on performance and immunomodulatory functions. *Poult Sci*. (2008) 87:1377–84. doi: 10.3382/ps.2007-00418
2. Shen YB, Piao XS, Kim SW, Wang L, Liu P, Yoon I, et al. Effects of yeast culture supplementation on growth performance, intestinal health, and immune response of nursery pigs. *J Anim Sci*. (2009) 87:2614–24. doi: 10.2527/jas.2008-1512
3. Swanson KS, Grieshop CM, Flickinger EA, Merchen NR, Fahey GC. Effects of supplemental fructooligosaccharides and mannanoligosaccharides on colonic microbial populations, immune function and fecal odor components in the canine. *J Nutr*. (2002) 132:1717–19. doi: 10.1093/jn/132.6.1717S
4. Middelbos IS, Godoy MR, Fastinger ND, Fahey GC. A dose-response evaluation of spray-dried yeast cell wall supplementation of diets fed to adult dogs: effects on nutrient digestibility, immune indices, and fecal microbial populations. *J Anim Sci*. (2007) 85:3022–32. doi: 10.2527/jas.2007-0079
5. Stercova E, Kumprechtova D, Auclair E, Novakova J. Effects of live yeast dietary supplementation on nutrient digestibility and fecal microflora in beagle dogs. *J Anim Sci*. (2016) 94:2909–18. doi: 10.2527/jas.2016-0584
6. Lin C-Y, Alexander C, Steelman AJ, Warzecha CM, De Godoy MRC, Swanson KS. Effects of a *Saccharomyces cerevisiae* fermentation product on fecal characteristics, nutrient digestibility, fecal fermentative end-products, fecal microbial populations, immune function, and diet palatability in adult dogs. *J Anim Sci*. (2019) 97:1586–99. doi: 10.1093/jas/skz064
7. Santos JPF, Aquino AA, Glória MBA, Avila-Campos MJ, Oba PM, Santos K de M, et al. Effects of dietary yeast cell wall on faecal bacteria and fermentation products in adult cats. *J Anim Physiol Anim Nutr*. (2018) 102:1091–101. doi: 10.1111/jpn.12918
8. Zhang AW, Lee BD, Lee SK, Lee KW, An GH, Song KB, et al. Effects of yeast (*Saccharomyces cerevisiae*) cell components on growth performance, meat quality, and ileal mucosa development of broiler chicks. *Poult Sci*. (2005) 84:1015–21. doi: 10.1093/ps/84.7.1015
9. Wu X, Vallance BA, Boyer L, Bergstrom KSB, Walker J, Madsen K, et al. *Saccharomyces boulardii* ameliorates *Citrobacter rodentium*-induced colitis through actions on bacterial virulence factors. *Am J Physiol Liver Physiol*. (2008) 294:295–306. doi: 10.1152/ajpgi.00173.2007
10. Generoso SV, Viana M, Santos R, Martins FS, Machado JAN, Arantes RME, et al. *Saccharomyces cerevisiae* strain UFMG 905 protects against bacterial translocation, preserves gut barrier integrity and stimulates the immune system in a murine intestinal obstruction model. *Arch Microbiol*. (2010) 192:477–84. doi: 10.1007/s00203-010-0574-8
11. Swanson KS, Grieshop CM, Flickinger EA, Bauer LL, Healy H-P, Dawson KA, et al. Supplemental fructooligosaccharides and mannanoligosaccharides influence immune function, ileal and total tract nutrient digestibilities, microbial populations and concentrations of protein catabolites in the large bowel of dogs. *J Nutr*. (2002) 132:980–9. doi: 10.1093/jn/132.5.980
12. Zhu C, Wang L, Wei S, Chen Z, Ma X, Zheng C, et al. Effect of yeast *Saccharomyces cerevisiae* supplementation on serum antioxidant capacity, mucosal sIgA secretions and gut microbial populations in weaned piglets. *J Integr Agric*. (2017) 16:2029–37. doi: 10.1016/S2095-3119(16)61581-2
13. Laflamme DP. Development and validation of a body condition score system for dogs: a clinical tool. *Canine Pract*. (1997) 25:10–5.
14. Association of American Feed Control Officials (AAFCO). *Official Publication*. Champaign, IL: AAFCO (2017).
15. Vilson Å, Hedhammar Å, Reynolds A, Spears J, Satyaraj E, Pelker R, et al. Immunoglobulins in dogs: correspondence and maturation in 15 litters of German shepherd dogs and their dams. *Vet Rec Open*. (2016) 3:e000173. doi: 10.1136/vetreco-2016-000173
16. Association of Official Analytical Chemists (AOAC). *Official Methods of Analysis*. 17th ed. Gaithersburg, MD: Association of Official Analytical Chemists (2006).
17. American Association of Cereal Chemists (AACC). *Approved Methods*. 8th ed. St Paul, MN: American Association of Cereal Chemists (1983).
18. Budde EF. The determination of fat in baked biscuit type dog foods. *J Assoc Off Agric Chem*. (1952) 35:799–805. doi: 10.1093/jaoac/35.3.799
19. Prosky L, Asp NG, Furda I, DeVries JW, Schweizer TF, Harland BF. Determination of total dietary fiber in foods and food products: collaborative study. *J Assoc Off Anal Chem*. (1985) 68:677–9. doi: 10.1093/jaoac/68.4.677

20. Bourquin LD, Garleb KA, Merchen NR, Fahey GC. Effects of intake and forage level on site and extent of digestion of plant cell wall monomeric components by sheep. *J Anim Sci*. (1990) 68:2479–95. doi: 10.2527/1990.6882479x
21. Anderson JW, Chen WJL. Plant fiber. Carbohydrate and lipid metabolism. *Am J Clin Nutr*. (1979) 32:346–63. doi: 10.1093/ajcn/32.2.346
22. Cummings JH. Short chain fatty acids in the human colon. *Gut*. (1981) 22:763–79. doi: 10.1136/gut.22.9.763
23. Grellet A, Heilmann RM, Lecoindre P, Feugier A, Day MJ, Peeters D, et al. Fecal calprotectin concentrations in adult dogs with chronic diarrhea. *Am J Vet Res*. (2013) 74:706–11. doi: 10.2460/ajvr.74.5.706
24. Heilmann RM, Berghoff N, Mansell J, Grützner N, Parnell NK, Gurtner C, et al. Association of fecal calprotectin concentrations with disease severity, response to treatment, and other biomarkers in dogs with chronic inflammatory enteropathies. *J Vet Intern Med*. (2018) 32:679–92. doi: 10.1111/jvim.15065
25. Hudgins LC, Parker TS, Levine DM, Gordon BR, Saal SD, Jiang XC, et al. A single intravenous dose of endotoxin rapidly alters serum lipoproteins and lipid transfer proteins in normal volunteers. *J Lipid Res*. (2003) 44:1489–98. doi: 10.1194/jlr.M200440-JLR200
26. Schumann RR. Old and new findings on lipopolysaccharide-binding protein: a soluble pattern-recognition molecule. *Biochem Soc Trans*. (2011) 39:989–93. doi: 10.1042/BST0390989
27. Brandtzaeg P. History of oral tolerance and mucosal immunity. *Ann N Y Acad Sci*. (1996) 778:1–27. doi: 10.1111/j.1749-6632.1996.tb21110.x
28. Corthesy B, Kraehenbuhl JP. Antibody-mediated protection of mucosal surfaces. *Curr Top Microbiol Immunol*. (1999) 236:93–111. doi: 10.1007/978-3-642-59951-4_6
29. Volman JJ, Ramakers JD, Plat J. Dietary modulation of immune function by β-glucans. *Physiol Behav*. (2008) 94:276–84. doi: 10.1016/j.physbeh.2007.11.045
30. Samuelsen ABC, Schrezenmeir J, Knutsen SH. Effects of orally administered yeast-derived beta-glucans: a review. *Mol Nutr Food Res*. (2014) 58:183–93. doi: 10.1002/mnfr.201300338
31. Stier H, Ebbeskotte V, Gruenwald J. Immune-modulatory effects of dietary yeast beta-1,3/1,6-D-glucan. *Nutr J*. (2014) 13:38. doi: 10.1186/1475-2891-13-38
32. Brown GD, Herre J, Williams DL, Willment JA, Marshall ASJ, Gordon S. Dectin-1 mediates the biological effects of β-glucans. *J Exp Med*. (2003) 197:1119–24. doi: 10.1084/jem.20021890
33. Sheng K-C, Pouniotis DS, Wright MD, Tang CK, Lazoura E, Pietersz GA, et al. Mannan derivatives induce phenotypic and functional maturation of mouse dendritic cells. *Immunology*. (2006) 118:372–83. doi: 10.1111/j.1365-2567.2006.02384.x
34. Shashidhara RG, Devegowda G. Effect of dietary mannan oligosaccharide on broiler breeder production traits and immunity. *Poult Sci*. (2003) 82:1319–25. doi: 10.1093/ps/82.8.1319
35. Che TM, Johnson RW, Kelley KW, Dawson KA, Moran CA, Pettigrew JE. Effects of mannan oligosaccharide on cytokine secretions by porcine alveolar macrophages and serum cytokine concentrations in nursery pigs. *J Anim Sci*. (2012) 90:657–68. doi: 10.2527/jas.2011-4310
36. Gmez-Verduzco G, Cortes-Cuevas A, Lpez-Coello C, Vila-González E, Nava GM. Dietary supplementation of mannan-oligosaccharide enhances neonatal immune responses in chickens during natural exposure to Eimeria spp. *Acta Vet Scand*. (2009) 51:11. doi: 10.1186/1751-0147-51-11
37. Grieshop CM, Flickinger EA, Bruce KJ, Patil AR, Czarnecki-Maulden GL, Fahey GC. Gastrointestinal and immunological responses of senior dogs to chicory and mannan-oligosaccharides. *Arch Anim Nutr*. (2004) 58:483–93. doi: 10.1080/00039420400019977
38. Firon N, Ofek I, Sharon N. Carbohydrate specificity of the surface lectins of *Escherichia coli*, *Klebsiella pneumoniae*, and *Salmonella typhimurium*. *Carbohydr Res*. (1983) 120:235–49. doi: 10.1016/0008-6215(83)88019-7
39. Marks SL, Kather EJ, Kass PH, Melli AC. Genotypic and phenotypic characterization of *Clostridium perfringens* and *Clostridium difficile* in diarrheic and healthy dogs. *J Vet Intern Med*. (2002) 16:533–40. doi: 10.1111/j.1939-1676.2002.tb02383.x
40. Weese JS, Staempfli HR, Prescott JF, Kruth SA, Greenwood SJ, Weese HE. The roles of *Clostridium difficile* and enterotoxigenic *Clostridium perfringens* in diarrhea in dogs. *J Vet Intern Med*. (2001) 15:374–8. doi: 10.1111/j.1939-1676.2001.tb02332.x
41. Marks SL, Rankin SC, Byrne BA, Weese JS. Enteropathogenic bacteria in dogs and cats: diagnosis, epidemiology, treatment, and control. *J Vet Intern Med*. (2011) 25:1195–208. doi: 10.1111/j.1939-1676.2011.00821.x
42. Gouveia EMMF, Silva IS, Nakazato G, Onselem VJV, Corrêa RAC, Araujo FR, et al. Action of phosphorylated mannanoligosaccharides on immune and hematological responses and fecal consistency of dogs experimentally infected with enteropathogenic *Escherichia coli* strains. *Brazilian J Microbiol*. (2013) 44:499–504. doi: 10.1590/S1517-83822013000200027

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Rabbit Carcasses for Use in Feline Diets: Amino Acid Concentrations in Fresh and Frozen Carcasses With and Without Gastrointestinal Tracts

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Whole-prey diets for exotic feline species are common, and this practice has also increased in popularity for domestic cats. However, prior analyses of prey indicate possible essential amino acid inadequacy, and dilated cardiomyopathy from taurine deficiency was reported in cats fed whole ground rabbit. Crude protein, body water, and amino acid concentrations were evaluated in fresh and frozen ground rabbits with ($n = 10$) or without ($n = 10$) gastrointestinal tracts. Amino acids were greater in fresh samples without gastrointestinal tracts ($p < 0.05$) except taurine, glycine, and cysteine. When normalized for protein content, only glutamate, alanine, methionine, isoleucine, tyrosine, lysine, histidine, and arginine were greater in fresh rabbits without gastrointestinal tracts (g/16 g N basis; $p < 0.05$). Freezing at −18°C for 30 days had no effect on crude protein or body water content. After freezing, only methionine was lower and only proline was higher when gastrointestinal tracts were omitted (g/16 g N basis; $p < 0.05$). Regardless, all essential amino acids except taurine exceeded Association of American Feed Control Officials and National Research Council nutrient recommendations for all feline life stages. In contrast, there was minimal impact of treatment on taurine concentrations. However, although feline taurine requirements for prey and other raw or fresh food diets remain undefined, none of the rabbit samples met any recommendation for taurine concentrations for commercial canned or dry extruded diets, ranging from 20 to 90% of the minimum values. Taurine supplementation is recommended when feeding rabbit to cats. Determination of taurine requirements of cats fed whole-prey diets is warranted.

Keywords: rabbit, amino acids, nutrition, feline, taurine, carcass, rabbit (lagomorph), prey

INTRODUCTION

The domestic cat (*Felis silvestris catus*) has been a successful hunter throughout its evolution and typically consumes a wide range of small prey (1–4). Accordingly, compared to dogs, cats have increased protein and amino acid (AA) requirements, differences in carbohydrate metabolism, and certain nutrients with limited endogenous synthesis remain essential (5). In particular, taurine deficiency can lead to dilated cardiomyopathy (DCM), reproductive abnormalities, and central retinal degeneration in both domestic and exotic felids (5–7). Whole-prey diets are increasing in

popularity for domestic cats (8, 9) and are common for captive exotic felids. However, taurine requirements, known to vary by diet type, remain undefined for whole-prey diets. Greater than 50% of required dietary taurine replaces taurine degraded by intestinal microbes (5); however, many dietary and processing factors influence the amount required (10–20). Rabbits are a popular whole-prey item; however, information on nutrient composition is limited or conflicting (8, 9, 21–24), and DCM secondary to taurine deficiency was reported in cats fed frozen whole ground raw rabbit (25). Additionally, analyses of various frozen whole-prey samples indicate possible insufficiencies in select essential AA (9). It is unknown how post-slaughter handling (i.e., dressing carcasses, grinding, freezer storage) affects nutrient concentration or availability of nutrients, including taurine. When an entire carcass is ground, taurine-rich organs and muscles are exposed to GIT (gastrointestinal tract) contents containing potentially taurine-degrading bacteria. Therefore, taurine concentrations could be affected by exposure to taurine-degrading GIT microbes and subsequent freezer storage. The purpose of this study was to evaluate the concentrations of essential AA in fresh, skinned ground rabbits with and without GIT and then determine if these concentrations are affected by freezing for 30 days. It was hypothesized that the presence of the GIT would reduce taurine concentrations and that these effects would be exacerbated by freezer storage.

MATERIALS AND METHODS

Experimental procedures were approved by the University of California-Davis Institutional Animal Care and Use Committee (Protocol #19040).

Rabbits

Twenty-one freshly slaughtered 45- to 65-day-old Californian/New Zealand cross rabbit carcasses, raised with intention for sale as meat and consuming exclusively commercial rabbit pellets (A.L. Gilbert Farmer's Best Feed Rabbit Pellets), were purchased from a local producer (Penryn Rabbit Farm; Penryn, CA) in three groups of five and one group of six. The carcasses were each randomly assigned a number; placed into a sealed, water-tight plastic bag; and stored in a cooler on ice until same-day processing. Rabbit carcasses were individually weighed before they were skinned in the traditional manner (removal of pelt with ears and feet below carpal/tarsal joints) and then reweighed. Ten rabbits were eviscerated (removing the GIT and urinary bladder), then reweighed, and 10 rabbits were left intact. One additional rabbit was dissected for individual tissue samples.

Processing and Analysis

Ten skinned, eviscerated rabbits (without GIT) and 10 skinned, intact rabbits (with GIT) were ground (including bones, head, internal organs) and mixed repeatedly using a Weston #22 meat grinder with 7 and 4.5 mm grinder plate until visually homogenous. The ground mixture from each rabbit was divided into two roughly equal portions by volume and placed into labeled, sealed commercial freezer bags. One of these was stored at −80°C for long-term preservation. Several sections were randomly removed from throughout the remaining bag (visually estimated to be about a palm-sized amount total), mixed, and checked for visual homogeneity. From this, aliquots (range 19.5–24.1 g) were distributed into duplicate labeled Falcon tubes. Three separated GITs with contents were homogenized as much as possible using a blender (after cutting the GITs into small pieces) and then sampled into aliquots as described above (range 16.2–17.1 g). In addition, entire organs (brain, heart, lung, liver, kidney) and thigh muscles were collected from a separate rabbit.

One set of samples was sealed with a cap and stored in a freezer (−18°C for 30 days; aliquots of 10 rabbits with and 10 without GIT in duplicate, and aliquots of three separated GIT in duplicate), and one set (as above plus entire organ samples) was kept for immediate processing.

For the first step of processing, homogenized sample dry matter (DM) was determined by freeze-drying the entire duplicate samples stored in Falcon tubes after overnight storage at −80°C using a SAVANT SpeedVac Concentrator SVC200H with Refrigerated Condensation Trap RT490) for 24–48 h until constant weight was achieved. Freeze-dried samples were then finely ground and well mixed using a standard coffee bean grinder. Total nitrogen (N) concentration of the freeze-dried samples (∼1 g of each was submitted in a closed, labeled centrifuge tube) from fresh and frozen ground rabbits and individual organs of one additional rabbit (only 0.1 g submitted from these) was determined by the UC Davis Analytical Laboratory using AOAC Official Method 990.03: protein (crude) in animal feed, combustion method (26). Approximately 0.2 g was removed from each ground, freeze-dried sample for AA analysis. From this, three 0.05-g aliquots were removed. Analysis for 18 AA, including all essential feline AA except tryptophan, was performed on a Biochrom 30 AA Analyzer (Cambridge, UK) using methods described elsewhere (20) and including determination of methionine and cystine by use of performic acid oxidation with acid hydrolysis (AOAC Official Method 944.12: Amino acids in feeds) (27).

Statistical Analysis

Statistical analysis was performed using computer software (Microsoft Excel 2011, Microsoft Corporation) and web-based applications (Social Science Statistics. Mann–Whitney *U* Test Calculator and Wilcoxon Signed Rank Test Calculator). Unpaired groups (rabbits with and without GIT) were compared using Mann–Whitney tests and paired groups (before and after freezing for 30 days) were compared using Wilcoxon signed rank tests. Probability of $p \leq 0.05$ was accepted as statistically significant. Concentrations of AA are reported as both measured % DM and as g per 16 g N to allow for standardization relative to apparent protein concentration based on analyzed N.

RESULTS

The 20 rabbit carcasses collected in the present study had a median weight of 1.67 kg (range 1.34–2.23 kg). There was no difference in predressed body weights (BW) between rabbits left intact and those eviscerated ($p = 0.67448$; **Table 1**). The 10

TABLE 1 | Body weights of fresh carcasses and body water, nitrogen (N), and crude protein (CP) concentrations of ground rabbit samples analyzed fresh and after freezing for 30 days at −18°C, with and without gastrointestinal tracts plus contents (GIT); $n = 10$ each group.

	Fresh		Frozen	
	Rabbits with GIT	**Rabbits without GIT**	**Rabbits with GIT**	**Rabbits without GIT**
	Median (range)			
Weight, as is basis	1,675 (1,368–2,163)	1,671 (1,336–2,231)	–	–
Body water	73.85 (73.94–73.99)*	71.37 (69.20–72.56)*	73.76 (71.89–75.33)*	70.95 (69.11–72.86)*
% N, DM basis	9.21 (8.53–10.16)	10.05 (9.37–10.72)	9.00 (8.46–10.57)	10.01 (9.39–10.71)
% CP, DM basis	57.54 (53.31−63.5)[a]	62.82 (58.56–67.00)[a]	56.25 (52.88–66.06)[b]	62.57 (58.69–66.94)[b]

*Significant comparisons of with and without GIT within each treatment of fresh vs. frozen ($p < 0.05$).
[a]Significant comparisons between fresh and frozen samples with GIT ($p < 0.05$).
[b]Significant comparisons between fresh and frozen samples without GIT ($p < 0.05$).

TABLE 2 | Amino acid concentrations of ground rabbit samples analyzed fresh and after freezing for 30 days at −18°C, with and without gastrointestinal tracts plus contents (GIT) and normalized for crude protein content; $n = 10$ each group.

	Fresh		Frozen	
Amino acid	**Rabbits with GIT**	**Rabbits without GIT**	**Rabbits with GIT**	**Rabbits without GIT**
	g/16 g N; median (range)			
Taurine	0.10 (0.075–0.145)	0.11 (0.087–0.129)	0.10 (0.088–0.147)	0.11 (0.094–0.125)
L-Asp	8.69 (7.49–9.36)	8.92 (8.43–9.95)	8.69 (8.08–9.47)	8.81 (8.10–9.37)
L-Thr	4.42 (3.32–5.32)[a]	4.68 (4.13–5.13)	4.66 (4.20–5.07)[a]	4.82 (4.32–5.41)
L-Ser	3.26 (2.02–4.36)[a]	3.59 (2.54–3.84)	3.81 (2.92–4.37)[a]	3.85 (3.54–4.12)
L-Glu	14.07 (12.46–15.22)*	14.77 (14.03–16.50)*[b]	13.91 (12.94–15.03)	14.29 (13.26–15.30)[b]
Gly	6.33 (5.96–7.41)[a]	6.36 (5.79–6.60)	5.66 (5.16–6.49)[a]	6.10 (5.58–6.47)
L-Ala	5.63 (5.46–6.21)*	5.87 (5.65–6.39)*	5.57 (5.21–6.10)	5.81(5.33–6.38)
L-Val	4.84 (4.04–4.99)	4.86 (4.56–5.47)	4.81 (4.32–5.18)	4.83 (4.39–5.51)
L-Cys	0.92 (0.70–1.41)[a]	1.02 (0.67–1.20)[b]	1.37 (1.02–1.69)[a]	1.58 (1.49–2.34)[b]
L-Met	2.16 (1.84–2.27)*	2.28 (2.17–2.57)*[b]	2.09 (1.96–2.31)*	1.94 (1.65–2.24)*[b]
L-Ile	4.00 (3.28–4.19)*	4.15 (3.91–4.66)*[b]	3.86 (3.56–4.16)	3.84 (3.41–4.08)[b]
L-Leu	7.71 (6.50–8.22)	8.01 (7.49–9.01)[b]	7.63 (6.96–8.25)	7.22 (6.53–7.94)[b]
L-Tyr	3.22 (2.65–3.38)*[a]	3.36 (3.18–3.78)*[b]	3.05 (2.81–3.18)[a]	2.98 (2.74–3.35)[b]
L-Phe	4.16 (3.54–4.48)	4.25 (3.99–4.75)[b]	3.93 (3.54–4.20)	3.90 (3.59–4.40)[b]
L-Lys	7.68 (6.41–8.22)*	8.14 (7.70–9.15)*[b]	7.34 (6.77–7.93)	7.64 (6.75–8.22)[b]
L-His	2.55 (2.15–2.78)*[a]	2.69 (2.48–2.99)*	2.39 (1.94–2.70)[a]	2.56 (2.25–2.91)
L-Arg	6.35 (5.93–7.01)*	6.76 (6.48–7.41)*[b]	6.27 (5.45–6.85)	6.32 (5.87–6.64)[b]
L-Pro	4.90 (4.70–5.44)[a]	4.97 (4.71–5.22)[b]	12.93 (4.34–15.06)*[a]	15.41 (13.86–16.28)*[b]

*Significant comparisons of with and without GIT within each treatment of fresh vs. frozen ($p < 0.05$).
[a]Significant comparisons between fresh and frozen samples with GIT ($p < 0.05$).
[b]Significant comparisons between fresh and frozen samples without GIT ($p < 0.05$).

removed GITs had a median weight of 410 g as is (range 282–496 g as is), which represented 21.1% of the total predressed BW (including pelt and feet; range 15.7–26.8%). Average of duplicate analyses of the 3 samples of only GIT plus contents showed relatively higher water content (79.4–79.7% water for fresh samples and 78.9–80.1% for frozen) and relatively low crude protein (CP) concentrations compared to samples of rabbits with or without GIT (36.9–39.% CP DM for fresh samples and 35.9–37.8% CP DM for frozen).

When AA concentrations of fresh ground rabbits with GITs were compared to those without GITs on a g/16 g N basis, 8 of 18 AA (glutamate, alanine, methionine, isoleucine, tyrosine, lysine, histidine, and arginine), five of which are essential, were greater in samples without GITs (**Table 2**; $p < 0.05$). However, when compared on a % DM basis, all 18 AA concentrations were higher in fresh samples without GITs, 15 of which were significantly greater (all except taurine, glycine, and cysteine; **Table 3**; $p < 0.05$). Taurine concentration did not greatly differ between groups with or without GITs and was only significantly higher in samples without GITs when expressed as % DM ($p = 0.046$).

AA concentrations, water, and CP content, and fresh organ weights of kidney, thigh muscle, heart, brain, lung, and liver from the single additional rabbit are presented in **Supplementary Table 1**.

TABLE 3 | Amino acid concentrations of ground rabbit samples analyzed fresh and after freezing for 30 days at −18°C, with and without gastrointestinal tracts plus contents (GIT) and normalized for water content; $n = 10$ each group.

Amino acid	Fresh		Frozen	
	Rabbits with GIT	Rabbits without GIT	Rabbits with GIT	Rabbits without GIT
	% DM; median (range)			
Taurine	0.05 (0.04–0.09)	0.07 (0.06–0.08)	0.05 (0.05–0.09)*	0.07 (0.06–0.07)*
L-Asp	4.91 (4.40–5.46)*	5.50 (5.35–6.01)*	4.85 (4.56–5.96)*	5.43 (5.05–6.20)*
L-Thr	2.55 (1.95–2.95)*[a]	2.98 (2.50–3.14)*	2.60 (2.34–3.20)*[a]	2.99 (2.75–3.58)*
L-Ser	1.85 (1.10–2.32)*[a]	2.27 (1.54–2.47)*	2.09 (1.63–2.56)*[a]	2.32 (2.14–2.67)*
L-Glu	8.03 (7.31–9.03)*	9.16 (8.79–9.97)*	7.86 (7.31–9.57)*	8.82 (7.95–10.12)*
Gly	3.63 (3.32–4.35)[a]	3.97 (3.39–4.36)	3.25 (2.87–3.73)*[a]	3.75 (3.39–4.10)*
L-Ala	3.27 (3.05–3.68)*	3.67 (3.44–3.93)*	3.16 (2.95–3.71)*	3.60 (3.40–3.97)*
L-Val	2.70 (2.37–2.92)*	3.04 (2.89–3.30)*	2.71 (2.44–3.24)*	2.99 (2.58–3.44)*
L-Cys	0.53 (0.41–0.75)[a]	0.64 (0.41–0.76)[b]	0.76 (0.57–1.11)*[a]	0.99 (0.92–1.38)*[b]
L-Met	1.22 (1.08–1.44)*	1.43 (1.35–1.56)*[b]	1.17 (1.11–1.52)	1.22 (0.97–1.34)[b]
L-Ile	2.24 (1.93–2.53)*	2.60 (2.43–2.81)*[b]	2.18 (2.01–2.68)	2.36 (2.06–2.69)[b]
L-Leu	4.39 (3.81–4.76)*	4.96 (4.80–5.45)*[b]	4.33 (3.94–5.16)	4.46 (3.83–5.19)[b]
L-Tyr	1.78 (1.55–2.06)*[a]	2.10 (2.04–2.29)*[b]	1.66 (1.59–2.05)[a]	1.84 (1.65–2.15)[b]
L-Phe	2.38 (2.08–2.62)*[a]	2.65 (2.57–2.87)*[b]	2.20 (2.00–2.63)*[a]	2.44 (2.12–2.84)*[b]
L-Lys	4.35 (3.76–4.92)*	5.05 (4.79–5.53)*[b]	4.12 (3.83–5.23)*	4.69 (3.96–5.44)*[b]
L-His	1.47 (1.26–1.66)*	1.71 (1.61–1.81)*	1.36 (1.11–1.58)*	1.60 (1.44–1.82)*
L-Arg	3.73 (3.37–4.20)*	4.22 (3.97–4.52)*[b]	3.57 (3.11–4.15)*	3.88 (3.49–4.40)*[b]
L-Pro	2.85 (2.60–3.35)*[a]	3.13 (2.76–3.45)*[b]	7.46 (2.37–8.83)*[a]	9.49 (8.60–10.17)*[b]

*Significant comparisons of with and without GIT within each treatment of fresh vs. frozen ($p < 0.05$).
[a]Significant comparisons between fresh and frozen samples with GIT ($p < 0.05$).
[b]Significant comparisons between fresh and frozen samples without GIT ($p < 0.05$).

DISCUSSION

The presence or absence of the GIT strongly influenced many of the findings in the current study. The differential dilutional effect of the GIT on the results when normalized for CP simply reflects that the GITs contain much less protein overall compared to the entire carcass. In addition, the effect of the GITs with their contents suggests that they contain lower concentrations of most AA and/or differences in concentrations relative to total N. The contribution of nonprotein N present in GIT may lead to further overestimates of CP when using the standard conversion factor of 6.25. For example, although samples from hindgut fermenters, such as rabbits and horses, were not assessed, a previous study of cattle, pig, and chicken manure reported exact conversion values based on AA composition of 4.78–5.36 (28). In the present study, AA were also diluted on a DM basis in the samples with GIT. Although AA concentrations were always higher in samples without GITs, the specific AA that were apparently affected by GIT inclusion differed between the fresh and frozen groups. In the frozen group, three AA showed significant differences between samples with and without GIT (taurine, glycine, and cysteine), which were not apparent in fresh samples, and in the fresh group, four AA were significantly higher in samples without GITs but lost this effect after freezing (methionine, isoleucine, leucine, and tyrosine). This may reflect differential effects of freezer storage on individual AA or simply variability among samples. When normalized for protein content and compared on a g/16 g N basis, there were fewer differences in frozen vs. fresh samples. The AA concentrations of frozen samples with and without GITs were surprisingly stable; only methionine was lower and proline was higher in samples without GITs.

Freezing did not impact body water or CP content but appeared to have an effect on some AA when compared to fresh samples. Overall, cysteine, proline, glycine, and histidine followed the same pattern of change pre- and post-freezing in the samples with and without GITs, suggesting that these are likely affected by one or more aspects of handling or storage universal to all samples (i.e., freezing). However, threonine, serine, glycine, and histidine changed concentrations between fresh and frozen samples with GITs but not between those without, indicating some impact of the presence of the GIT and its contents. However, only threonine and histidine are essential AA. Surprisingly, seven other AA decreased in frozen samples of ground rabbit without GITs, indicating differential effects of freezing with or without the presence of the GIT. Likewise, when compared on % DM, for rabbits ground with GITs and for those without GITs, the findings were very similar except for a decrease in phenylalanine and no change in histidine for rabbits with GITs (**Table 2**). For those without GITs, the pattern of change was the same whether in g/16 g N or a percentage basis except that glutamate was not different.

Although glycine (on a g/16 g N basis) was also not different between groups with or without GITs when either fresh or frozen, it was higher in fresh samples with GITs compared to frozen. Glycine concentrations of gut contents are likely influenced by the exclusive conjugation of bile acids in the rabbit with this

AA (29), depending on the presence and quantity of bile acids in the sample. The stability of glycine as a bile conjugate vs. when present in peptides and proteins has not been described in the literature to the author's knowledge, but it is possible that degradation of glycine in GIT contents during freezer storage could explain the differences seen in the present study. As already described, the apparent dilutional effect of GIT contents may also have played a role as especially evident on an absolute basis.

Although a pattern was not consistently obvious, it appeared that AA concentrations of rabbits with GITs trended toward the high end of the range for the rabbit with the smallest proportional GIT plus contents and trended toward the lower end of the range for the rabbit with the largest GIT plus contents (as % BW, DM basis). Assuming the majority of GIT weight is from contents, this further indicates a dilutional effect from the GIT contents, tempered by the AA concentrations of the GIT itself.

Taurine is a sulfur-containing beta-AA vital for maintaining normal retinal function (30), reproduction (31–33), and cardiac function (6). Cats obligatorily use taurine for bile acid conjugation (34), but have inadequate endogenous synthesis, making overall production insignificant compared to fecal losses (35). Many animal tissues (and presumably the evolutionary feline diet) contain high concentrations of taurine (20). Dietary taurine requirements in cats largely depend on the extent of losses associated with imperfect entero-hepatic recycling, which are further affected by microbial degradation in the gut as well as multiple dietary factors (10–20). Both the National Research Council (NRC) and the Association of American Feed Control Officials (AAFCO) recommend minimum taurine concentration for all feline life stages at 0.1% DM for extruded kibble diets; however, this is higher for canned diets (0.17 and 0.2% DM per NRC and AAFCO, respectively) (36, 37).

When compared to dietary concentration guidelines for all feline life stages per NRC and AAFCO (36, 37), all samples well exceeded the recommendations for all measured essential AA regardless of processing with or without GITs or whether measured fresh or post-freezing with the notable exception of taurine, which was an unexpected finding. In fact, median taurine concentrations in all four groups provided only 50–70% of the recommended 0.1% DM for extruded kibble diets and much lower compared with that for canned diets (0.17–0.2% DM). None of the whole ground rabbit samples met any recommendation for taurine concentrations for commercial diets (**Table 2**). Some samples would have met the NRC recommended minimum for "highly digestible purified diets" (0.04–0.053% DM depending on life stage) (37); however, these diets are typically only used in research settings, generally provide taurine in the crystalline form, and are likely of limited relevance to the needs of pet cats eating other types of diets. Although the requirement for taurine of cats consuming prey remains unknown, it seems unlikely that it would be less than that for commercial kibble diets due to the indigestible nature of certain components of the carcass. For example, dietary fiber plays a role in feline taurine needs (19), and several lines of evidence support that fur, collagen, or other indigestible components of prey carcasses function similarly to fiber (38–40). Further, diet type affects taurine metabolism (10, 11, 13, 18, 41), and previous studies in other species note differences in the gut microbial population with changes to feed texture (42, 43).

We had expected low taurine concentrations in rabbit GITs and that there would be, therefore, a strong effect of lower concentrations in rabbits ground with GITs when normalized for CP content, but this prediction was not supported by our findings. The ingredient list of the rabbit feed used by the producer did not include a source of purified taurine (**Supplementary Table 2**). Further, the primary components of rabbit feed are of plant origin and are not expected to contain taurine. We had also hypothesized that the presence of the GIT and exposure to taurine-degrading gut microbes would result in decreased concentrations in those samples; however, this was not the case. Perhaps prompt freezer storage was sufficient to interrupt microbial action, together with limited and controlled thawing time prior to analysis. This suggests that changes in other AA concentrations may occur by mechanisms other than microbial action or that there are other variables not yet elucidated.

Diet and age of rabbits also affect AA concentrations although effect on taurine was previously unknown (44, 45). A more recent study obtained rabbits (stillborn, 30–45 days of age, and >65 days of age) as frozen entire carcasses and then determined AA concentrations as well as AA digestibility in lyophilized, ground samples using the precision-fed cecectomized rooster assay (9). Those authors reported taurine concentrations that were well below or just meeting the lowest NRC and AAFCO recommendations for commercial feline diets (0.1% DM) in the 30- to 45- (0.01% DM) and the >65-day-old rabbits (0.1% DM); however, the stillborn rabbits, which came from a different source, were much higher in taurine content (0.29% DM) (9). The higher reported value for the stillborn rabbits is inconsistent with our findings (range 0.04–0.09% DM) as well as with previously published taurine concentrations for rabbits. Rabbits with only 0.06% taurine DM (measured from ground subsamples) were fed intact (not ground, not reported if previously frozen) to 10 cheetahs for 4 weeks, and normal serum taurine concentrations were maintained during this relatively short time (24); however, ideally, concurrent plasma and whole blood taurine measurements are preferred over serum due to potential variability in taurine content of serum secondary to sample-handling factors, such as clotting times and separation methods. Taurine deficiency leading to DCM was previously reported in 70% of 22 young domestic cats fed entire ground rabbit (unskinned, undressed) for a longer period of 10 months (25). The age of the rabbits and taurine concentrations were not reported although the authors did report low dietary vitamin E, which can contribute to taurine losses during processing (46). Subsequent publications by different authors cite a dietary concentration of 0.13% taurine DM for the diet used in the Glasgow et al. study (25), but the source of this value is unclear (9, 47). This concentration (0.13% DM) is greater than those documented in the current study (0.04–0.09% DM) as well as both AAFCO or NRC recommendations for dry extruded diets (0.1% DM), but it is lower than the recommended concentrations for canned diets (0.2% DM AAFCO; 0.17% DM NRC) (36, 37). It is unclear if the differences in measured taurine concentrations

or outcomes among reports are due to variations in study length, small numbers of rabbit samples with natural interindividual variability, differences in feeding methods (intact vs. ground could affect taurine measurements or metabolism), direct vs. indirect determination of taurine concentrations, presence or absence of the pelt (possible fiber-like effects in the gut), age or source of rabbits or other factors.

It is known that certain organs contain more taurine than others (20). In order to investigate possible differential distribution among the various organs, single samples of selected organs were collected from one additional rabbit. Only the heart and lung (but not thigh muscle, brain, liver, or kidney) contained adequate concentrations of taurine compared to NRC and AAFCO recommendations (**Supplementary Table 1**). This was despite the high amount of CP, especially in thigh muscle (89% DM). Given that rabbits exclusively conjugate bile acids with glycine, it is not surprising the liver had low concentrations of taurine (<25% of thigh muscle, 10% of kidney or brain, and 1–3% of heart and lung concentrations). The myocardial necessity of taurine for normal metabolism is a likely reason for its high concentration in the heart; however, a similar statement cannot be made for lung. The finding of adequate taurine in these two organs was somewhat unexpected in the face of apparently low global taurine. We did not have multiple samples for comparison, and this is an area in need of further research.

In summary, the GIT of the rabbit, inclusive of its contents, can constitute a large percentage of rabbit BW and likely impacts the concentration of AA on both a compositional and dilutional basis although individual differences are difficult to elucidate given the variables and multifactorial nature of the effect. Freezing ground rabbit appears to impact the concentrations of only select AA (cysteine, proline, glycine, and histidine) independent of the presence or absence of the GIT and its contents. The marked increase in proline that occurred with freezing remains unexplained, especially because glutamate concentrations did not decrease (therefore, conversion of glutamate to proline is not the apparent mechanism); however, it is of limited practical significance given it is a nonessential AA. Regardless, changes in essential AA concentrations in this study due to freezing or processing would not be expected to have physiological consequences as all essential alpha-AA in all four groups remained in excess of NRC recommended allowances for all feline life stages. The effects of extended periods of freezer or refrigerator storage on AA concentrations, as may occur in some retail or household situations, remains unknown.

The low concentrations of taurine in rabbit, regardless of sample type and storage conditions, would very likely be physiologically significant if fed in large proportions or as the sole diet. It remains to be seen if there are differences in taurine concentrations between wild and domestically raised rabbits. It is possible that all lagomorphs are inherently low in taurine regardless of diet. This could result in negative clinical consequences if cats are fed unsupplemented rabbit exclusively or as a large proportion of the diet but may not be of consequence in a wild-type setting as cats do not consume rabbit exclusively in the wild even when they constitute a larger percentage of the diet (48). Perhaps the consumption of additional and varied prey species is sufficient to meet overall needs. In addition, it would be interesting to characterize AA concentrations in rabbit carcasses after industrial processing either for use in commercial pet food or for human consumption (including complete evisceration and removal of the head). Finally, assessment of rabbits of different breeds and from different producers would enable a wider perspective on the body composition of domestically raised rabbits.

It remains unknown if supplementation of taurine to concentrations recommended for either dry extruded or canned diets is sufficient to maintain normal taurine status of cats consuming whole-prey diets generally or rabbit specifically. Overall, the physiologic effect of whole-prey diets (and the various forms they can assume) remains uncharacterized and may be impacted by inclusion of the pelt or whether the prey is ground or intact. It is noteworthy that ecological studies have confirmed the consumption of rabbits or other lagomorphs by wild cats by examination of scat or stomach samples rather than direct observation; therefore, it is undocumented if cats consume rabbits with a particular pattern of preference and/or if this may vary with rabbit size (e.g., entire consumption of small/neonatal/young rabbits or only specific parts of larger rabbits).

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author/s.

ETHICS STATEMENT

The animal study was reviewed and approved by University of California-Davis Institutional Animal Care and Use Committee (Protocol #19040).

AUTHOR CONTRIBUTIONS

JAL, AJF, and CCC conceived the study, wrote the grant, and provided logistical support and graduate student mentorship. TJO provided input on study design, arranged and conducted the experiment, and conducted the statistical analysis. All authors contributed to data interpretation and manuscript preparation.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2020.592753/full#supplementary-material

REFERENCES

1. Hewson R. The food of wild cats (*Felis silvestris*) and red foxes *(Vulpes vulpes*) in west and north-east Scotland. *J Zool.* (1983) 200:283–9. doi: 10.1111/j.1469-7998.1983.tb05790.x
2. Sarmento P. Feeding ecology of the European wildcat Felis silvestris in Portugal. *Acta Theriol.* (1996) 41:409–14. doi: 10.4098/AT.arch.96-39
3. Biro Z, Lanszki J, Szemethy L, Heltai M, Randi E. Feeding habits of feral domestic cats (*Felis catus*), wildcats (*Felis silvestris*) and their hybrids: trophic niche overlap among cat groups in Hungary. *J Zool.* (2005) 266:187–96. doi: 10.1017/S0952836905006771
4. Plantinga EA, Bosch G, Hendriks WH. Estimation of the dietary nutrient profile of free-roaming feral cats: possible implications for nutrition of domestic cats. *Br J Nutr.* (2011) 106(Suppl. 1):S35–48. doi: 10.1017/S0007114511002285
5. Morris JG. Idiosyncratic nutrient requirements of cats appear to be diet-induced evolutionary adaptations. *Nut Res Rev.* (2002) 15:153–68. doi: 10.1079/NRR200238
6. Pion PD, Kittleson MD, Rogers QR, Morris JG. Myocardial failure in cats associated with low plasma taurine: a reversible cardiomyopathy. *Science.* (1987) 237:764–8. doi: 10.1126/science.3616607
7. Ofri R, Barishak RY, EshRar G, Aizenberg I. Feline central retinal degeneration in captive cheetahs (*Acinonys jubatus). J Zoo Wildl Med.* (1996) 27:101–8.
8. Kerr KR, Kappen KL, Garner LM, Swanson KS. Commercially available avian and mammalian whole prey diet items targeted for consumption by managed exotic and domestic pet felines: macronutrient, mineral, and long-chain fatty acid composition. *Zoo Biol.* (2014) 33:327–35. doi: 10.1002/zoo.21147
9. Kerr KR, Kappen KL, Garner LM, Utterback PL, Parsons CM, Swanson KS. Commercially available avian and mammalian whole prey diet items targeted for consumption by managed exotic and domestic pet felines: true metabolizable energy and amino acid digestibility using the precision-fed cecectomized rooster assay. *J Anim Sci.* (2014) 92:4478–85. doi: 10.2527/jas.2013-7246
10. Hickman MA, Rogers QR, Morris JG. Effect of processing on the fate of dietary (^{14}C) taurine in cats. *J Nutr.* (1990) 120:995–1000. doi: 10.1093/jn/120.9.995
11. Hickman MA, Rogers QR, Morris JG. Taurine balance is different in cats fed purified and commercial diets. *J Nutr.* (1992) 122:553–9. doi: 10.1093/jn/122.3.553
12. Anantharaman-Barr G, Ballèvre O, Gicquello P, Bracco-Hammer I, Vuichoud J, Montigon F, et al. Fecal bile acid excretion and taurine status in cats fed canned and dry diets. *J Nutr.* (1994) 124(12 Suppl.):S2546–51. doi: 10.1093/jn/124.suppl_12.2546S
13. Backus RC, Rogers QR, Morris JG. Microbial degradation of taurine in fecal cultures from cats given commercial and purified diets. *J Nutr.* (1994) 124(12 Suppl.):2540S−5S. doi: 10.1093/jn/124.suppl_12.2540S
14. Morris JG, Rogers QR, Kim SW, Backus RC. Dietary taurine requirement of cats is determined by microbial degradation of taurine in the gut. *Adv Exp Med Biol.* (1994) 359:59–70. doi: 10.1007/978-1-4899-1471-2_7
15. Backus RC, Rogers QR, Rosenquist GL, Calam J, Morris JG. Diets causing taurine depletion in cats substantially elevated postprandial plasma cholecystokinin concentration. *J Nutr.* (1995) 125:2650–7.
16. Kim SW, Morris JG, Rogers QR. Dietary soybean protein decreases plasma taurine in cats. *J Nutr.* (1995) 125:2831–7.
17. Kim SW, Rogers QR, Morris JG. Dietary antibiotics decrease taurine loss in cats fed a canned heat-processed diet. *J Nutr.* (1996) 126:509–15. doi: 10.1093/jn/126.2.509
18. Kim SW, Rogers QR, Morris JG. Maillard reaction products in purified diets induce taurine depletion in cats which is reversed by antibiotics. *J Nutr.* (1996) 126:195–201. doi: 10.1093/jn/126.1.195
19. Stratton-Phelps M, Backus RC, Rogers QR, Fascetti AJ. Dietary rice bran decreases plasma and whole-blood taurine in cats. *J Nutr.* (2002) 132(6 Suppl.):1745S−7S. doi: 10.1093/jn/132.6.1745S
20. Spitze AR, Wong DL, Rogers QR, Fascetti AJ. Taurine concentrations in animal feed ingredients: cooking influences taurine content. *J Anim Physiol Anim Nutr.* (2003) 87:251–62. doi: 10.1046/j.1439-0396.2003.00434.x
21. Dierenfeld ES. Nutrition of captive cheetahs: food composition and blood parameters. *Zoo Biol.* (1993) 12:143–50. doi: 10.1002/zoo.14301201113
22. Bechert U, Mortenson J, Dierenfeld ES, Cheeke P, Keller M, Holick M, et al. Diet composition and blood values of captive cheetahs (*Acinonyx jubatus*) fed either supplemented meat or commercial food preparations. *J Zoo Wild Med.* (2002) 33:16–28. doi: 10.1638/1042-7260(2002)033[0016:DCABVO]2.0.CO;2
23. Dierenfeld ES, Alcorn HL, Jacobsen KL, Krista L. Nutrient composition of whole vertebrate prey (excluding fish) fed in zoos. In: *A.Z.A. Nutrition Advisory Group Fact Sheet: A Review*. Beltsville, MD: U.S. Dept. of Agriculture, Agricultural Research Service, National Agricultural Library, Animal Welfare Information Center (2002). p. 1–20. Available online at: https://archive.org/details/CAT11124570
24. Depauw S, Hesta M, Whitehouse-Tedd K, Stagegaard J, Buyse J, Janssens JP. Blood values of adult captive cheetahs (*Acinonyx jubatus*) fed either supplemented beef or whole rabbit carcasses. *Zoo Biol.* (2012) 31:629–41. doi: 10.1002/zoo.20427
25. Glasgow AG, Cave NJ, Marks SL, Pedersen NC. Role of diet in the health of the feline intestinal tract and in inflammatory bowel disease. *Cat Fanciers' Almanac.* (2002) 19:78–80. Available online at: https://ccah.sf.ucdavis.edu/sites/g/files/dgvnsk4586/files/inline-files/role-of-diet-feline-health-Glasgow_0.pdf
26. AOAC Official Method 990.03. Protein (crude) in animal feed, combustion method. In: Horwitz W, Latimer G, editors. *Official Methods of Analysis of AOAC International*, 18th ed. Gaithersburg, MD: AOAC International (2005). p. 30–1.
27. AOAC Official Method 994.12. Amino acids in feeds. In: Horwitz W, Latimer G, editors. *Official Methods of Analysis of AOAC International*, 18th ed. Gaithersburg, MD: AOAC International (2005). p. 9–19.
28. Chen X, Zhao G, Zhang Y, Han L, Xiao W. Nitrogen-to-protein conversion factors for crop residues and animal manure common in China. *J Agric Food Chem.* (2017) 65:9186–90. doi: 10.1021/acs.jafc.7b03441
29. Vessey DA. The biological basis for the conjugation of bile acids with either glycine or taurine. *Biochem J.* (1978) 174:621–6. doi: 10.1042/bj1740621
30. Hayes KC, Carey RE, Schmidt SJ. Retinal degeneration associated with taurine deficiency in the cat. *Science.* (1975) 188:949–51. doi: 10.1126/science.1138364
31. Sturman JA, Gargano AD, Messing JM, Imaki H. Feline maternal taurine deficiency: effect on mother and offspring. *J Nutr.* (1985) 116:655–67. doi: 10.1093/jn/116.4.655
32. Sturman JA, Palackal T, Imaki H, Moretz RC, French J, Wisniewski HM. Nutritional taurine deficiency and feline pregnancy and outcome. *Adv Exp Med Biol.* (1987) 217:113–24. doi: 10.1007/978-1-4899-0405-8_11
33. Sturman JA, Moretz RC, French JH, Wisniewski HM. Taurine deficiency in the developing cat: persistence of the cerebellar external granule cell layer. *J Neurosci Res.* (1985) 13:405–16. doi: 10.1002/jnr.490130307
34. Rabin AR, Nicolosi RJ, Hayes KC. Dietary influence of bile acid conjugation in the cat. *J Nutr.* (1976) 106:1241–6. doi: 10.1093/jn/106.9.1241
35. Park T, Rogers QR, Morris JG. High dietary protein and taurine increase cysteine desulfhydration in kittens. *J Nutr.* (1999) 129:2225–30. doi: 10.1093/jn/129.12.2225
36. Association of American Feed Control Officials. Chapter 4: model bill and regulations. In: Lueders D, editor. *Association of American Feed Control Officials Official Publication.* Oxford, IN: Association of American Feed Control Officials (2019). p. 107–232.
37. National Research Council. *Nutrient Requirements of Dogs and Cats*. Washington, DC: The National Academy Press (2006).
38. Sunvold GD, Fahey GC Jr, Merchen NR, Reinhart GA. *In vitro* fermentation of selected fibrous substrates by dog and cat fecal inoculum: influence of diet composition on substrate organic matter disappearance and short-chain fatty acid production. *J Anim Sci.* (1995) 73:1110–22. doi: 10.2527/1995.7341110x
39. Depauw S, Bosch G, Hesta M, Whitehouse-Tedd K, Hendriks WH, Kaandorp J, et al. Fermentation of animal components in strict carnivores: a comparative study with cheetah fecal inoculum. *J Anim Sci.* (2012) 90:2540–8. doi: 10.2527/jas.2011-4377

40. Depauw S, Hesta M, Whitehouse-Tedd K, Vanhaecke L, Verbrugghe A, Janssens GP. Animal fibre: the forgotten nutrient in strict carnivores? First insights in the cheetah. *J Anim Physiol Anim Nutr.* (2013) 97:146–54. doi: 10.1111/j.1439-0396.2011.01252.x
41. Crissey SD, Swanson JA, Lintzenich BA, Brewer BA, Slifka KA. Use of a raw meat-based diet or a dry kibble diet for sand cats (Felis margarita). *J Anim Sci.* (1997) 75:2154–60. doi: 10.2527/1997.7582154x
42. Mikkelsen LL, Naughton PJ, Hedemann MS, Jensen BB. Effects of survival of *Salmonella enterica* serovar Typhimurium in the pig gastrointestinal tract. *Appl Environ Microbiol.* (2004) 70:3485–92. doi: 10.1128/AEM.70.6.3485-3492.2004
43. Huang DS, Li DF, Xing JJ, Ma YX, Li ZJ, Lv SQ. Effects of feed particle size and feed form on survival of *Salmonella typhimurium* in the alimentary tract and cecal *S. typhimurium* reduction in growing broilers. *Poult Sci.* (2006) 85:831–6. doi: 10.1093/ps/85.5.831
44. Pla M. A comparison of the carcass traits and meat quality of conventionally and organically produced rabbits. *Livest Sci.* (2008) 115:1–12. doi: 10.1016/j.livsci.2007.06.001
45. Bivolarski B, Vachikova E, Ribarski S, Uzunova K, Pavlov D. Amino acid content and biological value of rabbit meat proteins, depending on weaning age. *Bulg J Vet Med.* (2011) 14:94–102.
46. Lambert IH, Nielsen JH, Andersen HJ, Ortenblad N. Cellular models for induction of drip loss in meat. *J Agric Food Chem.* (2001) 49:2225–30. doi: 10.1021/jf010121y
47. Hedberg GE, Dierenfeld ES, Rogers QR. Taurine and zoo felids: considerations of dietary and biological tissue concentrations. *Zoo Biol.* (2007) 26:517–31. doi: 10.1002/zoo.20158
48. Malo AF, Lozano J, Huertas DL, Virgó E. A change in the diet from rodents to rabbits (*Oryctolagus cuniculus)*. Is the wildcat (Felis silvestris) a specialist predator? *J Zool.* (2004) 263:401–7. doi: 10.1017/S0952836904005448

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Early Life Modifiable Exposures and Their Association With Owner Reported Inflammatory Bowel Disease Symptoms in Adult Dogs

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Background: Inflammatory bowel disease (IBD) is an idiopathic multifactorial disease in humans and dogs, usually assigned to the interactions between genes, gut microbiota, diet, environment, and the immune system. We aimed to investigate the modifiable early life exposures associated with IBD in dogs.

Materials and Methods: The study data was extracted from the validated owner-reported DogRisk food frequency questionnaire. This was a cross-sectional questionnaire-based study that tested 21 different early life dietary and environmental, demographic and genetic variables for their association with IBD or not, in adult dogs. A total of 7,015 dogs participated in this study. The study covered early life periods; prenatal, neonatal, early, and late postnatal periods. Two feeding patterns, a non-processed meat-based diet (NPMD) and an ultra-processed carbohydrate-based diet (UPCD) were studied. Data was analyzed using logistic regression analysis with a backward stepwise deletion.

Results: From the final models we found that the NPMD during early and late postnatal periods were significantly associated with lower IBD risk later in life. The UPCD during the same periods was associated with a higher risk of IBD incidence. Also, the maternal diet during the neonatal period showed a non-significant trend of lower IBD risk in the offspring with the NPMD and a higher IBD risk with the UPCD. Additionally, the normal body weight of puppies during the first 6 months of age was associated with a lower risk of IBD in adulthood while, slim puppies associated significantly with IBD in adulthood. From the non-modifiable background variables, we identified the maternal history of IBD as the strongest risk factor for later incidence of IBD. Furthermore, male dogs were twice as likely to develop IBD as female dogs were.

Conclusions: It is reassuring for owners to know that they themselves can have an impact on their dog's health. A high-fat, low-carbohydrate NPMD exposure during early life, and a normal body condition in puppyhood were significantly associated with less IBD in adult dogs. The opposite was true for UPCD exposure and abnormal body condition score in 6 month old puppies.

Keywords: chronic enteropathies, canine, diet, microbiome, gut, immune, prenatal

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INTRODUCTION

Canine inflammatory bowel disease (IBD) is a group of chronic idiopathic enteropathies in dogs characterized by persistent and/or recurrent gastrointestinal symptoms (1–3). In this study we will refer to these enteropathies as IBD. The disease usually affects middle-aged dogs with no gender predisposition (4, 5); although intermittent symptoms have been noted even in puppies < 6 months of age (5). Clinical phenotypes of IBD have been identified and used to define specific forms of IBD with varying breed predisposition [(1, 5, 6), **Table 1**]. To date, the etiopathogenesis of idiopathic IBD, both in humans and canines, is not fully understood. Current literature supports the concept that IBD is usually assigned to the interactions between genetics, gut microenvironment (dietary microbiota and diet composition), and the host immune system (1, 2). The genetic component of the disease has been recognized using both genome-wide association and candidate gene approaches in humans (14, 15) and dogs (6, 16, 17).

Mounting evidence from human epidemiological studies suggests that it is wise to focus on exploring the role that early life exposures have on influencing the gut microbiome and immune modulation, as it in turn can modify the disease risk (18–21). Several theories encourage identifying the role of environmental stimulants, including diet, in triggering the inflammatory response. The most prominent of them is the hygiene hypothesis which states that an increased frequency of immune disorders can be attributed to a reduction in enteric microbiota during early life. This has been presumed to be due to exaggerated sanitation, which results in an untrained, and therefore malfunctioning, immune system (22, 23). The newborn immune maturation is mainly driven by the early life exposure to microbes (24). The gut microbiota is the central source of the postnatal microbial exposure (25). The early life diet has a profound effect on the neonate gut microbiota and thereby also on immune regulation (26). Dietary patterns are a fundamental part of a healthy lifestyle and diets can influence gut microbial ecosystems, promoting gut health in dogs and humans. Recently, diet composition has been shown to substantially impact the abundance and modulation of gut microbiome in dogs and humans (27–34). Moreover, the diet processing; whether the diet is offered as a non-processed/raw diet or as an ultra-processed diet, has been observed to impact human and canine health (35, 36).

Research regarding the role of the early diet on IBD incidence in small animals is scarce. One study on cats analyzed the role that early life events and diet had on gastrointestinal symptoms that developed later in life (37). They found that when owners reported diarrhea, vomiting, and/or decreased consumption of commercial diets before their cat was 16 weeks of age, it was also associated with gastrointestinal symptoms occurring at least twice between 6 and 30 months of age (37). Although the disease is frequently presented at animal clinics, there is no data on the true prevalence of IBD in dogs (4). Currently there are no studies that identify early life exposures that might act as risk factors for chronic IBD development in dogs. Furthermore, the possible influence of a non-processed meat based diet and an ultra-processed carbohydrate based diet on the prevalence of IBD in dogs has not been previously assessed. Only one canine study found a significant risk of developing gastrointestinal diseases if a dog had previously suffered from a parvovirus infection (38). Therefore, it is important to investigate modifiable early life risk factors for canine IBD that might have an impact on future immune system stimulation. The main aim of the present study was to investigate possible associations of modifiable early life exposures, dietary and environmental, with owner-reported IBD incidence in later life. In addition, we aimed to test previous known risk factors of the disease, such as maternal history, gender, and breed.

TABLE 1 | Inflammatory bowel disease prone breeds.

IBD phenotypes	IBD prone breeds
Inflammatory bowel disease	Akita[1], Bernese mountain dog[1], Dalmatian[1], English setter[1], German shepherd[1,2,7], Golden retriever[1,2], Irish setter[1], Pointer[1], Rottweiler[1,2], Soft-coated wheaten terrier[1,3,7], Labrador retriever[2], Border colli[2], Boxer[2,7], Staffordshire bull terrier[2], Cocker spaniel[2], West highland white terrier[2], Weimaraner[2], Jack Russell terrier[2], Basenjis[3,7], Mixed breeds[2], French bull dog[3], Doberman pinscher[3], Mastiff[3], Alaskan malamute[3], Shar pei[7]
Intestinal malabsorption	Akita[1], Basenji[1], Chinese Shar pei[1], Chow chow[1], French bull dog[1], Irish setter[1], Old English sheep dog[1], Peruvian inca orchid[1], Rottweiler[1], Shiloh shepherd[1], Soft-coated wheaten terrier[1]
Gluten-sensitive enteropathy	Irish setter[1,2]
Ulcerative colitis	Akbash[1], Boxers[1,4,5,7], German shepherd[1], Skye terrier[1], French bull dog[5,7], Mastiff[5,7], Alaskan malmalute[5,7], Dobermann pinscher[5,7]
Tylosin responsive diarrhea	Irish setter[6], Basenji[6], Lundehund[6], Yorkshire terrier[6], German shepherd[6], Boxer[6], French bull dog[6], Shar-pei[6], Rottweiler[6], Soft-coated wheaten terrier[6]
Antibiotic responsive diarrhea	German shepherds[8]

[1–8]*cited from Dodds (7), Kathrani et al. (8), Cerquetella et al. (2), Davies et al. (9), Stokes et al. (10), Westermarck et al. (11), Hall (12), Hall (13) respectively.*

MATERIALS AND METHODS

The Questionnaire and Study Population

The study data was extracted from the DogRisk food frequency questionnaire (FFQ) data. This FFQ is an online validated ongoing questionnaire (39) http://www.ruokintakysely.fi/. It has been available online for dog owners since 2009, when it was launched by us at the University of Helsinki, Finland. The whole questionnaire is in Finnish language. As mentioned elsewhere (36, 39, 40) the FFQ includes 50 questions generating 1,332 data points, reported by the dog owners. It aims to gather information on the owner's dog's diets and lifestyle at different

Abbreviations: IBD, inflammatory bowel disease; NPMD, non-processed meat based diet; UPCD, ultra-processed carbohydrate based diet; FFQ, food frequency questionnaire.

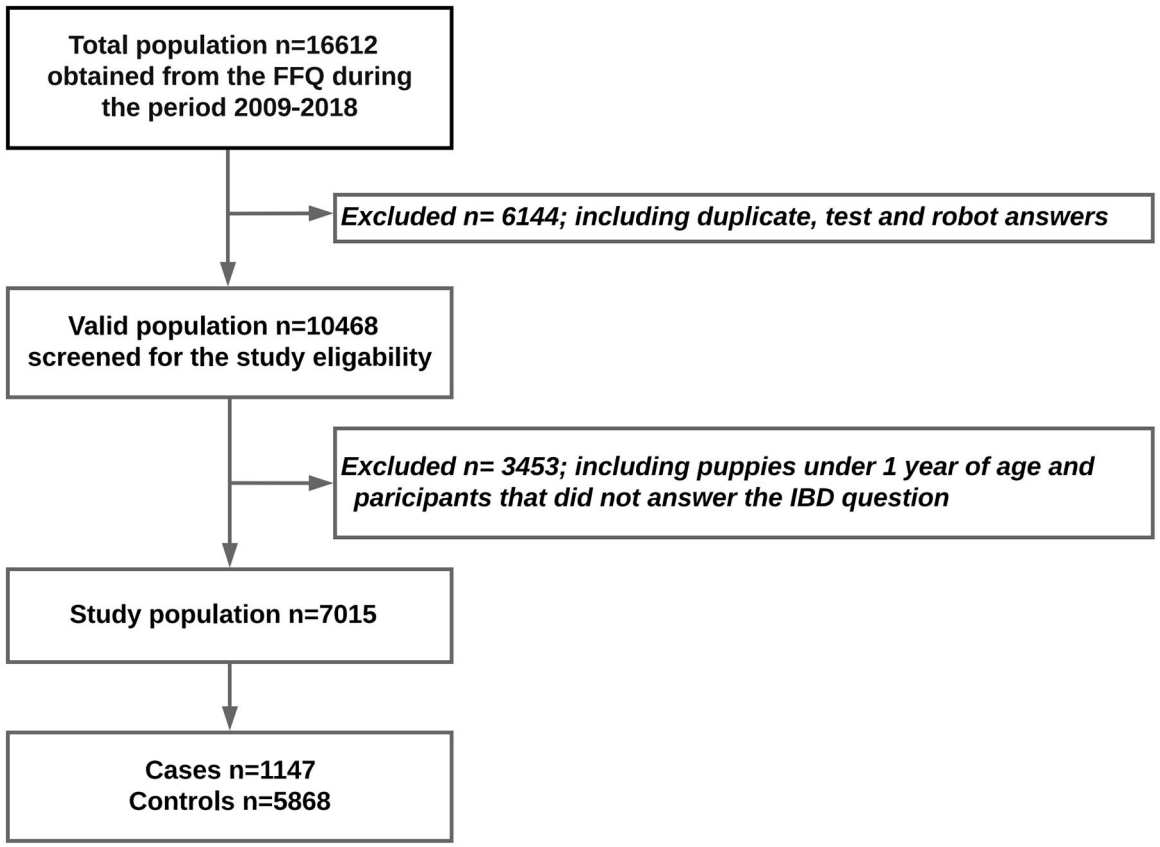

FIGURE 1 | Flow chart of the study population.

time points throughout its life, as well as data on the dog's health conditions, background, and demographic information. In addition to gathering information on the dog itself, it also contains several questions concerning the dogs' mothers' early maternal diets and their diseases. The FFQ has an ethical approval (29.4.2016) from the University of Helsinki Viikki campus ethical board.

The questionnaire received 16,559 responses between 2009 and 2019. The dogs under 1 year of age were excluded from this study in order to avoid reverse causality. Also, participants who had not answered the question about whether their dogs had been suffering from IBD or not were excluded. Otherwise, all breeds and both sexes were included. After all questionnaire test answers, robot answers, and duplicates had been removed, 7,015 participants were eligible for the study (**Figure 1**).

Study Design and Tested Variables

This is a once-answered, and in that respect cross-sectional, questionnaire-based study asking about multiple exposures at different time points, therefore also making it longitudinal. This study has been carried out to investigate the association between early life modifiable exposures (dietary and environmental) and the incidence of owner-reported IBD later in life. In addition, we tested the associations between non-modifiable genetic and demographic variables and IBD incidence. The study tested only one dependent categorical dichotomous variable, concerning IBD, for association with 21 different independent categorical and continuous variables (**Table 2**). The dependent variable obtained by responding either "Yes" or "No" to the question: "Has your dog suffered from inflammatory bowel disease (IBD), chronic bowel symptoms, chronic bowel 'allergies'?" The study cases were obtained when the owners responded "Yes," while those who responded "No" were chosen as study controls.

We analyzed four early life periods, prenatal (pregnancy period), neonatal (the 1st 3–4 weeks of life, i.e., the lactation period), early postnatal (from 1 to 2 months of age) and late postnatal periods (from 2 to 6 months of age) as shown in **Figure 2**.

The modifiable early life variables that were tested covered nutritional, environmental and lifestyle factors. We compared two common feeding patterns, the non-processed meat-based diets (NPMD) and the ultra-processed carbohydrate-based diets (UPCD) during the following early life periods: the maternal diet during pregnancy, the maternal diet during lactation, the young puppy first solid diet and the puppy diet in the period between 2 and 6 months of age. The NPMD is a diet rich in fresh animal proteins and fats (red meat, poultry, fish, organs, bones, lard, fish oils etc.) and low in carbohydrates but including raw vegetables,

TABLE 2 | Frequencies of the tested variables in the cases, controls, and total study cohort.

Variables	Categories	Inflammatory bowel disease %(n)		
		Cases, % (*n* = 1,147)	Controls, % (*n* = 7,015)	Total, % (*n* = 7,015)
Non- modifiable; genetic and demographic factors				
Maternal History of IBD	Mothers without IBD	90.0 (260)	98.8 (2,135)	97.7 (2,395)
	Mothers with IBD	10.0 (29)	1.2 (27)	2.3 (56)
Dog breed	IBD prone breeds	55.5 (562)	50.4 (2,583)	51.2 (3,145)
	IBD non-prone breeds	44.5 (450)	49.6 (2,546)	48.8 (2,996)
Dog gender	Males	60.3 (675)	43.4 (2,480)	46.2 (3,155)
	Females	39.7 (444)	56.6 (3,232)	53.8 (3,676)
Dog color	White >50%	21.8 (240)	20.0 (1,107)	20.3 (1,347)
	White <50%	78.2 (862)	80.0 (4,441)	79.7 (5,303)
Dog age, years (mean ± SD)*		5.10 ± 3.06	5.04 ± 3.12	5.05 ± 3.11
Modifiable factors				
Prenatal period (pregnancy)				
Maternal gestation diet	NPMB	6.0 (22)	9.1 (178)	8.6 (200)
	UPCD	94.0 (346)	90.9 (1,788)	91.4 (2,134)
Was the mother dewormed during/just before pregnancy?	Yes	95.9 (446)	96.2 (2,717)	96.2 (3,163)
	No	4.1 (19)	3.8 (107)	3.8 (126)
Was the mother vaccinated during/just before pregnancy?	Yes	59.5 (150)	48.7 (830)	50.1 (980)
	No	40.5 (102)	51.3 (873)	49.9 (975)
Neonatal period (lactation)				
Maternal lactation diet	NPMB	6.2 (21)	9.2 (172)	8.7 (193)
	UPCD	93.8 (319)	90.8 (1,706)	91.3 (2,025)
Early postnatal period (puppy 1–2 months of age)				
Puppy's first solid diet	NPMD	5.9 (22)	10.3 (198)	9.6 (220)
	UPCD	94.1 (353)	89.7 (1,720)	90.4 (2,073)
Frequency of outdoor activity	Many times/day	51.4 (331)	60.0 (2,105)	58.6 (2,436)
	Once/day	15.5 (100)	15.7 (552)	15.7 (652)
	A few times/week	14.4 (93)	11.5 (405)	12.0 (498)
	A few times/month	7.3 (47)	5.0 (176)	5.4 (223)
	Not at all	11.3 (73)	7.8 (273)	8.3 (346)
Rest, hours/day (mean ± SD)*		15.99 ± 3.97	15.98 ± 3.80	15.98 ± 3.82
Type of flooring	Slippery flooring	9.7 (66)	10.9 (399)	10.7 (465)
	Non-slippery flooring	26.3 (179)	27.4 (1,006)	27.2 (1,185)
	Dirt flooring	5.9 (40)	7.5 (275)	7.2 (315)
	Newspaper flooring	30.0 (204)	27.8 (1,023)	28.2 (1,227)
	Carpet flooring	28.1 (191)	26.5 (973)	26.7 (1,164)
Body condition score	Obese puppy	14.6 (109)	14.6 (573)	14.6 (682)
	Normal weight puppy	75.1 (561)	75.9 (2,977)	75.8 (3,538)
	Slim puppy	10.3 (77)	9.5 (371)	9.6 (448)
Late postnatal period (puppy 2–6 months)				
Puppy diet	NPMD	15.1 (45)	22.2 (310)	20.9 (355)
	UPCD	84.9 (254)	77.8 (1,088)	79.1 (1,342)
Outdoor activity, hours/day	< 0.5	1.2 (9)	2.2 (89)	2.1 (98)
	0.5–1.0	28.3 (219)	25.5 (1,019)	26.0 (1,238)
	1.0–2.0	51.8 (401)	51.5 (2,057)	51.6 (2,458)
	> 2.0	18.7 (145)	20.7 (826)	20.4 (971)
Rest, hours/day (mean ± SD)*		14.52 ± 3.21	14.43 ± 3.28	14.45 ± 3.27
Type of flooring	Slippery flooring	23.4 (191)	24.5 (1,040)	24.3 (1,231)

(Continued)

TABLE 2 | Continued

Variables	Categories	Inflammatory bowel disease %(n)		
		Cases, % (n = 1,147)	Controls, % (n = 7,015)	Total, % (n = 7,015)
	Non-slippery flooring	24.9 (203)	25.1 (1,064)	25.0 (1,267)
	Dirt flooring	1.0 (8)	2.0 (85)	1.8 (93)
	Newspaper flooring	0.5 (4)	0.3 (11)	0.3 (15)
	Carpets flooring	12.0 (98)	13.5 (572)	13.2 (670)
Body condition score	Obese puppy	7.3 (59)	6.4 (266)	6.5 (325)
	Normal weight puppy	61.6 (495)	69.0 (2,883)	67.8 (3,378)
	Slim puppy	31.1 (250)	24.7 (1,032)	25.7 (1,282)
Was the puppy vaccinated 2–4 times under 1 year of age?	Yes	98.1 (1,050)	98.7 (5,493)	98.6 (6,543)
	No	1.9 (20)	1.3 (72)	1.4 (92)
Was the puppy dewormed 2–10 times under 1 year of age?	Yes	98.9 (1,018)	98.8 (5,303)	98.8 (6,321)
	No	1.1 (11)	1.2 (64)	1.2 (75)

*(n): the number of dogs, IBD, inflammatory bowel disease; NPMD, non-processed meat based diet; UPCD, ultra-processed carbohydrate based diet. *Scale variables presented as (mean ± SD).*

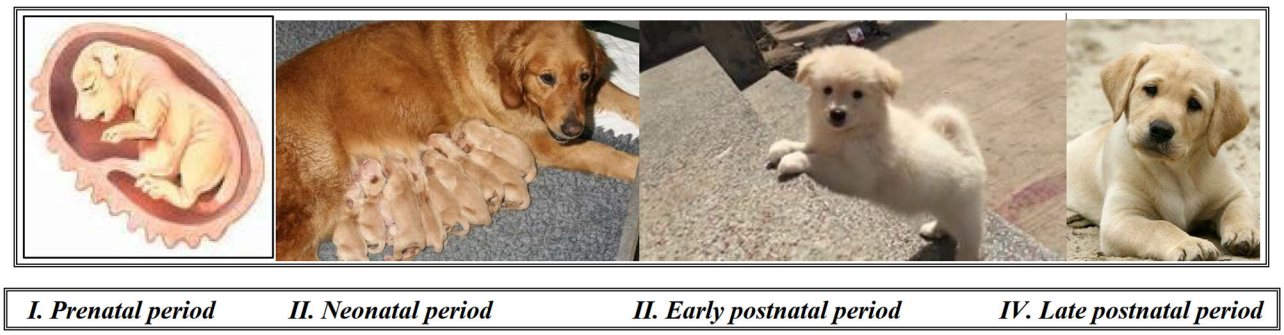

FIGURE 2 | Pathway of the study variables at different time points. Prenatal period image adapted from https://dogs.lovetoknow.com/wiki/Canine_Gestation, neonatal period image adapted from https://www.yorkbeach.co.uk/puppies/daisy_2016.html, early postnatal period image adapted from https://i.ytimg.com/vi/8nt7M12CTa0/maxresdefault.jpg, and late postnatal period image adapted from https://www.pets4homes.co.uk/pet-advice/what-to-expect-of-your-puppy-at-4-to-6-months-old.html.

fruits or berries for micronutrients and vegetal fibers. The NPMD is either commercial or prepared at home by chopping, grating, mixing and freezing the ingredients. The UPCD refers to commercially produced extruded kibble. Typically, 40–60% of the contents of the UPCD are processed carbohydrates (mostly grains, rice, potatoes etc. but also some vegetable pulp). The two diets; NPMD and UPCD, exhibit different macronutrient profiles but may also differ in many other ways.

We tested four environmental and lifestyle factors during the early and late postnatal periods including outdoor activity, rest hours, type of flooring, and the body condition score. The body condition score (BCS) was a 5-category scale where the owner could choose between 1 = very slim, 2 = slim, 3 = normal, 4 = round/fat, and 5 = obese.

The questions about vaccination and deworming programs of the pregnant dams and puppies under 1 year of age were also tested for the association with the dependent variable. The question regarding the dog's mother was: "Did you vaccinate/deworm the dam while she was pregnant or just before?" The question regarding the puppy asked whether or not it had received 2–4 vaccinations before the age of 1 year? The answers of these questions were, "yes," "no" or "I do not know/I do not remember." Only the yes and no answers were used in the analyses.

The final five questions regarded the non-modifiable genetic and demographic variables, namely: maternal history of IBD, dog age, dog gender, dog color (specifically wanting to know the amount of white in the coat) and if the dog breed is prone to IBD or not prone to IBD (**Table 1**). The variable of IBD prone breed or not, was gathered from the literature (**Table 1**).

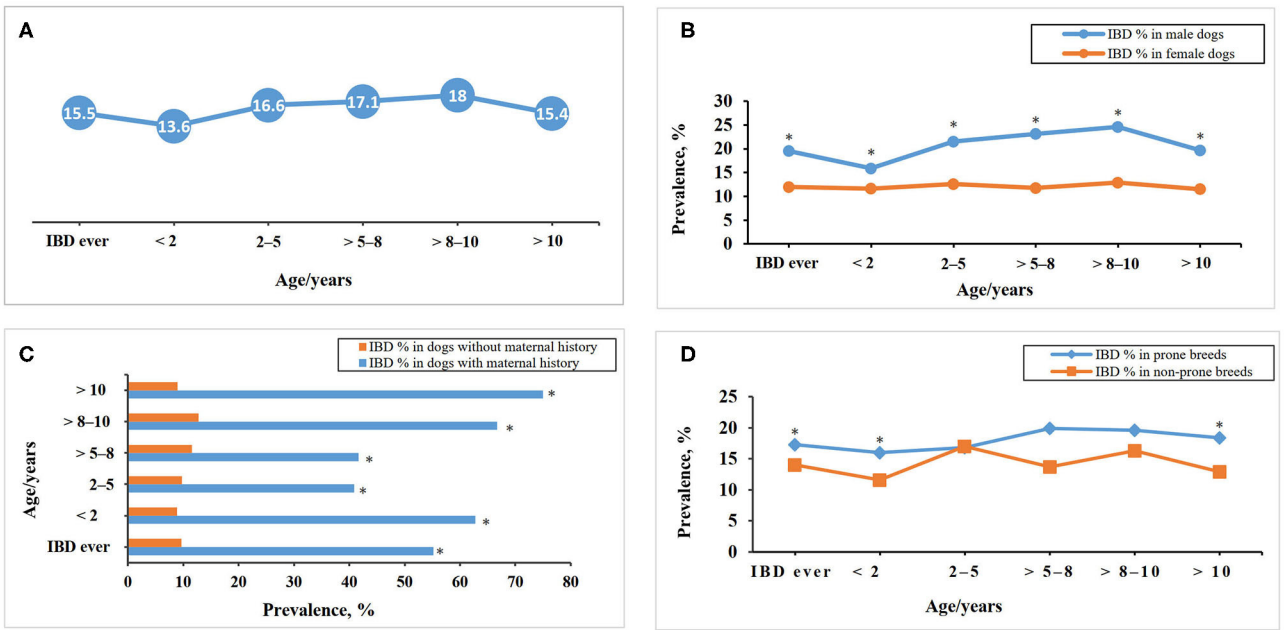

FIGURE 3 | Inflammatory bowel disease prevalence within different age groups in the DogRisk food frequency questionnaire population, $n = 10468$, **(A)** and when the population was stratified for gender **(B)**, maternal history **(C)**, and disease predisposition in the different breeds **(D)**. *The difference between the two groups within each age group is significant at $P < 0.05$.

Statistical Methods

The categorical and discrete variables were presented as frequencies n (%) using cross-tabulation for the cases, controls and the total study population, while the continuous variables were summarized as means and standard deviations (mean ± SD). The disease prevalence was calculated using cross-tabulation as ratios of the diseased dogs in the total population and within each age group. Moreover, the prevalence was calculated after stratifying the FFQ cohort for gender, maternal history, and disease predisposition in the different breeds.

The association of the tested independent variables with IBD symptoms in adult dogs was calculated using logistic regression analyses. Firstly, the variables were analyzed using univariate logistic regression and the variables with a $p < 0.2$ were used for final modeling. Five final models were created using the backward stepwise regression method. The first model included the demographic non-modifiable variables, the other four models contained the early life modifiable exposures during prenatal life, neonatal life, early postnatal life and late postnatal life, respectively. The five models were adjusted for age and the statistical significance was considered for p-values lower than 0.05 (*), 0.01 (**), and 0.001 (***). To help the veterinarian or nutritionist reader make an informed choice regarding the type of food that they usually prescribes in their practice, we report our regression analysis using two dummy variables that were created for the dichotomous variables instead of one. The missing values were not imputed. To test the fitness of the regression models an Omnibus test p-value should be lower than 0.05, a Hosmer and Lemeshow test p-value should be larger than 0.05 and the Nagelkerke's R should be as big as possible (41, 42). The statistical analyses were performed using SPSS version 25. The visualization of odds ratios was carried out using the forest plot package (43) in R software version 3.5.1 (44).

RESULTS

Inflammatory Bowel Disease Prevalence (IBD) in the Finnish DogRisk Food Frequency Questionnaire (FFQ) Population

The prevalence of IBD in the FFQ population differs between the age groups and when the population was stratified for gender, history of maternal disease, white color coat ratio, and IBD prone breeds as shown in **Figure 3**.

Variables Characteristics

The distribution of the tested variables within the cases, controls and the total study cohort are presented in **Table 2** as frequencies (%) and numbers (n) for the categorical variables, while the scale variables are presented as means ± SDs (**Table 2**).

Regression Analysis

The univariate logistic regression analysis showed 10 significant associations ($p < 0.05$). Additionally, six variables ($p < 0.2$) were also included in the final models (**Table 3**).

From the multivariate logistic regression models, four early-life exposures showed a significant association with canine IBD incidence in adulthood and one early-life exposure showed a non-significant "trend" as shown in **Figure 4**. The final models'

TABLE 3 | Associations of early life covariates with inflammatory bowel disease in dogs based on univariate logistic regression analyses.

Covariates	Crude effect estimates		Multivariate models
	cOR (95% CI)	*P*-value	
Non- modifiable; genetic and demographic factors			
Maternal history of IBD			Included in model I
Non-IBD mothers vs. IBD mothers	0.113 (0.066–0.195)	**<0.001*** **	
IBD vs. non-IBD mothers	8.820 (5.141–15.131)	**<0.001*** **	
Dog breed			Included in model I
Non-IBD prone vs. IBD prone	0.812 (0.709–0.930)	**0.003****	
IBD prone vs. non-IBD prone	1.231 (1.075–1.410)	**0.003****	
Dog gender			Included in model I
Female vs. male	**0.505 (0.443–0.575)**	**<0.001*** **	
Male vs. female	1.981 (1.739–2.258)	**<0.001*** **	
Dog color			Included in model I
> 50% white coat vs. < 50%	1.117 (0.954–1.307)	**0.169**	
< 50% white coat vs. > 50%	0.895 (0.765–1.048)	**0.169**	
Dog age[§]	1.007 (0.987–1.027)	0.505	Included in model I
Modifiable factors			
I. Prenatal period maternal factors			
Mother's diet during pregnancy			Included in model II
NPMD vs. UPCD	0.639 (0.404–1.009)	**0.055**	
UPCD vs. NPMD	1.566 (0.991–2.474)	**0.055**	
Was the mother dewormed during/just before pregnancy?			Not included
Yes vs. no	0.924 (0.562–1.521)	0.757	
No vs. yes	1.082 (0.657–1.780)	0.757	
Was mother vaccinated during/just before pregnancy?			Included in model II
Yes vs. no	1.547 (1.182–2.024)	**0.001****	
No vs. yes	0.647 (0.494–0.846)	**0.001****	
II. Neonatal period (0–1 month of age)			
Mother's diet during lactation			Included in model III
NPMD vs. UPCD	0.653 (0.409–1.043)	**0.075**	
UPCD vs. NPMD	1.532 (0.959–2.447)	**0.075**	
III. Early postnatal period (1–2 months of age)			
Puppy's first solid diet			Included in model IV
NPMD vs. UPCD	0.541 (0.343–0.853)	**0.008****	
UPCD vs. NPMD	1.847 (1.172–2.912)	**0.008****	
Frequency of outdoor activity			Included in model IV
Many times/day vs. not at all	0.588 (0.443–0.780)	**<0.001*** **	
Once/day vs. not at all	0.677 (0.485–0.947)	**0.023***	
A few times/week vs. not at all	0.859 (0.610–1.210)	0.384	
A few times/month vs. not at all	0.999 (0.661–1.509)	0.995	
Type of flooring			Included in model IV
Slippery vs. dirt flooring	1.137 (0.746–1.734)	0.550	
Non-slippery vs. dirt flooring	1.223 (0.847–1.767)	0.283	
Newspaper vs. dirt flooring	1.371 (0.953–1.973)	**0.089**	
Carpets vs. dirt flooring	1.350 (0.936–1.946)	**0.109**	
Rest, hours/day	1.001 (0.958–1.045)	0.977	Not included
Body condition Score			Not included
Slim vs. normal weight puppies	1.101 (0.848–1.431)	0.469	
Obese vs. normal weight puppies	1.009 (0.807–1.263)	0.934	
IV. Late postnatal period (2–6 months of age)			

(Continued)

TABLE 3 | Continued

Covariates	Crude effect estimates		Multivariate models
	cOR (95% CI)	*P*-value	
Puppy diet			Included in model V
NPMD vs. UPCD	0.622 (0.442–0.875)	**0.006****	
UPCD vs. NPMD	1.608 (1.143–2.262)	**0.006****	
Outdoor activity, hours/day			Included in model V
0.5–1 vs. < 0.5	2.125 (1.054–4.284)	**0.035***	
1–2 vs. < 0.5	1.928 (0.963–3.858)	**0.064**	
> 2 vs. < 0.5	1.736 (0.855–3.524)	**0.127**	
Rest, hours/day	1.008 (0.983–1.034)	0.528	Not included
Type of flooring			Included in model V
Slippery vs. dirt flooring	1.951 (0.930–4.094)	**0.077**	
Non-slippery vs. dirt flooring	2.027 (0.967–4.250)	**0.061**	
Newspaper vs. dirt flooring	3.864 (0.997–14.973)	**0.051**	
Carpets vs. dirt flooring	1.820 (0.855–3.876)	**0.120**	
More than two types vs. dirt flooring	2.249 (1.078–4.690)	**0.031***	
Body condition score			Included in model V
Slim vs. normal weight puppies	1.411 (1.193–1.669)	**<0.001*****	
Obese vs. normal weight puppies	1.292 (0.959–1.740)	**0.092**	
Was the puppy vaccinated 2–4 times under 1 year of age?			Included in model V
Yes vs. no	0.688 (0.417–1.134)	**0.143**	
No vs. yes	1.453 (0.882–2.395)	**0.143**	
Was the puppy dewormed 2–10 times under 1 year of age?			Not included
Yes vs. no	1.117 (0.587–2.125)	0.736	
No vs. yes	0.895 (0.471–1.703)	0.736	

*cOR, Crude odds ratio; CI, Confidence intervals; IBD, Inflammatory bowel disease; NPMD, Non-processed meat based diet; UPCD, Ultra-processed carbohydrate based diet; vs., versus. *p < 0.05, **p < 0.01, ***p < 0.001, Bolded values indicate p < 0.2,* §*scale variable measured in years.*

odds ratios for the associations of the non-modifiable and the early life modifiable factors with IBD in adult dogs are presented in **Figure 4**. A $p < 0.05$ and an OR > 1 means increased risk for IBD while $p < 0.05$ and OR < 1 means decreased risk.

DISCUSSION

A growing trove of literature regarding genes and the microbiome confirmed by data from human epidemiological studies advocates the importance of early life exposures in modulating the risk of IBD incidence (18, 45–48). However, the impact that early life environmental and nutritional exposures have on IBD risk in dogs has not been previously examined. This is the first study to investigate the association between modifiable early life exposures and the incidence of IBD in adult dogs. Exploring the most pertinent early life exposures provides clues to the etiopathogenesis of this complex disease and allows for the development of a primary prevention strategy for IBD in dogs. At the same time, as a dog's pregnancy and early life is shorter than a human's, but otherwise similar in both terms of environment and diet, this study might also provide insight regarding the prevention of human IBD. Our results are divided into the non-modifiable factors that cannot be altered, and the modifiable factors, where the owners' choices can have an impact on their dog's future health.

Non-modifiable Exposures and How They Related to Owner Reported IBD

Our analysis of the non-modifiable background related factors showed that the maternal history of IBD was directly associated with IBD incidence in the offspring in later life. To the best of our knowledge, the family history of IBD in dogs and the risk of the disease morbidity in the offspring later, has not been demonstrated before. The role that the family disease history has on the offspring's subsequent IBD development in adulthood, has been studied in several human studies (18, 49–53). Our findings are similar to a human study, where they found that the greatest risk factor for IBD was having one or more affected first-degree relative (52). In the case of having two parents with IBD, the long-term risk of developing the disease in the offspring was over 30% (53). The increased risk of IBD in dogs with a maternal history of it supports the proposed genetic component of the disease (6). However, other theories exist: Freud et al. (54) concluded that long-term morbidity of pediatric diseases in the offspring up to 18 years of age (such as cardiovascular,

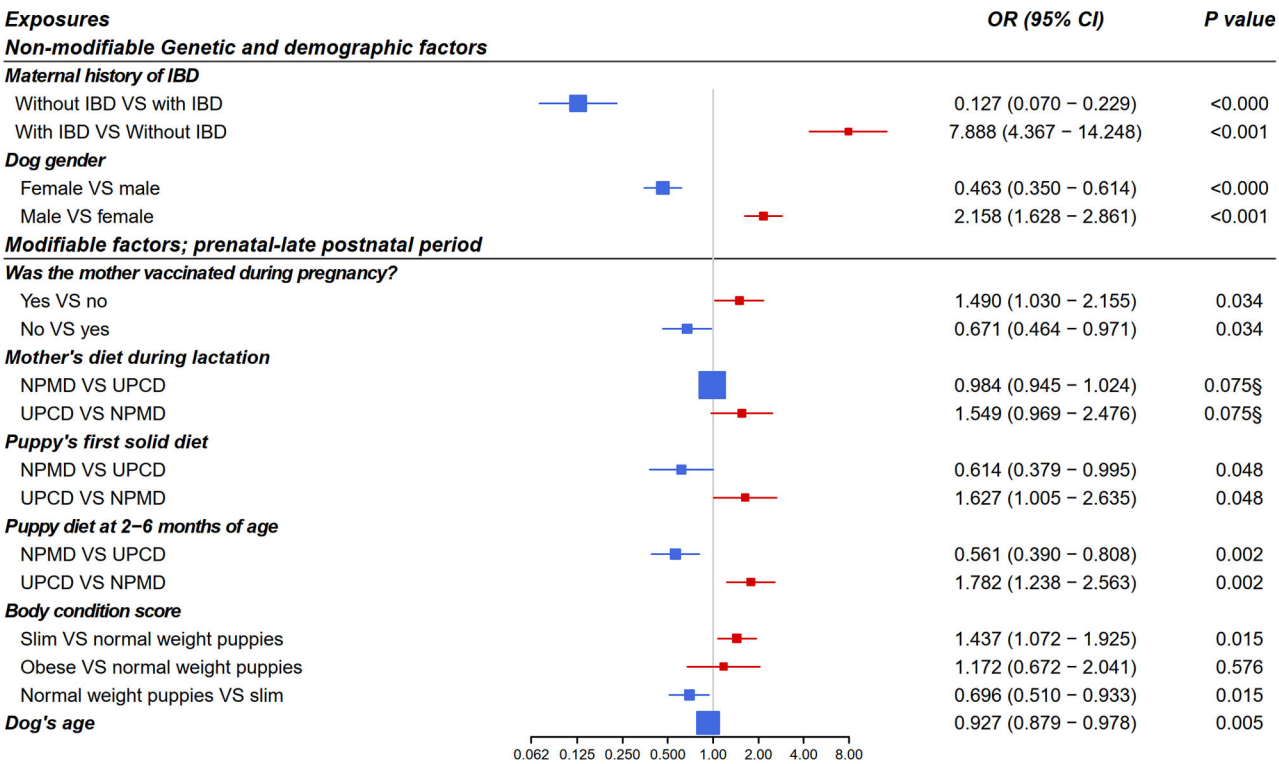

FIGURE 4 | Forest plot of odds ratios for the association of early life exposures and inflammatory bowel disease in adult dogs based on multivariate logistic regression analysis ($n = 7015$, adjusted for dog age). Included dogs for each model: model 1 ($n = 1976$), model 2 ($n = 1022$), model 3 ($n = 2218$), model 4 ($n = 1830$), and model 5 ($n = 1532$). Modifiable exposures included models from 2 to 5 as following model 2: prenatal period, model 3: neonatal period, model 4: early postnatal period, model 5: late postnatal period. OR, odds ratio; CI, confidence interval; IBD, inflammatory bowel disease; NPMD, non-processed meat based diet; UPCD, ultra-processed carbohydrate based diet; VS, versus. §non-significant $p > 0.05$.

endocrinal, respiratory, hematological, neurological, urinary, and gastrointestinal diseases) were not affected by maternal IBD during pregnancy. This suggests that genetics alone cannot explain the increased prevalence of IBD, instead it synergizes with other potential risk factors, especially diet (1, 2).

In the present study, the male dogs exhibited a higher risk of developing IBD than females did. Similarly, a Japanese study that studied the prevalence of chronic enteropathy in dogs through using an insurance-based population found that there was a slightly higher disease prevalence in males compared to female dogs (55). On the other hand, some studies have reported that there is no gender predisposition of IBD development in dogs (5, 56). As IBD is often used as an umbrella term for different diseases, the different types of IBD and the different breeds studied may be the reason for this discrepancy between different studies.

In our study, there was a significant association between the incidence of IBD and the dog's age. We found that the highest IBD prevalence within different age groups was in middle-aged dogs, from 5 to 10 years of age. This finding is in accordance with one study (5), and with anecdotal knowledge that IBD is mainly a disease of middle-aged dogs.

The incidence of IBD showed a significant difference between the IBD-prone and non-prone breed cohorts (**Figure 3**). Breed predisposition has been recognized for IBD [(7, 8), **Table 1**]. Gluten sensitive enteropathy has been reported with Irish setters (7, 8), while ulcerative colitis has been found to be most common in boxers (7, 9, 10, 12). Chronic enteropathy or protein-losing enteropathy is the most common form of IBD. This form can affect many breeds and mixed-breed dogs (2, 7, 8, 12). German shepherd dogs are more prone to develop antibiotic responsive diarrhea (13), especially tylosin responsive diarrhea (11).

Modifiable Exposures and How They Related to Owner Reported IBD

Next we present the modifiable prenatal exposures that were significantly associated with IBD prevalence in adulthood. Surprisingly, the maternal vaccination during or just before pregnancy was significantly associated with more IBD in the offspring in adulthood, while not vaccinating the dam was associated with less IBD in the offspring at adult age. Literature on the risks of prenatal exposure to vaccines and incidence of IBD later in dogs remains limited. However, our finding is in line with studies of adjuvants such as aluminum salts and mercury-containing compounds such as thimerosal, that have been reported to be involved in the development of inflammatory disorders (57, 58) and stimulation of the immune system in

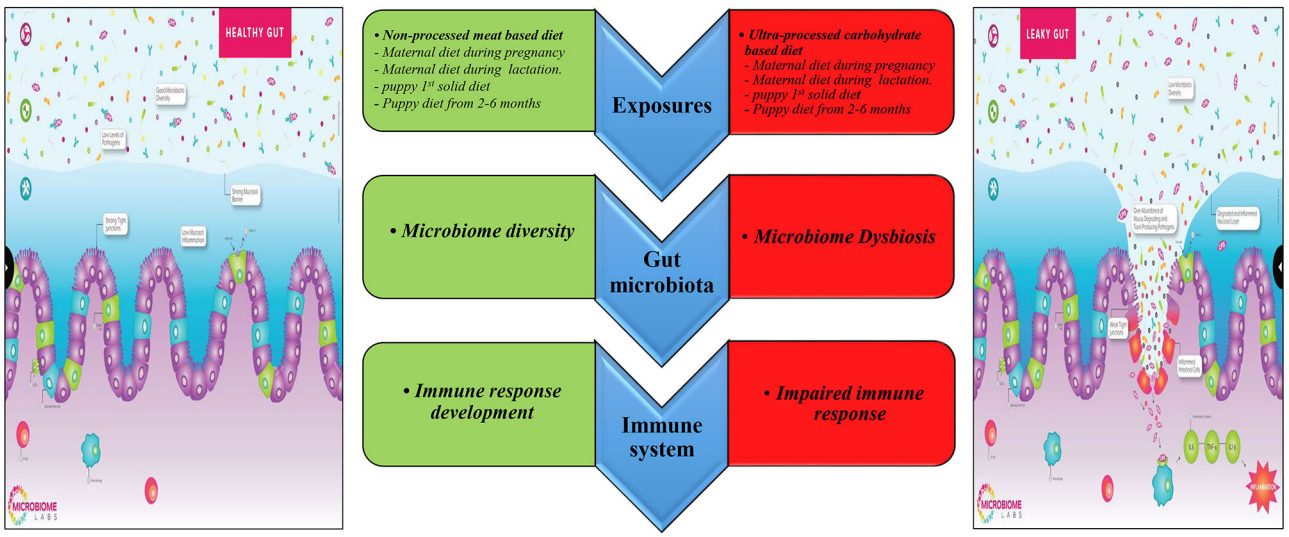

FIGURE 5 | Prospective mechanisms underlining the relationship between early diet and IBD in adult dogs. Edited from the Microbiome Labs (https://microbiomelabs.com/).

humans (59). A human study testing the effect of childhood vaccination and risk of IBD later found an association between an early life measles vaccination and the risk of IBD later (60), while two other studies found no association between measles vaccines in early life and the risk of IBD (61, 62). Moreover, a meta-analysis showed that the majority of childhood vaccinations against different infectious diseases do not increase the risk for subsequent IBD development (63). We also analyzed the associations between early life puppy vaccinations and IBD and found that these vaccinations had no association with IBD. As there results are controversial and our data only suggestive, we recommend further studies looking at this before any conclusions are drawn.

From the modifiable postnatal exposures, the maternal diet during the lactation/neonatal period appeared to be an important modifiable factor in our study, although it did not reach significance in our final models. Evidence proposes that the maternal gut microbiome may be translocated intracellularly to the mammary glands through the systemic circulation (64). This has been studied in humans, where the authors concluded that the maternal diet in the neonatal period alters the gut microbiome of the offspring, which subsequently modulates the risk of related diseases through breastfeeding (65). The role of the maternal diet during lactation has been confirmed to shape the lifelong health of the newborn human child (66). However, the direct impact of the maternal diet on the milk microbiome during pregnancy and lactation was not established in our study. Future research is needed to test how different feeding patterns in dogs during pregnancy and lactation affects the neonate gut microbiota diversity.

During the early and late postnatal periods, there were significant negative associations between the NPMD and incidence of IBD for the same dogs later in life. These findings are in accordance with several studies, which stated that a raw meat-based diet stimulated the growth of a balanced gut microbiome in healthy dogs which improved their gut function in comparison to dogs fed an extruded dry food (28, 30, 36, 67). Our observations are in accordance with the hygiene hypothesis, which states that the more microbial exposures in the early life, the more developed immune system in adulthood (22, 23). This confirms the role of the NPMD in developing the immune system when given in the early life (14, 68).

Besides the interaction between diet, gut microbiome, and the immune system discussed above (**Figure 5**), there are additional factors that may explain the effect of the early life diet on the offspring's risk of IBD in adulthood. First, the maternal and postnatal diet can permanently modify the epigenetic programming in the newborn during its formative early life. A recent review suggested that the individual becomes resistant or susceptible to diseases by altering inflammatory molecular pathways and immunity via epigenetic modification (69). However, the underlying mechanisms are not clear. Evidence suggests that the maternal gut microbiome can affect the neonate gut microbiome by causing a particular epigenetic signature that can influence the intestinal barrier's properties against inflammatory diseases (69).

Next we discuss the effects that distinct dietary components may have on gut health and physiology. The UPCD contains a high ratio of (carbohydrate and gluten-rich) processed cereal, and hence may increase the dogs' risk of developing gluten sensitive enteropathy (70–72). Furthermore, in mouse studies, the authors found that the consumption of diets rich in refined carbohydrates increased intestinal dysbiosis, permeability, and inflammation (73, 74). As sugars are highly absorbable and carbohydrates are chains of short sugar molecules, a high-carbohydrate diet may cause a high sugar concentration in

the intestinal lumen. This in turn may supply excessive energy for the microbiota and hence lead to intestinal bacterial overgrowth (75). The UPCD also contains a low dietary fiber content. Dietary fibers, also called prebiotics, are non-digestible carbohydrates responsible for maintaining normal intestinal homeostasis through encouraging bacterial diversity, protecting mucosal barriers, and increasing the synthesis of short chain fatty acids (76, 77). Furthermore, the processing methods, which the UPCD or their ingredients, are exposed to, such as rendering, milling, fermentation, heat treatment, and extrusion negatively affects the bioavailability of key minerals and vitamins present in the diet (78, 79). Feed additives, e.g., dietary emulsifiers which are used to improve kibble texture have been found to contribute to the increased incidence of inflammatory diseases both by reducing the gut microbiota diversity and by reducing the thickness of the mucus layer (80). Since dogs have no requirement for carbohydrates (81), this underlines the fact that carbohydrates should not be a main ingredient in a dog's diet (82). As carnivores, dogs have evolved to eat fresh meat-based diets rich in animal proteins, fats, and animal fibers (83, 84). A canine study showed that a raw meat-based diet was both highly palatable and highly digestible when compared to an extruded diet. Moreover, although including lots of fats and proteins, the NPMD decreased blood triglyceride levels, maintained normal serum chemistry and high fecal quality, and altered the fecal microbiota and metabolite concentration (85).

The body condition score during the late puppyhood period revealed that there was a significantly negative association between IBD development and dogs with normal body weight, while puppies with a lower body weight associated positively with developing the disease.

Prior research on IBD shows that there is an association between increase in obesity in the population and IBD prevalence (86). Obesity is a known perpetual factor of systemic low-grade inflammation (87), generating a pro-inflammatory state and immune dysregulation in obese puppies (88). Furthermore, a review also established an association between increased IBD morbidity and malnutrition (89). Additionally, a study found that young IBD patients have been demonstrated to have weight loss prior to diagnosis (90), where 57% of patients with Crohn's disease and 51% of patients with ulcerative colitis exhibited a significantly low body mass index prior to diagnosis. The association of a low body condition score during puppyhood with IBD incidence later in life maybe a reflection of active undiagnosed disease at young age. The loss in weight prior to or at the time of the disease diagnosis is attributed to many factors. As IBD is an inflammatory disease, it stimulates catabolism, alternates metabolic hormones levels, increases the gastrocolic reflux leading to loss of appetite, and causes nutrient malabsorption (90). Although incidences of IBD in puppies $<$ 6 months old have been recorded (5), it remains unclear whether the loss in body weight during puppyhood is a cause or a consequence of IBD. Further research is needed to understand the physiological disturbance underlying the association between the body condition score and active IBD.

Limitations and Strengths of the Study

The present study has some limitations. Firstly, the study design is an owner-reported cross-sectional study which makes it prone to recall bias. In order to overcome this limitation, a thorough questionnaire of the activity and dietary habits of 2,000 different aged Finnish dogs was conducted prior to this FFQ (not published). Based on these results, we constructed this DogRisk internet-based FFQ where all of the foods that were mentioned by owners in the first questionnaire were included as multiple choice questions in the new one. Two empty rows were also given to the owner, if there still would have been things missing. It is considered better to give options to owners that they can choose from when they see them written, that that they should remember all things by themselves. Another limitation is that the FFQ is based on frequencies and not quantity. Also, due to the lack of details regarding the ingredients of the food variables, we were not able to examine the nutrient profiles of the diets. Moreover, the study cases and controls were reported by the owners. We did not ask the owners for a veterinary visit wrap-up, including the diagnosis. To some extent, this has been overcome by validating different types of diseases from the FFQ in a previous validation study (39). Another limitation is that the data has a lot of missing values. This was overcome by retaining a reasonable sample size ($n = 7{,}015$).

The present study also has strengths. It is the first study to investigate early life environmental and nutritional exposures and their association with IBD incidence in dogs. It is also the first study to investigate the associations between genetic and demographic variables on IBD incidence in dogs. A wide range of covariates were covered and validated data was used (39), both which favor the study's validity, reliability and reproducibility. Furthermore, our data included information about four early life periods, pre- and postnatal, which covered 8 months of the dog's life starting from conception till the age of 6 months. Finally, reverse causality was considered and addressed in this study when choosing the age limits of the cases and controls.

CONCLUSIONS AND RECOMMENDATIONS

In conclusion, our study identified many modifiable early life risk and protective factors for canine IBD incidence in adulthood. We conclude that a raw feeding pattern, which typically features a moderate protein, high fat, and low carbohydrate macronutrient profile, during neonatal, early and late postnatal periods, as maternal and puppy diets, was associated with a decreased risk of IBD later in life. Conversely, a dry extruded feeding pattern, which mostly includes a moderate protein, low fat, and high carbohydrate macronutrient profile, during the same periods, was associated with an increased risk of developing IBD later in life. In addition, maternal vaccination during or just prior to pregnancy was significantly associated with a higher risk of IBD incidence in the offspring later. Furthermore, a normal body condition score was associated with a decreased risk of IBD development, while being abnormal in weight (very lean) was associated with an increased risk of IBD at adult age. As foretold,

the identified non-modifiable risk factors from the current study such as dogs with a history of maternal IBD, male dogs, middle-aged dogs, and dogs from breeds prone to IBD development, were all associated with an increased risk of IBD prevalence. Our novel findings regarding the modifiable risk factors provide clues for further research in the disease prevention. The study findings suggest a causal relationship but does not prove it. Future prospective longitudinal dietary intervention studies are needed to confirm our findings, as well as to develop primary strategies for IBD prevention in dogs.

DATA AVAILABILITY STATEMENT

Being funded by commercial sources has not altered our adherence to Frontiers policies on sharing data and materials. The data is still used for theses and will be totally disclosed later. However, for research purposes the data can be obtained upon request from the authors: anna.hielm-bjorkman@helsinki.fi.

ETHICS STATEMENT

The animal study was reviewed and approved by Ethical approval (29.4.2016) from the University of Helsinki Viikki campus ethical board. Written informed consent was obtained from the owners for the participation of their animals in this study.

AUTHOR CONTRIBUTIONS

MH and AH-B planned, designed, and drafted the study. AH-B organized the database. MH and AH-B performed the data extraction and did the statistical analysis together with KV who created the figures from statistical software. All authors wrote sections of the manuscript and edited it, contributed to manuscript revision, read, and approved the submitted version.

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REFERENCES

1. Simpson KW, Jergens AE. Pitfalls and progress in the diagnosis and management of canine inflammatory bowel disease. *Vet Clin North Am Small Anim Pract.* (2011) 41:381–98. doi: 10.1016/j.cvsm.2011.02.003
2. Cerquetella M, Spaterna A, Laus F, Tesei B, Rossi G, Antonelli E, et al. Inflammatory bowel disease in the dog: differences and similarities with humans. *World J Gastroenterol.* (2010) 16:1050–6. doi: 10.3748/wjg.v16.i9.1050
3. Jergens AE. Inflammatory bowel disease: current perspectives. *Vet Clin North Am Small Anim Pract.* (1999) 29:501–21. doi: 10.1016/S0195-5616(99)50032-6
4. Dandrieux JRS, Mansfield CS. Chronic enteropathy in canines: prevalence, impact and management strategies. *Vet Med.* (2019) 10:203–14. doi: 10.2147/VMRR.S162774
5. Yogeshpriya S, Veeraselvam M, Krishnakumar S, Arulkumar T, Jayalakshmi K, Saravanan M, et al. Technical review on inflammatory bowel disease in dogs and cats. *Int J Sci Environ Technol.* (2017) 6:1833–42.
6. Peiravan A, Bertolini F, Rothschild MF, Simpson KW, Jergens AE, Allenspach K, et al. Genome-wide association studies of inflammatory bowel disease in German shepherd dogs. *PLoS ONE.* (2018) 13:e0200685. doi: 10.1371/journal.pone.0200685
7. Dodds WJ. *Guide to Congenital and Deritable Disorders in Dogs. hsVma.* Davis, CA: The Humane Society Veterinary Medical Association (2011). Available online at: https://www.hsvma.org/assets/pdfs/guide-to-congenital-and-heritable-disorders.pdf
8. Kathrani A, Werling D, Allenspach K. Canine breeds at high risk of developing inflammatory bowel disease in the south-eastern UK. *Vet Rec.* (2011) 169:635. doi: 10.1136/vr.d5380
9. Davies DR, O'Hara AJ, Irwin PJ, Guilford WG. Successful management of histiocytic ulcerative colitis with enrofloxacin in two Boxer dogs. *Aust Vet J.* (2004) 82:58–61. doi: 10.1111/j.1751-0813.2004.tb14643.x
10. Stokes JE, Kruger JM, Mullaney T, Holan K, Schall W. Histiocytic ulcerative colitis in three non-boxer dogs. *J Am Anim Hosp Assoc.* (2001) 37:461–5. doi: 10.5326/15473317-37-5-461
11. Westermarck E, Skrzypczak T, Harmoinen J, Steiner JM, Ruaux CG, Williams DA, et al. Tylosin-responsive chronic diarrhea in dogs. *J Vet Intern Med.* (2005) 19:177–86. doi: 10.1111/j.1939-1676.2005.tb02679.x
12. Hall EJ. Breed-specific intestinal disease. In: *World Small Animal Veterinary Association 29th World Congress Proceedings*. Rhodes (2004).
13. Hall EJ. Antibiotic-responsive diarrhea in small animals. *Vet Clin Small Anim.* (2011) 41:273–86. doi: 10.1016/j.cvsm.2010.12.004
14. Andersena V, Olsenc A, Carbonneld F, Tjønnelandc A, Vogel U. Diet and risk of inflammatory bowel disease. *Dig Liver Dis.* (2012) 44:185–94. doi: 10.1016/j.dld.2011.10.001
15. Jostins L, Ripke S, Weersma RK, Duerr RH, McGovern DP, Hui KY, et al. Host-microbe interactions have shaped the genetic architecture of inflammatory bowel disease. *Nature.* (2012) 491:119–24. doi: 10.1038/nature11582

16. Kathrani A, Lee H, White C, Catchpole B, Murphy A, German A, et al. Association between nucleotide oligomerisation domain two (Nod2) gene polymorphisms and canine inflammatory bowel disease. *Vet Immunol Immunopathol.* (2014) 161:32–41. doi: 10.1016/j.vetimm.2014.06.003
17. Karlsson EK, Baranowska I, Wade CM, Salmon HNH, Zody MC, Anderson N, et al. Efficient mapping of mendelian traits in dogs through genome-wide association. *Nat Genet*. (2007) 1321–8. doi: 10.1038/ng.2007.10
18. van der Sloot KWJ, Weersma RK, Dijkstra G. Development and validation of a web-based questionnaire to identify environmental risk factors for inflammatory bowel disease: the Groningen IBD Environmental Questionnaire (GIEQ). *J Gastroenterol.* (2019) 54:238–48. doi: 10.1007/s00535-018-1501-z
19. Ananthakrishnan AN. Epidemiology and risk factors for IBD. *Nat Rev Gastroenterol Hepatol.* (2015) 12:205–17. doi: 10.1038/nrgastro.2015.34
20. van der Sloot KJW, Amini M, Peters V, Dijkstra G, Alizadeh BZ. Inflammatory bowel diseases: review of known environmental protective and risk factors involved. *Inflamm Bowel Dis.* (2017) 23:1499–509. doi: 10.1097/MIB.0000000000001217
21. Koloski NA, Jones M, Weltman M, Kalantar J, Bone C, Gowryshankar A, et al. Identification of early environmental risk factors for irritable bowel syndrome and dyspepsia. *Neurogastroenterol Motil.* (2015) 27:1317–25. doi: 10.1111/nmo.12626
22. Strachan DP. Hay fever, hygiene, and household size. *BMJ.* (1989) 299:1259–60. doi: 10.1136/bmj.299.6710.1259
23. Klement E, Lysy J, Hoshen M, Avitan M, Goldin E, Israeli E. Childhood hygiene is associated with the risk for inflammatory bowel disease: a population-based study. *Am J Gastroenterol.* (2008) 103:1775–82. doi: 10.1111/j.1572-0241.2008.01905.x
24. Nash MJ, Frank DN, Friedman JE. Early microbes modify immune system development and metabolic homeostasis-the "Restaurant" hypothesis revisited. *Front Endocrinol.* (2017) 8:349. doi: 10.3389/fendo.2017.00349
25. Rodríguez JM, Murphy K, Stanton C, Stanton C, Ross RP, Kober OI, et al. The composition of the gut microbiota throughout life, with an emphasis on early life. *Microb Ecol Health Dis.* (2015) 26:26050. doi: 10.3402/mehd.v26.26050
26. Parigi SM, Eldh M, Larssen P, Gabrielsson S, Villablanca EJ. Breast milk and solid food shaping intestinal immunity. *Front Immunol.* (2015) 6:415. doi: 10.3389/fimmu.2015.00415
27. Deng P, Swanson KS. Gut microbiota of humans, dogs and cats: current knowledge and future opportunities and challenges. *Br J Nutr.* (2015) 113:S6–17. doi: 10.1017/S0007114514002943
28. Sandri M, Dal Monego S, Conte G, Sgorlon S, Stefanon B. Raw meat based diet influences faecal microbiome and end products of fermentation in healthy dogs. *BMC Vet Res.* (2016) 13:65. doi: 10.1186/s12917-017-0981-z
29. David LA, Maurice CF, Carmody RN, Gootenberg DB, Button JE, Wolfe BE, et al. Diet rapidly and reproducibly alters the human gut microbiome. *Nature.* (2013) 505:559–63. doi: 10.1038/nature12820
30. Sandri M, Sgorlon S, Conte G, Serra A, Dal Monego S, Stefanon B. Substitution of a commercial diet with raw meat complemented with vegetable foods containing chickpeas or peas affects faecal microbiome in healthy dogs. *Ital J Anim Sci.* (2019) 18:1205–14. doi: 10.1080/1828051X.2019.1645624
31. Kerr KR, Forster G, Dowd SE, Ryan EP, Swanson KS. Effects of dietary cooked navy bean on the fecal microbiome of healthy companion dogs. *PLoS ONE.* (2013) 8:e74998. doi: 10.1371/journal.pone.0074998
32. Middelbos IS, Vester Boler BM, Qu A, White BA, Swanson KS, Fahey GCJ. Phylogenetic characterization of fecal microbial communities of dogs fed diets with or without supplemental dietary fiber using 454 pyrosequencing. *PLoS ONE.* (2010) 5:e9768. doi: 10.1371/journal.pone.0009768
33. Panasevich MR, Kerr KR, Dilger RN, Fahey GC Jr, Guérin-Deremaux L, Lynch GL, et al. Modulation of the faecal microbiome of healthy adult dogs by inclusion of potato fibre in the diet. *Brit J Nutr.* (2015) 113:125–33. doi: 10.1017/S0007114514003274
34. Stercova E, Kumprechtova D, Auclair E, Novakova J. Effects of live yeast dietary supplementation on nutrient digestibility and fecal microflora in beagle dogs. *J Anim Sci.* (2016) 94:2909–18. doi: 10.2527/jas.2016-0584
35. Monteiro CA, Cannon G, Moubarac JC, Levy RB, Louzada MLC, Jaime PC. The UN decade of nutrition, the NOVA food classification and the trouble with ultra-processing. *Public Health Nutr.* (2018) 21:5–17. doi: 10.1017/S1368980017000234
36. Hemida M, Vuori KA, Salin S, Moore R, Anturaniemi J, Hielm-Björkman A. Identification of modifiable pre- and postnatal dietary and environmental exposures associated with owner-reported canine atopic dermatitis in Finland using a web-based questionnaire. *PLoS ONE.* (2020) 15:e0225675. doi: 10.1371/journal.pone.0225675
37. Kathrani A, Blackwell EJ, Williams JL, Gruffydd-Jones T, Murray JK, Hezzell M, et al. Exploring early life events including diet in cats presenting for gastrointestinal signs in later life. *Vet Rec.* (2019) 185:144. doi: 10.1136/vr.105040
38. Kilian E, Suchodolski JS, Hartmann K, Mueller RS, Wess G, Unterer S. Long-term effects of canine parvovirus infection in dogs. *PLoS ONE.* (2018) 13:e0192198. doi: 10.1371/journal.pone.0192198
39. Roine J, Uusitalo L, Hielm-Bjorkman A. Validating and reliability testing the descriptive data and three different disease diagnoses of the internet-based DOGRISK questionnaire. *BMC Vet Res.* (2016) 12:30. doi: 10.1186/s12917-016-0658-z
40. Anturaniemi J, Uusitalo L, Hielm-Björkman A. Environmental and phenotype-related risk factors for owner-reported allergic/atopic skin symptoms and for canine atopic dermatitis verified by veterinarian in a Finnish dog population. *PLoS ONE.* (2017) 12:e0178771. doi: 10.1371/journal.pone.0178771
41. Dohoo I, Martin W, Stryhn H, Hilbe J, Anthony J. *Methods in Epidemiologic Research.* Charlottetown: VER Inc. (2012). p. 499–500.
42. Hosmer DW, Lemeshow S. *Applied Logistic Regression*. 2nd ed. New York, NY: Wiley (2000). doi: 10.1002/0471722146
43. Gordon M, Lumley T. Advanced forest plot using 'grid' graphics. *R Package Version 1.9.* (2019). Available online at: https://CRAN.R-project.org/package=forestplot
44. R Core Team. *R: A Language and Environment for Statistical Computing. R Foundation for Statistical Computing*. Vienna (2017). Available online at: https://www.R-project.org/
45. Vedamurthy A, Ananthakrishnan AN. Influence of environmental factors in the development and outcomes of inflammatory bowel disease. *Gastroenterol Hepatol.* (2019) 15:72–82.
46. Guo AY, Stevens BW, Wilson RG, Russell CN, Cohen MA, Sturgeon HC, et al. Early life environment and natural history of inflammatory bowel diseases. *BMC Gastroenterol.* (2014) 14:216. doi: 10.1186/s12876-014-0216-8
47. Roberts SE, Wotton CJ, Williams JG, Griffith M, Goldacre MJ. Perinatal and early life risk factors for inflammatory bowel disease. *World J Gastroenterol.* (2011) 17:743–49. doi: 10.3748/wjg.v17.i6.743
48. Kostic AD, Xavier RJ, Gevers D. The microbiome in inflammatory bowel disease:current status and the future ahead. *Gastroenterology.* (2014) 146:1489–99. doi: 10.1053/j.gastro.2014.02.009
49. Probert CS, Jayanthi V, Hughes AO, Thompson JR, Wicks AC, Mayberry JF. Prevalence and family risk of ulcerative colitis and Crohn's disease: an epidemiological study among Europeans and South Asians in Leicester-shire. *Gut.* (1993) 34:1547–51. doi: 10.1136/gut.34.11.1547
50. Halme L, Turunen U, Helio T, Paavola P, Walle T, Miettinen A, et al. Familial and sporadic inflammatory bowel disease: comparison of clinical features and serological markers in a genetically homogeneous population. *Scand J Gastroenterol.* (2002) 37:692–8. doi: 10.1080/00365520212511
51. Yang H, McElree C, Roth MP, Shanahan F, Targan SR, Rotter JI. Familial empirical risks for inflammatory bowel disease: differences between Jews and non-Jews. *Gut.* (1993) 34:517–24. doi: 10.1136/gut.34.4.517
52. Peeters M, Nevens H, Baert F, Hiele M, de Meyer AM, Vlietinck R, et al. Familial aggregation in Crohn's disease: increased age-adjusted risk and concordance in clinical characteristics. *Gastroenterology.* (1996) 111:597–603. doi: 10.1053/gast.1996.v111.pm8780562
53. Halme L, Paavola-Sakki P, Turunen U, Lappalainen M, Farkkila M, Kontula K. Family and twin studies in inflammatory bowel disease. *World J Gastroenterol.* (2006) 12:3668–72. doi: 10.3748/wjg.v12.i23.3668
54. Freud A, Beharier O, Walfisch A, Sergienko R, Landau D, Sheiner E. Maternal inflammatory bowel disease during pregnancy is not a risk factor for long-term morbidity of the offspring. *J Crohns Colitis.* (2016) 10:1267–72. doi: 10.1093/ecco-jcc/jjw083

55. Inoue M, Hasegawa A, Hosoi Y, Sugiura K. Breed, gender and age pattern of diagnosis for veterinary care in insured dogs in Japan during fiscal year 2010. *Prev Vet Med.* (2015) 119:54–60. doi: 10.1016/j.prevetmed.2015.02.010
56. Hall EJ, German AJ. Malattia infiammatoria intestinale. In: Steiner JM, editor. *Gastroenterologia del Cane e del Gatto*. Milano: Elsevier. (2009). p. 296–311.
57. Pineton de Chambrun G, Body-Malapel M, Frey-Wagner I, Djouina M, Deknuydt F, Atrott K, et al. Aluminum enhances inflammation and decreases mucosal healing in experimental colitis in mice. *Mucosal Immunol.* (2014) 7:589–601. doi: 10.1038/mi.2013.78
58. Geier DA, King PG, Hooker BS, Dórea JG, Kern JK, Sykes LK, et al. Thimerosal:clinical, epidemiologic and biochemical studies. *Clin Chim Acta.* (2015) 444:212–20. doi: 10.1016/j.cca.2015.02.030
59. Lerner A. Aluminum is a potential environmental factor for Crohn's disease induction: extended hypothesis. *Ann N Y Acad Sci.* (2007) 1107:329–45. doi: 10.1196/annals.1381.035
60. Thompson NP, Montgomery SM, Pounder RE, Wakefield AJ. Is measles vaccination a risk factor for inflammatory bowel disease? *Lancet.* (1995) 345:1071–4. doi: 10.1016/S0140-6736(95)90816-1
61. Davis RL, Kramarz P, Bohlke K, Benson P, Thompson RS, Mullooly J, et al. Measles-mumps-rubella and other measles-containing vaccines do not increase the risk for inflammatory bowel disease: a case-control study from the vaccine safety datalink project. *Arch Pediatr Adolesc Med.* (2001) 155:354–9. doi: 10.1001/archpedi.155.3.354
62. Shaw SY, Blanchard JF, Bernstein CN. Early childhood measles vaccinations are not associated with paediatric IBD: a population-based analysis. *J Crohns Colitis.* (2015) 9:334–8. doi: 10.1093/ecco-jcc/jjv029
63. Pineton de Chambrun G, Dauchet L, Gower-Rousseau C, Cortot A, Colombel JF, Peyrin-Biroulet L. Vaccination and risk for developing inflammatory bowel disease: a meta-analysis of case–control and cohort studies. *Clin Gastroenterol Hepatol.* (2015) 13:1405–15. doi: 10.1016/j.cgh.2015.04.179
64. Latuga MS, Stuebe A, Seed PC. A review of the source and function of microbiota in breast milk. *Semin Reprod Med.* (2014) 32:68–73. doi: 10.1055/s-0033-1361824
65. Chu DM, Meyer KM, Prince AL, Aagaard KM. Impact of maternal nutrition in pregnancy and lactation on offspring gut microbial composition and function. *Gut Microbes.* (2016) 7:459–70. doi: 10.1080/19490976.2016.1241357
66. Zhou X, Du L, Shi R, Chen Z, Zhou Y, Li Z. Early-life food nutrition, microbiota maturation and immune development shape life-long health. *Crit Rev Food Sci Nutr.* (2019) 59:S30–8. doi: 10.1080/10408398.2018.1485628
67. Sallander M, Adolfsson J, Bergvall K, Hedhammar Å, Nodtvedt A. The effect of early diet on canine atopic dermatitis (CAD) in three high-risk breeds. *Open Dermatol J.* (2009) 3:73–80. doi: 10.2174/1874372200903010073
68. Dixon LJ, Kabi A, Nickerson KP, McDonald C. Combinatorial effects of diet and genetics on inflammatory bowel disease pathogenesis. *Inflamm Bowel Dis.* (2015) 21:912–22. doi: 10.1097/MIB.0000000000000289
69. Indrio F, Martini S, Francavilla R, Corvaglia L, Cristofori F, Mastrolia SA, et al. Epigenetic matters: the link between early nutrition, microbiome, and long-term health development. *Front Pediatr.* (2017) 5:178. doi: 10.3389/fped.2017.00178
70. Hall EJ, Batt RM. Dietary modulation of gluten sensitivity in a naturally occurring enteropathy of Irish setter dogs. *Gut.* (1992) 33:198–205. doi: 10.1136/gut.33.2.198
71. Hall EJ, Batt RM. Delayed introduction of dietary cereal may modulate the development of gluten-sensitive enteropathy in Irish setter dogs. *J Nutr.* (1991) 121:S152–3. doi: 10.1093/jn/121.suppl_11.S152
72. Hall EJ, Batt RM. Differential sugar absorption for the assessment of canine intestinal permeability: the cellobiose/mannitol test in gluten-sensitive enteropathy of Irish setters. *Res Vet Sci.* (1991) 51:83–7. doi: 10.1016/0034-5288(91)90036-N
73. Martinez-Medina M, Denizot J, Dreux N, Robin F, Billard E, Bonnet R, et al. Western diet induces dysbiosis with increased E coli in CEABAC10 mice, alters host barrier function favouring AIEC colonisation. *Gut.* (2014) 63:116–24. doi: 10.1136/gutjnl-2012-304119
74. Kamada N, Kim YG, Sham HP, Vallance BA, Puente JL, Martens EC, et al. Regulated virulence controls the ability of a pathogen to compete with the gut microbiota. *Science.* (2012) 336:1325–9. doi: 10.1126/science.1222195
75. Steinhoff-Wagner J, Zitnan R, Schonhusen U, Pfannkuche H, Hudakova M, Metges CC, et al. Diet effects on glucose absorption in the small intestine of neonatal calves: Importance of intestinal mucosal growth, lactase activity, and glucose transporters. *J Dairy Sci.* (2014) 97:6358–69. doi: 10.3168/jds.2014-8391
76. Andoh A, Bamba T, Sasaki M. Physiological and anti-inflammatory roles of dietary fiber and butyrate in intestinal functions. *JPEN J Parenter Enteral Nutr.* (1999) 23:S70–3. doi: 10.1177/014860719902300518
77. Looijer-van LMA, Dieleman LA. Prebiotics in chronic intestinal inflammation. *Inflamm Bowel Dis.* (2009) 15:454–62. doi: 10.1002/ibd.20737
78. Satpute M, Annapure U. Approaches for delivery of heat sensitive nutrients through food systems for selection of appropriate processing techniques: a review. *J Hyg Eng Design.* (2013) 4:71–92.
79. Reddy MB, Love M. The impact of food processing on the nutritional quality of vitamins and minerals. In: Jackson LS, Knize MG, Morgan JN, ediors. *Impact of Processing on Food Safety. Advances in Experimental Medicine and Biology.* Boston, MA: Springer (1999). p. 459. doi: 10.1007/978-1-4615-4853-9_7
80. Chassaing B, Koren O, Goodrich JK, Poole AC, Srinivasan S, Ley RE, et al. Dietary emulsifiers impact the mouse gut microbiota promoting colitis and metabolic syndrome. *Nature.* (2015) 519:92–6. doi: 10.1038/nature14232
81. National Research Council. *Nutrient Requirements of Dogs*. Washington, DC: The National Academies Press (1974).
82. Hilton J. Carbohydrates in the nutrition of dog. *Can Vet J.* (1990) 31:128–9.
83. Coppinger R, Coppinger L. Dogs. In: *A startling New Understanding of Canine Origin, Behavior and Evolution. Prentice, Hall and IBD.* New York, NY: Scribner (2001).
84. Brown S. The canine ancestral diet. In: *Unlocking the Canine Ancestral Diet: Healthier Dog Food the ABC Way*. Wenatchee, WA: Dogwise Publishing (2010). p. 5–11.
85. Algya KM, Cross TL, Leuck KN, Kastner ME, Baba T, Lye L, et al. Apparent total-tract macronutrient digestibility, serum chemistry, urinalysis, and fecal characteristics, metabolites and microbiota of adult dogs fed extruded, mildly cooked, and raw diets. *J Anim Sci.* (2018) 96:3670–83. doi: 10.1093/jas/sky235
86. Ng SC, Zeng Z, Niewiadomski O, Tang W, Bell S, Kamm MA, et al. Early course of inflammatory bowel disease in a population-based inception cohort study from 8 countries in Asia and Australia. *Gastroenterology.* (2016) 150:86–95. doi: 10.1053/j.gastro.2015.11.019
87. Ellulu MS, Patimah I, Khaza'ai H, Rahmat A, Abed Y. Obesity and inflammation: the linking mechanism and the complications. *Arch Med Sci.* (2017) 13:851–63. doi: 10.5114/aoms.2016.58928
88. Cortese L, Terrazzano G, Pelagalli A. Leptin and immunological profile in obesity and its associated diseases in dogs. *Int J Mol Sci.* (2019) 20:2392. doi: 10.3390/ijms20102392
89. Waitzberg DL, Ravacci GR, Raslan M. Hospital hyponutrition. *Nutr Hosp.* (2011) 26:254–64. doi: 10.1590/S0212-16112011000200003
90. Elsherif Y, Alexakis C, Mendall M. Determinants of weight loss prior to diagnosis in inflammatory bowel disease: a retrospective observational study. *Gastroenterol Res Pract.* (2014) 2014:762191. doi: 10.1155/2014/762191

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CASE REPORT
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Case Report: Application and Limitations of a Plant-Based Diet Formulated for a Cat With Feline Lower Urinary Tract Disease

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A 2-year-old male castrated domestic shorthair cat was presented for recommendations for dietary management of chronic FLUTD using a strictly plant-based diet as per the stipulations of the cat's owner. The cat had a history of urethral obstruction of unknown etiology, persistent marked struvite crystalluria, and persistent inappropriate elimination. Commercial plant-based products meeting the nutritional recommendations for maintenance of adult cats with the lowest concentration of struvite precursors were identified, but the cat would not eat them. At the request of the client, a homemade plant-based diet was formulated with the intention of increasing water intake and promoting acidic, dilute urine. Urine concentration was able to be decreased somewhat and struvite crystalluria resolved, but the urine remained more alkaline than intended. The cat clinically improved and no further FLUTD episodes were reported by the client.

Keywords: crystalluria, feline nutrition, struvite, urethral obstruction, vegan

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INTRODUCTION

Feline lower urinary tract disorders (FLUTD) are common, potentially life-threatening problems in cats (1). Nearly 20% may be attributable to urethral obstruction, with 10–30% of obstructions attributable to urolithiasis (1–3). One of the most common uroliths are magnesium ammonium phosphate (struvite) (4, 5). Struvite has also been reported to be a common component of urethral plugs (6). Risk factors for FLUTD include breed, sex, reproductive status, age, body condition score (BCS), indoor and outdoor access, activity, number of pets in the household, potential stressors, litter management, diet, and water intake (1, 7). The focus of the case presented here is on management of nutritional risk factors.

CASE PRESENTATION

A 2-year-old male castrated domestic shorthair cat was presented to his veterinarian for suspect urethral obstruction. On presentation, he was documented to have a BCS of 3 on a 5-point scale, weighing 4.85 kg. A full, hard bladder was palpated, and white, gritty material was noted at the tip of his penis. Pre-anesthetic bloodwork revealed few abnormalities (see **Table 1**). A catheter was passed with some resistance, allowing emptying of the bladder. Copious amounts of "sand" were present in the urine. Urine pH was 8.0, cocci bacteria and large quantities of struvite crystals were detected (**Table 2**). Due to the presence of struvite crystals in the urine and small uroliths visualized by ultrasound, a canned feline veterinary therapeutic diet formulated for dissolution and prevention

TABLE 1 | Hematology and serum biochemistry at time of urethral obstruction.

Parameter	Units	Result
Hematology		
RBC	$x10^{12}$/L	9.09
HCT	%	40.3
HGB	g/dL	11.8
MCV	fL	44.3
MCH	Pg	13.0
MCHC	g/dL	29.3
RDW	%	22.7
Retic	K/μL	8.2
WBC	$x10^9$/L	14.61
Neu	$x10^9$/L	11.01
Lym	$x10^9$/L	2.91
Mono	$x10^9$/L	0.31
Eos	$x10^9$/L	0.14
Baso	$x10^9$/L	0.24
PLT	K/ μL	397
MPV	fL	15.8
Plateletcrit	%	0.63
Biochemistry		
Glucose	mmol/L	19.26
Creatinine	mmol/L	122
Urea	mmol/L	7.0
Total protein	g/L	63
Albumin	g/L	32
Globulin	g/L	31
Albumin/globulin	Ratio	1.0
ALT	U/L	38
ALKP	U/L	38
Na	mmol/L	155
K	mmol/L	8.0
Na/K	Ratio	19
Cl	mmol/L	118
Osm	mmol/kg	330

of struvite uroliths was recommended (**Table 3**). Previously, the cat had been fed commercial plant-based diets (**Table 3**). The cat was discharged with amoxicillin-clavulanic acid (62.5 mg Q12h PO, 10 days), prazosin (0.5 mg Q12h PO, 15 days, then 0.5 mg Q24h, 15 days), buprenorphine (0.08 mg Q8h PO, 16 days, tapering to 0.04 mg Q24h as required), and robenacoxib (6 mg PO SID, 6 days). Alprazolam (0.5 mg Q12h, 14 days) was also prescribed for underlying anxiety. The cat re-presented twice over the following 5 weeks with acute difficulty urinating, vocalizing, straining, and urinating outside of the litter box. No abnormalities were detected on physical examinations and urinalyses revealed improvement in urine acidity with fewer struvite crystals. Against recommendations, the client discontinued the therapeutic food as it contained animal products, and the cat was returned to his previous commercial plant-based diet.

One month later, the cat was referred to the Ontario Veterinary College Health Sciences Centre Clinical Nutrition Service for consultation regarding plant-based dietary management for FLUTD. The owner was vegan and required that they feed the cat a plant-based diet in order to comply with their ethics. On presentation, the cat had a BCS of 5/9 (8), weighing 5.0 kg. Nutritional evaluation revealed risk factors based on the WSAVA Nutritional Evaluation Checklist (9), including living in a multi-cat house, ongoing urinary signs, and being fed an unconventional diet. The cat's resting energy requirement (RER) was estimated using: $(BW^{0.75} * 70 \text{ kcal}) = 250$ kcal (10). Given the cat's activity, current bodyweight and BCS, his daily energy intake was estimated to be $RER * 1.2 = 300$ kcal. A recommendation was made for a commercial feline veterinary therapeutic diet formulated to manage signs of FLUTD (**Table 3**), which was declined by the client as it contained animal products. After discussing the challenges related to management with a plant-based diet, commercial plant-based diets were identified as alternative strategies. While detailed nutrient profiles are not typically available for many commercial plant-based diets available in Canada, nutrient analyses had been performed as part of a research project conducted by the authors (11). Diets meeting the AAFCO adult maintenance recommendations with the lowest concentration of struvite precursors were identified (**Table 3**). Based on energy density, food doses were recommended to combine kibble and canned food in a 50:50 ratio. The recommendation was made for the cat to be fed 35 g (143 kcal) kibble and 150 g (148 kcal) canned food, allowing for 10 kcal (3% of daily energy intake) to be given as treats or snacks. The client was advised on the energy and nutrient content of suitable treats and snacks. Recommendations were made to encourage water intake, feed small, frequent meals, and to supplement the diet with eicosapentaenoic acid and docosahexaenoic acid (EPA+DHA) at a dose equivalent to 0.5% of the diet on a dry matter basis (7). Recommendations to increase water intake included adding water to food, adding palatants to water, providing multiple water dishes in different locations, different materials (e.g., ceramic or metal), offering wide dishes to avoid whisker stimulation, and drinking fountains.

Three months later, the cat re-presented to his veterinarian for inappetence and weight loss. His bodyweight was recorded at 4.30 kg, though BCS was still documented as 3 on a 5-point scale. According to dietary anamnesis the cat would not eat the canned diet, nor accept soaked kibble or the DHA+EPA supplement recommended by the Clinical Nutrition Service, and he had continued to be fed the commercial plant-based diet he had been fed prior to the urethral obstruction (**Table 3**). Urine was collected by the client using non-absorbent litter (**Table 2**). A veterinary therapeutic dissolution diet was again discussed with the client as being the most evidence-based solution to mitigate risk of recurrent FLUTD signs, but the client declined this recommendation. Mirtazapine (2 mg Q24–48h as required) was prescribed to stimulate food intake. Though no dietary change was implemented, recheck examination 3 weeks later revealed a slight increase in body weight from 4.30 to 4.43 kg. Four months later, the cat re-presented for decreased urine output. At that time his body weight was 4.51 kg and his BCS was documented

TABLE 2 | Selected urinalysis characteristics from time of urethral obstruction to end of follow-up.

Parameter	Date										
	12/3/19	**25/3/19**	**18/4/19**	**1/8/19**	**1/10/19**	**29/10/19**	**8/1/20**	**27/1/20**	**14/2/20**	**20/3/20**	**28/4/20**
Diet	Kibble B and C, Canned B	Therapeutic canned	Kibble B and C				Homemade diet		Kibble A		Kibble A, Formulated diet
Weight (kg)	4.85	NR	5.03	4.30			4.51			5.80	
BCS (/9)	5	NR	5	5			5			6	
Collection method	Catheterization	Free flow		Cystocentesis			Free flow				Cystocentesis
Urine specific gravity	1.040	1.032	1.018	1.030	1.065	1.025	1.060	1.060	1.060	1.040	1.038
pH	8.0	6.0	8.0	8.5	7.5	8.0	7.0	7.0	6.0	7.0	7.0
Protein	2+	2+	–	1+	1+	2+	1+	1+	1+	Trace	Trace
Blood	4+	–	–	3+	–	–	–	–	–	-	–
Crystals	10–16 S/hpf	Occasional S	10–20 S/hpf	1–5 S/hpf	3–5 S/hpf	Minor amorphous debris	10–20 S/hpf	>40 S/hpf	1–10 S/hpf	1–10 S/hpf	–
Red blood cells	4+	N	20–30/hpf	50–75/hpf	15–20/hpf	N	0–2/hpf	N	N	N	<1/hpf
White blood cells	1+	2–4/hpf	10–15	0–2/hpf	N	N	N	N	N	N	<1/hpf
Bacteria	1+ cocci	1+ cocci	Chains	N	N	N	N	1+ cocci	N	1+ cocci	N

–, negative; S, struvite; NR, not reported.

TABLE 3 | Profile of specific nutrients of interest in commercial plant-based feline diets, compared to the recommended therapeutic diet.

	Target	Recommended veterinary therapeutic diet	Initial diet			Recommended commercial diets		Homemade diets	
			Plant-based canned A	Plant-based kibble A	Plant-based kibble B	Plant-based canned C	Plant-based kibble C	Client's homemade diet	Formulated homemade diet
Energy (kcal/100 g)	–	81.5	109.9	386.1	441.3	407.5	98.7	101.6	123.1
Base excess (mmol/kg DM)	<250	422	−51	−180	−160	−172	−72	−45	−398
Nutrient (g/100 kcal)									
Moisture	Moist	100.0	64.6	16.1	11.8	75.5	10.9	76.3	54.5
Protein	7.0–9.0	9.8	7.7	8.3	6.7	8.6	7.0	6.1	8.2
Methionine	0.10–0.38	0.18	0.11	0.18	0.16	0.13	0.17	0.12	0.32
Methionine + Cystine	>0.2	0.31	0.15	0.24	0.24	0.16	0.25	0.15	0.54
Taurine	>0.05	0.11	0.03	0.03	0.05	0.03	0.06	0.06	0.24
Fat	2.3–4.6	4.3	2.3	3.0	4.9	2.2	3.4	5.1	4.2
EPA + DHA	> 0.05	0.17	0.00	0.01	0.01	0.00	0.01	0.01	0.16
Calcium	0.14–0.30	0.26	0.30	0.20	0.28	0.16	0.18	0.24	0.28
Phosphorus	0.13–0.26	0.23	0.26	0.18	0.22	0.16	0.13	0.14	0.26
Magnesium	0.01–0.02	0.01	0.05	0.04	0.04	0.05	0.02	0.04	0.02
Sodium	0.07–0.14	0.33	0.12	0.04	0.08	0.08	0.17	0.04	0.18
Potassium	0.15	0.15	0.44	0.17	0.28	0.44	0.16	0.03	0.39
Chloride	>0.20	0.36	0.25	0.14	0.25	0.20	0.26	0.06*	0.22*
Sulfur	>0.05	NM	0.09	0.11	0.09	0.11	0.12	0.15*	0.15*

**Likely underestimated as content is not reported in the USDA database or Canadian Nutrient File for most food products NM, not measured.*

as 5 on a 9-point scale. Free-flow urine was examined again (**Table 2**), and a feline veterinary therapeutic dissolution diet was recommended, but again declined. The client elected to try to increase water intake and began preparing a home-cooked plant-based diet (**Table 4**).

Three months later, the cat was re-presented to the Clinical Nutrition service for management of inappetence. Despite the client's concerns about the cat's intake, on presentation the cat weighed 5.8 kg with a BCS of 6 on a 9-point scale, his heaviest recorded weight. Evaluation of the home-cooked plant-based diet using diet formulation software (Balance IT®) revealed the diet to be deficient in numerous nutrients, most notably meeting 0% of the cat's arachidonic acid requirement, while it provided iodine over 8 times the maximum recommended dose (**Table 3**). On a daily basis, the cat was fed: 1 tbsp homemade food, 2 tbsp of vegetables (pumpkin, green lentils, peas, squash, green beans, or corn), ¾ cup of kibble B, 1/8 tsp enzyme supplement, 250 mg methionine, and 1/8 tsp psyllium. The diet recipe was reported to have been acquired from a friend and it was unknown where the dosing had been acquired for the supplementation of enzymes, methionine, or psyllium. Treats included commercial plant-based deli slices, non-dairy cheese, potato chips, coconut yogurt, non-dairy sour cream, and dried nori seaweed. The client was made aware that the cat was slightly overweight and that his estimated daily caloric intake was more than sufficient to maintain his current bodyweight. Concern for inappetence or inadequate intake was not necessary. As the cat would not eat a canned food or soaked kibble, it was suggested to combine the kibble with preferred plant-based treats and foods the cat would eat, while also increasing water intake. The client was advised not to feed more than 10% of the daily caloric allotment from foods other than the kibble. Additionally, the methionine dose was increased from 250 to 500 mg to acidify the urine.

One month later, the cat was eating the recommended food, but struvite crystalluria persisted and the owner was concerned about inability to increase water intake on the dry extruded diet. The cat was re-presented to the Clinical Nutrition Service, weighing 5.8 kg with a BCS of 6 on a 9-point scale. A homemade plant-based diet was designed using web-based formulation software[1] (**Tables 3**, **4**) to meet the AAFCO nutrient profile for adult maintenance with low concentrations of struvite precursors, and utilized ingredients intended to promote urine acidity (12). Acidification of the urine was predicted by calculation of the base excess of the diet (13). Base excess in the food was calculated using the equation: Base excess (mmol/kg DM) = 2[Ca]+2[Mg]+[Na]+[K]−2[P]−2[Met]−[Cl] (13). Plant-based fatty acid supplementation was recommended, with plant-based arachidonic acid and EPA+DHA. As the cat was slightly overweight, a conservative energy intake was recommended to just meet his RER of 250 kcal to prevent further weight gain. The next urinalysis performed revealed a stable USG and pH, though struvite crystals were absent. The

[1]Balance IT®.

TABLE 4 | Homemade feline diet recipe, ingredients listed from highest to lowest inclusion (by weight).

Client's homemade diet	Formulated diet
Firm tofu	Pumpkin
Brown rice	Tofu
Red lentils	Carrots
Sweet potato	Soy protein isolate
Carrots	Chestnuts
Celery	Beets
Pumpkin	Beyond beef®
Green peas	Nutritional yeast
Green beans	Tomato paste
Vegetable oil	Balance IT® Feline supplement
Apple cider vinegar	Sunflower oil
Olives	Pumpkin seeds
Kelp powder	Omega-3 fatty acid oil
Spirulina or chlorella	Olives
Nutritional yeast	Desiccated coconut
Dried parsley	Arachidonic acid supplement
Catnip	Basil
Flax seeds	
Vegecat™ supplement	

client was encouraged to continue regular rechecks with their family veterinarian, and feedback from the client 9 months later revealed the cat was doing well, he continued to eat a combination of the homemade food and commercial plant-based kibble, his urine was consistently free of struvite crystals, pH maintained between 6.5–7.0, and the USG below 1.040. The owner reported no further concerns with inappetence, no changes in bodyweight, and the cat had suffered no further FLUTD episodes.

DISCUSSION

The case report presented here was of a cat with chronic FLUTD of nearly 1-year duration. A complicating factor was the client's request for the diet to be plant-based. There was a lack of client adherence to veterinary therapeutic diet recommendations, resulting in multiple relapses. This case report proposes nutritional counseling and development of a nutrition support plan that fits both the patient's nutritional requirements as well as the owner's lifestyle, habits and beliefs.

Cats evolved consuming a diet comprised exclusively of prey, resulting in evolution of anatomical, physiological, and metabolic adaptations to utilize animal tissues (14, 15). These unique adaptations have resulted in the cat being classified as an obligate carnivore (16). With recent interest being shown in feeding plant-based diets to dogs and cats (17), there has been discussion as to the practical and ethical considerations of whether carnivorous animals require animal tissues, *per se*, or if their exceptional nutritional requirements can be met with animal-free diets enriched with key essential nutrients (18, 19). In addition to the challenges of meeting a cat's nutritional requirements from only plant, mineral, and synthetic ingredients, plant-based diets have also been proposed to be a potential risk factor for urinary alkalinization (20) and thus struvite urolithiasis, though this has yet to be substantiated.

In this case, the cat was first presented for urethral obstruction, though no underlying cause (urethral plug, urolith) was reported. The cat did not re-obstruct, but persistently demonstrated other signs of FLUTD. Signs of FLUTD include: dysuria, haematuria, pollakiuria, stranguria, inappropriate urination or obstruction (2, 3). There are multiple conditions that fall under the general term of FLUTD that may cause the aforementioned signs, including anatomic abnormalities, urolithiasis, urethral plugs, urethral obstruction, infection, neoplasia, idiopathic inflammation, obstruction, and reflex dyssynergia (21). Given that imaging revealed radiopaque debris in the bladder and urinalysis showed marked crystalluria, it was assumed that struvite, either as uroliths or as a component of a urethral plug, were the primary cause, though other causes of urethral obstruction were not ruled out.

The significance of struvite crystalluria in cats with FLUTD is not known, as healthy cats have been demonstrated to have asymptomatic struvite crystalluria (22, 23). However, a recent case report regarding a cat with feline idiopathic cystitis and marked struvite crystalluria (24) supported previous hypotheses that, in the absence of other aetiological agents, persistent urinary signs may be attributable to crystalluria (25–27).

Dietary factors, low water intake, and decreased urination frequency are considered the main risk factors for FLUTD (3, 5, 28). Thus, promoting large volumes of dilute urine, and simultaneously encouraging frequent urination are the key approaches for nutritional management. Addition of anti-inflammatory nutrients such as DHA and EPA may also be beneficial (7). In cases with urolithiasis, additional nutritional interventions are indicated. Considering that struvite crystals are composed of magnesium, ammonium and phosphate, high dietary levels of these nutrients are considered to be causative (1, 29). Additionally, other minerals that may affect urinary excretion of phosphorus, such as calcium and chloride may also contribute toward struvite crystalluria. For uroliths to form, the urine environment must favor precipitation of crystals and aggregation of crystals into stones. This requires a high concentration of solutes, urine pH conducive to precipitation of phosphorus and magnesium, presence of ammonia, and a relatively low urine volume (3). Thus, intervention for dissolution and prevention of struvite urolithiasis is targeted at reducing the formation of struvite crystals and/or impairing the ability of crystals to aggregate into stones (3).

Firstly, controlling intake, and thus, to a degree, excretion of precursor minerals can reduce the substrate for crystal formation (30). The contribution of urinary protein to struvite crystalluria has been less commonly reported but demonstrated *in vitro*, making consideration of dietary protein levels warranted to reduce protein excretion (31). Protein restriction is not required, but avoidance of protein in gross excess of the recommended intake may be prudent. Concentration of precursors is a component of struvite crystalluria. The presence of ammonia

and a pH > 6.0 are required for precipitation to occur, as high urinary pH reduces the solubility of phosphorus and magnesium in the urine, allowing the ammonia to bind the minerals leading to precipitation as struvite crystals (29, 31). Manipulation of the urinary pH to maintain a slightly acidic (pH < 6.5) environment thus increases solubility of struvite, reducing crystal precipitation (30, 32). Urine pH can be manipulated by inclusion of acidic sulfur-containing amino acids and minerals such as phosphorus, chloride and sulfur, and restriction of basic minerals, such as calcium, magnesium, sodium, and potassium. Water is also a key consideration in cases with crystalluria and urolithiasis. In order to mitigate aggregation of crystals into stones, urine volume can be increased to reduce urine concentration and saturation and increase frequency of voiding (3, 33). By increasing voiding frequency, urine spends less time within the bladder, reducing the time during which uroliths can form and grow (3). High moisture diets are considered a key strategy in prevention of urolithiasis, regardless of composition (34). Manipulation of dietary sodium can also be used to increase voluntary water intake (35).

In this case, veterinary therapeutic diets were unacceptable to the client, due to the presence of animal products. Initially, commercial plant-based products with low magnesium content and base excess supportive of acidic urine formation were identified as alternative recommendations (36). First, canned products were suggested as an effective method of increasing water intake in cats (3, 37). Moisture content >73% has been reported to increase total water intake and urine volume while decreasing urine specific gravity (38). The canned food had similar magnesium content, but greater protein, sulfur amino acid and moisture content, and a lower base excess than the canned food previously fed (**Table 3**). It was hypothesized this would lead to more acidic and less concentrated urine. The cat found this canned diet unpalatable and would not eat it, so next an extruded product higher in salt (sodium chloride) was identified to increase water intake (39, 40). Additionally, it was recommended to feed multiple smaller meals to encourage more frequent drinking and reduce the magnitude of the post-prandial alkaline tide (41, 42). Finally, plant-based EPA+DHA and arachidonic acid supplements were recommended. Anti-inflammatory effect have been demonstrated for EPA and DHA, while arachidonic acid may help maintain water balance in the kidney, aside from being an essential nutrient for cats (43).

Palatability of the plant-based diets was an issue; the cat consistently refused to consume adequate amounts of diets identified as low risk for FLUTD, but preferred diets unconducive to acidification or production of dilute urine. As a result, the client elected to feed a home-prepared diet. Though the cat preferred to eat the client's home-prepared diet, it was unsuitable not only for dietary management of FLUTD but was also inadequate for adult maintenance as a result of numerous nutrient imbalances (**Table 3**). A new home-prepared diet was formulated with a nutrient profile more conducive for mitigation of FLUTD. As with the commercial diets, appropriate dietary protein, sulfur amino acids, EPA+DHA, and salt, and lower magnesium were prioritized. Reduction of urine pH was anticipated by manipulating the base excess of the diet and including sulfur-rich acidifying ingredients (12), while increased urine production was expected by increasing salt and recommendations to add water to the food and stimulate voluntary water intake. Initially, the cat's acceptance of the homemade diet formulated by the nutrition service was also limited, and the owner reported inappetence or disinterest in eating the diet. Over a 2 week period, minor modifications, such as inclusion of olives, tomato paste, and basil per the client's experience, improved the palatability of the diet and eventually the cat accepted the diet and maintained an adequate intake. No further episodes of FLUTD were reported and the client was pleased with the outcome.

None of the commercial PBD were specifically formulated for dissolution or prevention of struvite urolithiasis, nor had any undergone feeding trials to demonstrate either efficacy in promoting urinary tract health or suitability for feline adult maintenance.

Most veterinary therapeutic urinary diets undergo relative supersaturation testing to determine efficacy of dissolution or prevention of struvite uroliths (38, 44), but this testing has not been performed for the commercial plant-based or homemade diets. Instead, plant-based diets were selected for nutrient profiles as close as possible to one of several available therapeutic diets. Though the homemade diet was formulated specifically for struvite prevention, no guarantee of efficacy could be made. With the exception of a single timepoint, urinary pH was consistently neutral (7), not acidic (6–6.5) as intended. The urine concentration fluctuated greatly, but began to trend toward dilution once the homemade diet was initiated. This was not surprising, however, since the homemade diet was fed in conjunction with kibble and assorted treats, potentially contributing toward urinary alkalization. Furthermore, timing of urine collection was not recorded, nor whether urine samples were fasted or fed, all factors that influence urine pH. Though failure to acidify the urine was a clear limitation in the application of the homemade diet, resolution of clinical signs and improvement in other urinary parameters suggests adequate dietary modification to prevent FLUTD in this cat, at least during the 9 months of follow-up.

Ideally, any cat with a history of recurrent FLUTD and crystalluria should be monitored closely with repeat urinalyses every 3–6 months. Specifically, changes in pH (particularly increases in pH), increase in urine specific gravity, presence of protein in the urine or increases in crystalluria would warrant investigation and potentially a change to the diet. For a cat fed an unconventional diet, including a plant-based diet, further monitoring at 6-monthly intervals would be desired to ensure the cat is able to maintain general wellness by routine physical examination, complete blood count, and serum biochemistry.

CONCLUSION

Management of FLUTD is complex and multifactorial, with appropriate nutrition and feeding a critical component. This case presented a greater challenge due to the client's preference for a plant-based diet, as there are no diets formulated and

tested for management of FLUTD that do not contain animal ingredients. Commercial plant-based diets with nutrient profiles closest to that recommended for FLUTD were trialed but not accepted, culminating in formulation of a homemade plant-based diet. Unlike veterinary therapeutic diets that have undergone RSS testing and/or clinical trials, there is no evidence to support the efficacy of homemade formulations for mitigation of FLUTD, crystalluria, or urolithiasis. Nevertheless, 9 months post-intervention, the cat was reported by the client to be healthy and well.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author/s.

ETHICS STATEMENT

Ethical review and approval was not required for the animal study because this case report describes clinical treatment of a privately owned animal. Written informed consent was obtained from the owners for the participation of their animals in this study.

AUTHOR CONTRIBUTIONS

SD was veterinarian directly managing this case with CG's support. AV and SA supervised and mentored SD. SD wrote the report with assistance and feedback from CG, AV, and SA. All authors contributed to the article and approved the submitted version.

REFERENCES

1. Lekcharoensuk C, Osborne C, Lulich J. Epidemiologic study of risk factors for lower urinary tract diseases in cats. *J Am Vet Med Assoc*. (2001) 218:1429–35. doi: 10.2460/javma.2001.218.1429
2. Kaul E, Hartmann K, Reese S, Dorsch R. Recurrence rate and long-term course of cats with feline lower urinary tract disease. *J Feline Med Surg.* (2020) 22:544–56. doi: 10.1177/1098612X19862887
3. Kerr K. Dietary management of feline lower urinary tract symptoms. *J Anim Sci.* (2013) 91:2965–75. doi: 10.2527/jas.2012-6035
4. Houston D, Vanstone N, Moore A, Weese H, Weese J. Evaluation of 21 426 feline bladder urolith submissions to the canadian veterinary urolith centre (1998-2014). *Can Vet J*. (2016) 75:196–201.
5. Osborne C, Lulich J, Kruger J, Ulrich L, Koehler L. Analysis of 451,891 canine uroliths, feline uroliths, and feline urethral plugs from 1981 to 2007: Perspectives from the minnesota urolith center. *Vet Clin North Am Small Anim Pract*. (2008) 39:183–97. doi: 10.1016/j.cvsm.2008.09.011
6. Escolar E, Bellanato J. Analysis of feline urinary calculi and urethral plugs by infrared spectroscopy and scanning electron microscopy. *Vet Rec*. (2003) 152:625–28. doi: 10.1136/vr.152.20.625
7. Forrester S, Kruger J, Allen T. Feline lower urinary tract diseases. In: Hand MS, Thatcher CD, Remillard RL, Roudebush P, Novotny BJ, editors. *Small Animal Clinical Nutrition*. 5th ed. Topeka, KS: Mark Morris Institute (2010). p. 925–76.
8. WSAVA. *Body Condition Score (Cat)*. Global Nutrition Toolkit (2013). Available online at: https://www.wsava.org/WSAVA/media/Arpita-and-Emma-editorial/Body-Condition-Score-Cat.pdf (accessed September 24, 2020).
9. WSAVA. *Nutritional Assessment Checklist*. (2013). Available online at: https://www.wsava.org/WSAVA/media/Arpita-and-Emma-editorial/Nutritional-Assessment-Checklist.pdf (accessed September 24, 2020).
10. Gross K, Yamka R, Khoo C, Friesen K, Jewell D, Schoenherr W, et al. Macronutrients. In: Hand MS, Thatcher CD, Remillard RL, Roudebush P, Novotny BJ, editors. *Small Animal Clinical Nutrition*. 5th ed. Topeka, KS: Mark Morris Institute (2010). p. 49–105.
11. Dodd S, Shoveller A, Ma D, Fascetti A, Yu Z, Verbrugghe A. Comparison of essential nutrients in plant-based pet foods to the estimated nutrient requirements of dogs and cats. In: *ACVIM Forum*. Baltimore, MD (2020).
12. Halfen D, Kessler AdM, Trevizan L, Jeremias J, Vendramini T, Santos J, et al. Different sources of sulfur in diets of adult cats on the urinary parameters and acid-base balance. *Ciênc Rural*. (2018) 48:e20180017. doi: 10.1590/0103-8478cr20180017
13. Kienzle E, Schuknecht A, Meyer H. Influence of food composition on the urine ph in cats. *J Nutr*. (1991) 121:S87–8. doi: 10.1093/jn/121.suppl_11.S87
14. Verbrugghe A, Bakovic M. Peculiarities of one-carbon metabolism in the strict carnivorous cat and the role in feline hepatic lipidosis. *Nutrients*. (2013) 5:2811–35. doi: 10.3390/nu5072811
15. Plantinga E, Bosch G, Hendriks W. Estimation of the dietary nutrient profile of free-roaming feral cats: possible implications for nutrition of domestic cats. *Br J Nutr*. (2011) 106:S35–48
16. Morris J. Idiosyncratic nutrient requirements of cats appear to be diet-induced evolutionary adaptations. *Nutr Res Rev*. (2002) 15:153–68. doi: 10.1079/NRR200238
17. Dodd S, Cave N, Adolphe J, Shoveller A, Verbrugghe A. Plant-based (vegan) diets for pets: a survey of pet owner attitudes and feeding practices. *PLoS ONE*. (2019) 14:e0210806. doi: 10.1371/journal.pone.0210806
18. Gray C, Sellon R, Freeman L. Nutritional adequacy of two vegan diets for cats. *J Am Vet Med Assoc*. (2004) 225:1670–75. doi: 10.2460/javma.2004.225.1670
19. Semp P. *Vegan nutrition of dogs and cats*. [Master's thesis]. Veterinary University of Vienna, Vienna, Austria (2014).
20. Knight A, Leitsberger M. Vegetarian versus meat-based diets for companion animals. *Animals*. (2016) 65:57. doi: 10.3390/ani6090057
21. Osborne C, Kruger J, Lulich J. Feline lower urinary tract disorers. Definition of terms and concepts. *Vet Clin North Am Small Anim Pract*. (1996) 26:169–79. doi: 10.1016/S0195-5616(96)50200-7
22. Gunn-Moore D. Feline lower urinary tract disease. *J Feline Med Surg*. (2003) 5:133–38. doi: 10.1016/S1098-612X(02)00129-8
23. Hostutler R, Chew D, DiBartola S. Recent concepts in feline lower urinary tract disease. *Vet Clin North Am Small Anim Pract*. (2005) 35:147–70. doi: 10.1016/j.cvsm.2004.08.006
24. Bell E, Lulich J. Marked struvite crystalluria and its association with lower urinary tract signs in a cat with feline idiopathic cystitis. *Austr Vet J*. (2015) 93:332–35. doi: 10.1111/avj.12353
25. Gevaert D, De Smet B, De Wilde R. Dietary treatment of progressive feline struvite crystalluria. *J Small Anim Pract*. (1994) 35:575–80. doi: 10.1111/j.1748-5827.1994.tb03823.x
26. Gerber B, Eichenberger S, Reusch C. Guarded long-term prognosis in male cats with urethral obstruction. *J Feline Med Surg*. (2008) 10:16–23. doi: 10.1016/j.jfms.2007.06.007
27. Lund H, Krontveit R, Halvorsen I, Eggertsdóttir A. Evaluation of urinalyses from untreated adult cats with lower urinary tract disease and healthy control cats: predictive abilities and clinical relevance. *J Feline Med Surg*. (2013) 15:1086–97. doi: 10.1177/1098612X13492739
28. Houston D, Moore A, Favrin M, Hoff B. Feline urethral plugs and bladder uroliths: A review of 5484 submissions 1998–2003. *Can Vet J*. (2003) 44:974–77.
29. Gomes V, Ariza P, Borges N, Schulz F Jr., Fioravanti M. Risk factors associated with feline urolithiasis. *Vet Res Commun*. (2018) 42:87–94. doi: 10.1007/s11259-018-9710-8

30. Tefft KM, Byron JK, Hostnik ET, Daristotle L, Carmella V, Frantz N. Effect of a struvite dissolution diet in cats with naturally occurring struvite urolithiasis. *J Feline Med Surg*. (2021) 23:269–77. doi: 10.1177/1098612X20942382
31. Matsumoto K, Funaba M. Factors affecting struvite (mgnh4po46h2o) crystallization in feline urine. *Biochimicha et Biophysica Acta*. (2008) 1780:233–39. doi: 10.1016/j.bbagen.2007.09.013
32. Bartges J, Kirk C, Cox S, Moyers T. Influence of acidifying or alkalinizing diets on bone mineral density and urine relative supersaturation with calcium oxalate and struvite in healthy cats. *Am J Vet Res*. (2013) 74:1347–52. doi: 10.2460/ajvr.74.10.1347
33. Torres-Henderson C, Bunkers J, Contreras E, Cross E, Lappin M. Use of purina pro plan veterinary diet ur urinary st/ox to dissolve struvite cystoliths. *Top Comp Anim Med*. (2017) 32:49–54. doi: 10.1053/j.tcam.2017.07.007
34. Lulich J, Berent A, Adams L, Westropp J, Bartges J, Osborne C. Acvim small animal consensus recommendations on the treatment and prevention of uroliths in dogs and cats. *J Vet Int Med*. (2016) 30:1564–74. doi: 10.1111/jvim.14559
35. Xu H, Laflamme D, Long G. Effects of dietary sodium chloride on health parameters in mature cats. *J Feline Med Surg*. (2009) 11:435–41. doi: 10.1016/j.jfms.2008.10.001
36. Lulich J, Kruger J, MacLeay J, Merrills J, Paetau-Robinson I, Albasan H, et al. Efficacy of two commercially available, low-magnesium, urine-acidifying dry foods for the dissolution of struvite uroliths in cats. *J Am Vet Med Assoc*. (2013) 243:1147–53. doi: 10.2460/javma.243.8.1147
37. Carciofi A, Bazolli R, Zanni A, Kihara L, Prada F. Influence of water content and the digestibility of pet foods on the water balance of cats. *Brazil J Vet Res Anim Sci*. (2005) 42:429–34. doi: 10.11606/issn.1678-4456.bjvras.2005.26401
38. Buckley C, Hawthorne A, Colyer A, Ae S.Effect of dietary water intake on urinary output, specific gravity and relative supersaturation for calcium oxalate and struvite in the cat. *Br J Nutr*. (2011) 106:S128–30. doi: 10.1017/S0007114511001875
39. Markwell P, Buffington C, Smith B. The effect of diet on lower urinary tract diseases in cats. *J Nutr*. (1998) 128:2753S—57S. doi: 10.1093/jn/128.12.2753S
40. Hawthorne A, Markwell P. Dietary sodium promotes increased water intake and urine volume in cats. *J Nutr*. (2004) 134:2128S—29S. doi: 10.1093/jn/134.8.2128S
41. Kirschvink N, Lhoest E, Leemans J, Delvaux F, Istasse L, Gustin P, et al. Effects of feeding frequency on water intake in cats. In: *ACVIM Forum*. Baltimore, MD: American College of Veterinary Internal Medicine Record (2005).p. 476–76.
42. Finke M, Litzenberger B. Effect of food intake on urine ph in cats. *J Small Anim Pract*. (1992) 33:261–65. doi: 10.1111/j.1748-5827.1992.tb01135.x
43. Hall J, Brockman J, Davidson S, MacLeay J, Jewell D. Increased dietary long-chain polyunsaturated fatty acids alter serum fatty acid concentrations and lower risk of urine stone formation in cats. *PLoS ONE*. (2017) 12:e0187133. doi: 10.1371/journal.pone.0187133
44. Houston D, Weese H, Evason M, Biourge V, van Hoek I. A diet with a struvite relative supersaturation less than 1 is effective in dissolving struvite stones *in vivo*. *Br J Nutr*. (2011) 106:S90–2. doi: 10.1017/S0007114511000894

Conflict of Interest: The authors declare that SD is the owner of Dodd Veterinary Services and provides both veterinary care and nutritional consultation to private clients and industry partners. CG holds the Nestlé Purina Professorship in Companion Animal Nutrition at the Ontario Veterinary College, is the owner of Grant Veterinary Nutrition Services and consults with Simmons Pet Food. SA is the owner of Sit, Stay Speak Nutrition LLC and provides nutrition consultation to industry partners. AV is the Royal Canin Veterinary Diets Endowed Chair in Canine and Feline Clinical Nutrition at the Ontario Veterinary College, serves on the Health and Nutrition Advisory Board for Vetdiet and has received honoraria and research funding from various pet food manufacturers and ingredient suppliers.

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ORIGINAL RESEARCH
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Evaluating the Potential Benefit of a Combined Weight Loss Program in Dogs and Their Owners

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Introduction: Little has been published on the psychological bond between the owner and the pet, and how this might influence shared habits that could lead to overweight and obesity. Another factor that could improve the effectiveness of a weight loss plan, is that the owner would see the dog as a weight loss partner and therefore this could increase the motivation to follow the assigned diet and exercise guidelines.

Objective: The aim of this research was to evaluate the potential mutual effects of weight loss programs for both dogs and dog owners.

Methods: Two studies were conducted: In the human-centered trial, 60 dog owners were enrolled, who signed up to receive dietary and exercise recommendations to lose weight themselves during an 8 week period, from which 29 were randomly assigned to also get recommendations for their dog. For the dog-centered trial, we selected 13 dog owners that wanted their dog to lose weight during a 6 week period, from which 7 were randomly assigned to also get recommendations for themselves. The average weight loss over the time period was recorded. A questionnaire was used to evaluate diet and exercise habits, as well as information about the relationship between the dog and owner.

Results: The average human weight loss was 2.6% in the owner+dog group ($n = 29$) and 2.3% in the owner only group ($n = 31$; $p > 0.05$). Forty percent (24/60) of the dogs in the human-centered trial were overweight. The overweight dogs in the owner+dog group ($n = 12/29$) lost 3.7% of their body weight, compared to 1.2% in the overweight dogs from the owner only group ($n = 12/31$; $p > 0.05$). In the dog-centered trial, the 7 dogs in the dog+owner group lost 8.0% of their body weight, vs. 8.3% in the six dogs in the dog only group ($p > 0.05$). The owners in the dog+owner group lost 2.5% of their body weight, compared to 0.5% in the dog only group ($p > 0.05$). In both trials owners' perceived responsibility for both their own and their dogs' weight significantly increased. In addition, habit strength regarding unhealthy feeding and exercise behaviors in relation to the dogs decreased, and self-efficacy in relation to providing the dog with healthy food and exercise increased.

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Conclusion: Active weight loss in either dog owner or dog, seemed to lead to passive weight loss in the other, especially when some tools or guidelines were provided. These findings support mutual benefits of weight loss programs for dogs and dog owners, and support future weight loss programs to be a One Health approach.

Keywords: canine, obesity, weight loss, human-animal bond, overweight

INTRODUCTION

Overweight has a high prevalence in people and pets in the western world. In the US, the prevalence of human obesity [i.e., a Body Mass Index (BMI) of ≥30] in 2017–2018 was 42.4% (1), and 56% of the dog population [i.e., a Body Condition Score (BCS) of ≥6 out of 9] was classified as overweight (2). In the same period in the UK, the prevalence of human obesity was 29% (3), and 59% of the dog population was overweight (4). An association between overweight in dogs and their owners has been demonstrated, as dogs rely on their owners for their food, snacks, and activity (5). Treatment of obesity consists of dietary and exercise recommendations in both people and pets, and medication and/or surgical procedures in more severe cases in people. A healthy diet for people is diverse and includes plenty of fruits and vegetables, whole-grain products, less free sugar, less salt, less saturated fats, more unsaturated fats (oils, nuts), and plenty of water (6). Exercise does not necessarily include doing sports every day, as walking 10,000 steps daily and avoiding sitting down for too long also improves physical health (7). Dogs also benefit from a combination of healthy diet and exercise. Nutritional recommendations by veterinarians include avoiding snacks, providing less food, increased dietary fiber, increased dietary protein, decreased amounts of fat, and providing plenty of water (8). Exercise for at least 30–60 min a day is recommended for dogs to improve physical health (9).

Currently, overweight and obesity in humans and in their pets are being treated separately. However, we propose that the problem of overweight/obesity in dogs and owners may benefit from a One Health approach, where both groups are targeted together. There are several indications that dog owners may experience benefit when their dogs are following a weight loss program at the same time, and vice versa. Previous research has already shown that dogs positively affect the health status of their owners. For example, one study showed that dog owners had an average of 55 min more physical activity per week compared to people without dogs (10). Another trial showed that dog owners were more likely to achieve their own walking goals (11). Beyond the obvious explanation that dogs simply need to be walked and hence stimulate physical activity, researchers have proposed that positive health effects for dog owners also result from the commitment of the owner to the dog (12), as well as increased perceived social support (11). Indeed, dogs are typically considered an important source of social support (13), which could benefit overweight people, as social support is known to positively affect weight loss attempts (14). Mutual benefits can be suggested, as in a previous weight loss study in people and pets, dogs got more exercise due to their owners' active weight loss program (15).

The majority of publications on overweight and obesity in pets and owners explore the possible association between the lifestyle of the owner and the body condition of the pet, whereas little has been published on the psychological bond between the owner and the pet, and how this might influence shared habits that could lead to overweight and obesity. Another factor that could improve the effectiveness of a weight loss plan, is that the owner would see the dog as a weight loss partner, and therefore this could increase the motivation to follow the assigned diet and exercise guidelines. The aim of this study was to evaluate the potential mutual effects of a weight loss program for both dogs and owners on each other. The primary objective was to demonstrate if an active weight loss trial in either dog or dog owners would lead to passive weight loss in the other, irrespective of offering tools and guidelines.

MATERIALS AND METHODS

All participants voluntarily applied to be enrolled in the trials and could withdraw at any time. According to Dutch legislation, all participants provided written informed consent, as required for ethical approval.

An active weight loss trial in dog owners (human-centered trial) as well as an active weight loss trial in dogs (dog-centered trial) were conducted. Both were randomized clinical trials with two-arms. Participants were recruited by social media (Facebook messages, newspapers, local news, local radio, University website, and Twitter) and distribution of posters in dog- or obesity-related areas (veterinary practices, pet stores, doggy day care centers, dog training centers, animal shelters, apartment complexes, and a physiotherapy practice).

Inclusion criteria for the human-centered trial were: being an adult owner and caretaker of a dog, having a BMI above 25.0, and agreeing to participate by signing an informed consent. The dog's BCS was not considered an inclusion criterion, as healthy-weight dogs should be able to provide peer support to a similar extent as overweight dogs. The additional inclusion criterion for the dog-centered trial was: the dog had to have a BCS of 6 or higher, and for this trial the BMI of the owner was not considered an inclusion criterion.

The only exclusion criterion for both trials was being unable to walk together with the dog for roughly one hour per day. Data was collected at the Faculty of Veterinary Medicine, Utrecht University, Utrecht, The Netherlands. Participants were invited to the clinic for personal appointments between one researcher

(JRN for the human-centered trial, TM for the dog-centered trial) and one participant with their dog per visit.

Human-Centered Trial

Prior to the first visit, all participants filled out a food diary for 1 day for themselves and their dogs, in order for the clinician/researcher to be able to provide personal advice at the end of the consultation. Participants were invited to the clinic and provided written informed consent. Thereafter, baseline measurements took place. All baseline and final measurements were taken by the same clinician/researcher that was not blinded to the group allocation. The dog owner's body weight, fat percentage, and waist circumference were measured, as this is common practice in human obesity evaluation (16). The weight and fat percentage were measured (to the nearest 0.1 cm or 0.1%, respectively) using a Soehnle Body Balance Shape F4 weighing scale. The waist circumference was measured between the pelvic bone and the last rib. Dogs were evaluated by determining their BCS, and measuring their body weight and height. The BCS was determined by palpation and observation of the ribs, waist, abdominal tuck, pelvic area, and lumbar vertebrae (17). The dog's body weight was measured using a veterinary weighing scale, and its height was measured at the withers. Baseline steps per day for dogs and their owners were determined the day after the first clinical visit (when pedometers were distributed). Owners were instructed to walk their usual amount for that particular day.

After these measurements, a questionnaire was distributed to determine participants' baseline values of parameters concerning their motivation for and expectations of the program, current eating- and exercise habits of both owner and dog, cognitive abilities such as laziness and ability to stick to long-term goals, and about the relationship between the dog and owner. All questions were answered using a Likert-scale of one to seven (1 = totally disagree/never, 4 = neutral, 7 = totally agree/always) (**Appendix A** in **Supplementary Material**). Participants were alternately assigned to owner only or owner+dog group, and got information about the group allocation (owner+dog or owner only, and the difference between the two) at the end of trial, only when they were interested. The researcher was not blinded for the group allocation. The participants were unaware of group allocation, only the general objective (i.e., effects of dogs on a human weight loss program) was explained. Participants were instructed to adhere to a dietary and exercise recommendations for an 8 week period during spring. In the owner+dog group, dietary and exercise recommendations were given for the dog owners, as well as their dogs, whereas in the owner only group, the dog owners only received diet and exercise recommendations for themselves. Owners of healthy-weight dogs in the owner+dog group were asked to strictly adhere to their dog's eating habits, as if the dog was on a diet.

Diet recommendations were given according to the information leaflet based on the recommendations of the Dutch Nutrition Center [i.e., a diverse diet including plenty of fruits and vegetables, whole-grain products, less free sugar, less salt, less saturated fats, more unsaturated fats (oils, nuts), and plenty of water (6)]. This leaflet provided information on healthy eating, limiting unhealthy foods and reducing portion sizes. Additionally, the participants were given exercise instructions [i.e., 10,000 steps per day, not sitting too long (7)].

For the owner+dog group, additional diet and exercise recommendations for the dogs were given. The dietary intervention for the dogs was based on an ideal Body Condition Score (BCS) of five out of nine and an expected weight loss of 0.5–2.0% body weight per week. To calculate the ideal body weight, each BCS point above 5 was equal to 10% excess body weight. The resting energy requirement (RER) for the ideal body weight was then calculated with the following formula: RER = (body weight in kg)^0.75 × 293 kJ. Once the energy density of the food had been calculated, the RER was divided by the energy density to give a number of grams to feed the dog per day. The energy density of treats were also calculated and owners were instructed to reduce the usual food by the same amount of energy to compensate for treats [adapted from (8)].

They were also instructed to walk their dogs at least 60 min per day (9). Pedometers were distributed (Yamax SW-650 for humans and Tractive Motion Pet Activity Tracker for dogs) to track their numbers of steps during trial. Every day, the participants recorded the number of steps of themselves and the number of steps of their dog in a diary. Two weeks later, participants received a phone call in which experience so far and any issues relating the pedometers were addressed. Six weeks later (8 weeks after the first appointment), the participants came in for the final appointment. The same parameters were measured by the same researcher using the same tools, and the pedometers were returned. The final questionnaire included questions about participants' abidance by the program, reflection on their expectations, experienced changes in eating- and exercise habits of both owner and dog, and several forms of peer support that may or may not have occurred (**Appendix B** in **Supplementary Material**).

The study was powered to detect a 30% difference in success between groups at 8 weeks follow-up, based on a body weight percentage loss of 5%, a power of 0.80, and 2-tailed alpha of 0.05. On the basis of these assumptions, we required 48 participants per group (96 in total per trial).

Dog-Centered Trial

For the dog-centered trial all measurements were similar. The only difference was that in the dog only group, the dog owners just received diet and exercise recommendations for their overweight dogs, while in the dog+owner group the owners additionally received diet and exercise recommendations for themselves. The diet and exercise recommendations were the same for the human-centered trial and the dog-centered trial. The dog-centered trial lasted for 42 days instead of 56 days for the human-centered trial.

Analysis of Questionnaires

Most questions used a Likert scale of 1–7, however, some used a scale of 1–5, therefore, after the trial the answers of the 5-point Likert scale were translated to the 7-point Likert scale, to allow for comparison. Multi-item scales for habit strength regarding unhealthy eating and exercise behaviors (toward themselves and/or their dog, depending on group allocation),

self-efficacy (toward themselves and/or their dog, depending on group allocation), human-animal bound, compliance (toward themselves and/or their dog, depending on group allocation), support from their dog, other sources of support, and if the instructions were helpful, were computed after verifying the scales' reliabilities (Cronbach's alpha $\geq$ 0.650). For the human-centered trial, all multi-item scales were checked for correlation using a reliability test, which resulted in a Kronbach's Alpha of $\leq$0.572. As this is below 0.650, the multi-scale items were not correlated and were evaluated separately. Only significant results are presented. For the dog-centered trial the multi-scale items were pooled together because of the small sample size, and median scores per multi-scale item were used for analyses and presentation. Some items are presented separately.

Data Analysis

For the human-centered trial, adjustments were performed in measured data to attribute for different duration of participation. Outcomes in these data were translated to a duration of 55 days (8 weeks minus the first day of the first clinic visit), as participation duration varied between 47 and 78 days with a mean of 57 days. The affected parameters were weight loss of humans and dogs, waist circumference loss of humans and fat percentage loss of humans. This assumed linear loss over time, so measured outcomes were divided by the amount of days participated and then multiplied by 55.

Data analysis was performed using IBM SPSS Statistics 25. Differences between groups in continuous outcomes were analyzed using a summary independent-samples *T*-test. Additionally, to assess attribution to weight loss from different support sources (friends/family, dog, and participation in research), we analyzed the data as a cohort (irrespective of group assignment) with two measurements to increase power (when no significant difference between groups was found). For these analyses we used a linear regression and chi-squared test. A *p*-value of $<$0.05 was set as the level of significance.

RESULTS

Human-Centered Trial

Of 165 interested people, 87 people appeared eligible, and were alternately allocated to the owner+dog group ($n = 44$) and owner only group ($n = 43$). Of these 87 people, 81 started the trial, and 60 finished (69%; 29 in the owner+dog group and 31 in the owner only group, respectively; **Figure 1**). The 21 participants that canceled their 8 weeks follow-up appointment, did so because according to them, the results were not as they expected, or they were unable to follow the given instructions. For that matter,

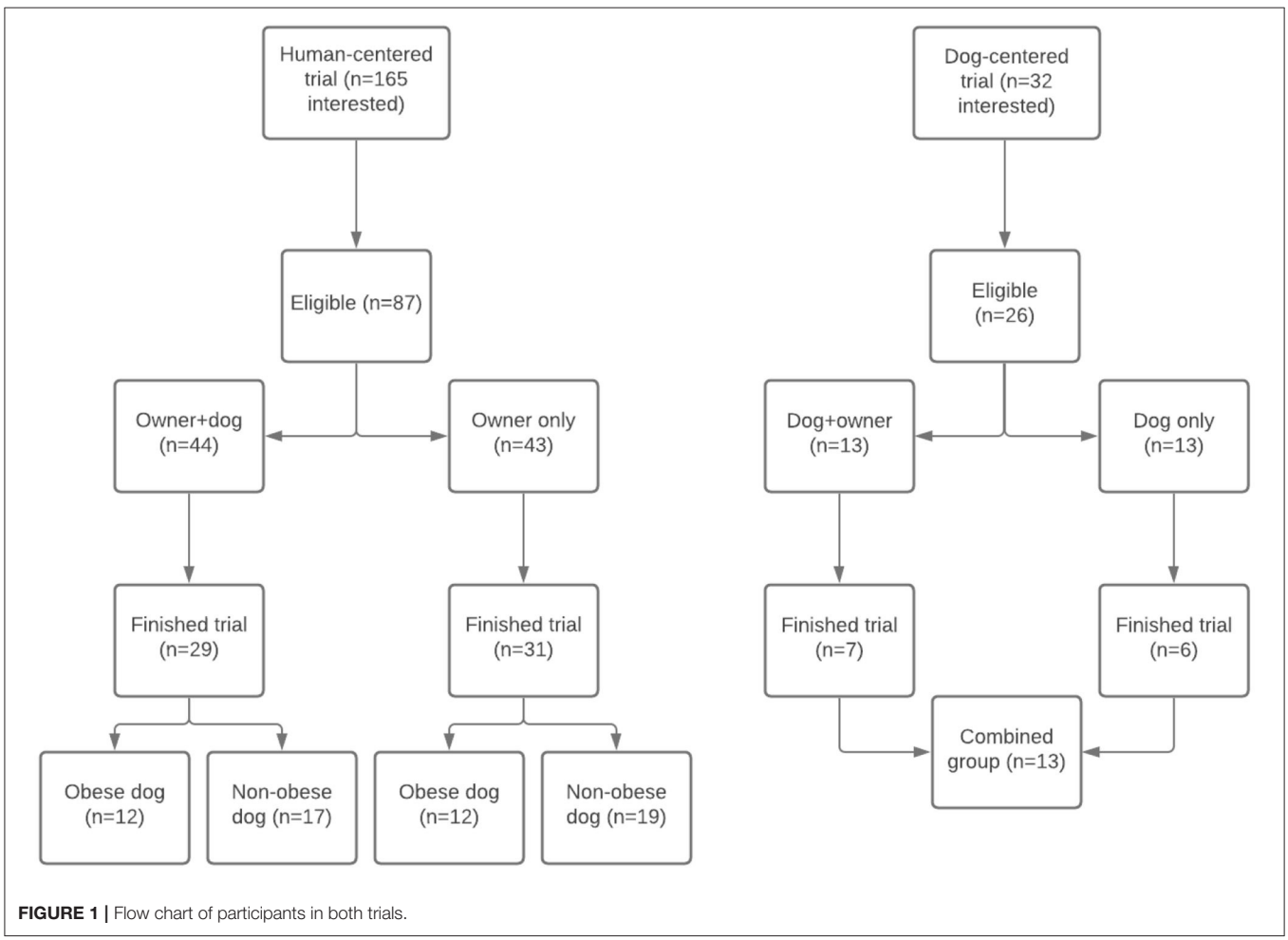

FIGURE 1 | Flow chart of participants in both trials.

our results need to be interpreted with caution, as we may have included mostly positive results. Only data of those who finished the intervention was included in data analysis. Comparability of both groups at intake was determined (**Table 1**). No large differences were found between group baseline characteristics, and these data confirmed that people in both groups needed to lose weight.

A mean weight loss of 2.6% (SD = 2.3) was achieved in the owner+dog group, and 2.3% (SD = 2.2) in the owner only group. Weight loss within groups was statistically significant, but did not differ significantly between groups (**Table 2**). The number of steps for both dog owners and dogs increased significantly in both groups, but there was no significant difference between groups. Dog owners in the owner+dog group increased their number of steps from 7,170 to 9,450 steps per day vs. 7,440 to 9,770 steps per day in the owner only group, and their dogs increased their number of steps from 3,890 to 4,450 steps per day vs. 3,380 to 3,960 steps per day, respectively.

Table 3 shows the other weight loss parameters, the effects of the interventions on the dogs, and the perceived peer support in both groups. Both men and women in both groups lost a significant amount of fat and waist circumference. No differences between groups/genders were found, only within groups. Both healthy-weight and overweight/obese dogs participated in trials. To evaluate the effect of weight loss, we only included the 24 overweight/obese dogs (12 in each group; **Figure 1**). Dogs in the intervention group lost a mean of 3.7% (SD = 5.1) body weight in 55 days, and dogs in the control group lost 1.2% (SD = 3.5). This weight loss resulted in a lower mean BCS for each group. No statistically significant difference was found between groups, but in the intervention group, the percentage of weight loss for the dogs was statistically significant, whereas in the control group it was not.

Questionnaire data of the whole group combined revealed a decrease in laziness from mean 3.7 (SD = 0.2) to 3.0 (SD = 0.2; $p = 0.006$). The unhealthy eating habits decreased [love for snacks from mean 5.4 (SD = 0.2) to 4.9 (SD = 0.2; $p = 0.002$); thoughtless snacking from mean 5.1 (SD = 0.2) to 4.2 (SD = 0.2; $p < 0.001$); snack frequency from mean 4.5 (SD = 0.2) to 3.2 (SD = 0.2; $p < 0.001$)], whereas healthy eating habits increased from mean 3.7 (SD = 0.2) to 4.9 (SD = 0.1; $p < 0.001$). Avoiding snack temptation increased from mean 2.6 (SD = 0.2) to 3.7 (SD = 0.2; $p < 0.001$). Healthy exercise habits increased from mean 4.6 (SD = 0.1) to 4.9 (SD = 0.1; $p = 0.004$). The final questionnaire revealed that in both groups dog owners experienced healthier food and exercise habits toward their dogs (mean 4.5 SD = mean 1.8, and 5.3 SD = 1.7, respectively).

Both groups of dog owners noted to have experienced peer support by their family/friends, dogs, and by the fact that they were participating in a research project, as all means are above neutral point 4 (**Table 3**). Family/friends scored lowest in both groups, followed by support from the dog, however, there was no statistically significance between these support sources. Considering no significant differences between groups for perceived peer support have been found, both groups have been combined for further analysis (**Table 4**). We found in linear regression analyses that only the motivation from participating in the research project significantly contributed to percentage weight loss ($p = 0.008$).

TABLE 1 | Baseline characteristics human-centered trial.

	Owner+dog group	Owner only group
Number of participants	29	31
Humans		
Men	31%	35.5%
Women	69%	64.5%
Age	45 y/o (range 22–71)	49 y/o (range 22–74)
BMI	33.6 kg/m^2 (SD 4.7)	31.3 kg/m^2 (SD 3.8)
Body weight	104.8 kg (SD 16.4)	95.6 kg (SD 14.0)
Fat percentage men	35.6% (SD 6.0)	31.2% (SD 4.6)
Fat percentage women	45.3 % (SD 8.1)	41.5% (SD 6.9)
Waist circumference men	122.6 cm (SD 9.3)	116.1 cm (SD 8.0)
Waist circumference women	110.0 cm (SD 12.1)	104.4 cm (SD 9.1)
Dogs		
BCS dog	5.4 (SD 1.3)	5.1 (SD 1.1)
Dogs with BCS 4–5	55.2%	51.6%
Self-reported on day 2		
Steps per day owner	7,166 steps (SD 3,287)	7,438 steps (SD 2,355)
Steps per day dog	3,887 steps (SD 1,001)	3,375 steps (SD 1,287)

Mean values are given with either range or standard deviation (SD). No significant differences were found between groups.

TABLE 2 | Weight loss results in dog owners of the human-centered trial.

Group	Start weight (SD)	End weight (SD)	Percentage lost (SD)
Owner+dog	104.8 (16.38)	102.2 (16.81)	2.6% (2.27)
Owner only	95.6 (14.05)	93.3 (13.37)	2.3% (2.17)

Weight loss results, in the owner+dog group and the owner only group, adjusted for 55 days of participation, mean and standard deviation (SD). Significant differences within groups, but not between groups.

Dog-Centered Trial

A total of 32 potential participants contacted the researcher. Of these potential candidates, 13 actually participated in the pilot, six requested more information, after which they stopped replying to emails, three agreed to participate but stopped replying before having made an appointment, four agreed to participate but changed their mind before having made an appointment due to personal circumstances, and six did not meet the inclusion criteria. Only data of those 13 candidates who finished the trials was included in exploratory analysis (**Figure 1**). Of the 13 owners that participated, 11 were female and 2 were male. The ages ranged from 24 to 61 years old with a median of 47.5 years. Of the 13 dogs that participated, six were female and seven were male. The median age was 5 years old, ranging between 2 and 9 years and the mean BCS was 7.7 (SD = 0.8). Four of the dogs were Labradors, five were mixed breeds, and there was one Golden Retriever, one Kooikerhondje, one Jack Russell, and one Tibetan Terrier. The measured weight loss of the dogs in percentages of initial body weight was significant within

TABLE 3 | Other weight loss parameters, effects on overweight dogs, and perceived peer support, human-centered trial.

Group	Fat percentage at start men (SD)	Fat percentage at end men (SD)	Percentage lost men (SD)	Fat percentage at start women (SD)	Fat percentage at end women (SD)	Percentage lost women (SD)
FAT PERCENTAGE LOSS						
Owner+dog (9 men, 20 women)	35.6 (6.05)	32.6 (4.53)	3.0 (5.0)	45.3 (8.06)	42.7 (7.94)	2.7 (3.13)
Owner only (11 men, 20 women)	31.2 (4.63)	28.3 (4.12)	2.9 (1.92)	41.5 (6.94)	40.0 (5.84)	1.5 (3.26)
Group	**Waist circumference at start men (SD)**	**Waist circumference at end men (SD)**	**Percentage lost men (SD)**	**Waist circumference at start women (SD)**	**Waist circumference at end women (SD)**	**Percentage lost women (SD)**
WAIST CIRCUMFERENCE LOSS						
Owner+dog (9 men, 20 women)	122.6 (9.33)	114.23 (15.76)	6.7 (10.99)	110.0 (12.07)	104.9 (13.85)	4.7 (6.40)
Owner only (11 men, 20 women)	116.1 (7.98)	109.3 (8.58)	5.8 (5.98)	104.5 (9.11)	93.2 (13.91)	10.7 (10.77)
Group	**Weight at start (range)**	**Weight at end (range)**	**Percentage lost (SD)**	**BCS at start (SD)**	**BCS at end (SD)**	**Percentage healthy weight dogs at the end (difference)**
EFFECTS OF THE INTERVENTIONS ON THE OVERWEIGHT DOGS						
Owner+dog (12)	22.1 (4.9–41.2)	21.1 (4.8–38.3)	3.69 (5.063)	6.8 (0.75)	5.8 (0.84)	75.9 (+20.7)
Owner only (12)	27.3 (3.6–64.0)	26.9 (3.7–63.6)	1.17 (3.503)	6.1 (0.30)	5.6 (0.52)	71.0 (+19.4)
Group	**Support from friends/family (SD)**	**Support from participating in research (SD)**	**Support from dog (SD)**	**Dog supported more than/equal to friends/family (SD)**		**Dog supported more than/equal to participating in research (SD)**
PERCEIVED PEER SUPPORT (QUESTIONNAIRE)						
Owner+dog (29)	4.6 (1.97)	5.5 (1.76)	4.9 (1.79)	5.0 (1.69)		4.6 (1.81)
Owner only (31)	4.2 (1.33)	5.6 (1.12)	4.8 (1.33)	4.9 (1.75)		4.4 (1.45)

Fat percentage loss, weight circumference loss and dog's weight loss have been adjusted for 55 days of participation, mean and range (Range) or standard deviation (SD). Perceived peer support was tested in the questionnaire on a scale of 1–7 (1 is totally disagree, 4 is neutral, and 7 is totally agree).

TABLE 4 | Comparison of sources of support (all participants combined), human-centered trial.

	Support friends/family	Support dog	Support research
Mean answer	4.4 (1.55)	4.8 (1.57)	5.5 (1.46)

The mean and standard deviation (SD) answers to the three sources of support: "dog," "friends/family," and "participating in research," on a scale of 1 to 7, where 1 is "totally disagree," 4 is "neutral," and 7 is "totally agree." All answers were included regardless of group allocation, as there was no significant difference between groups in any measured parameter.

groups, but not between groups, with a mean reduction of 6.0% (SD = 4.6) in the dog+owner group and 6.2% (SD = 4.0) in the dog only group. The reduced BCSs were also not different between groups, with a mean reduction of 0.8 points (SD = 0.6) in the dog+owner group, and 0.7 points (SD = 0.5) in the dog only group. The percentage of weight loss measured in the owners had a mean of 1.9% (SD = 2.0) in the dog+owner group and 0.4% (SD = 0.6) in the dog only group, which was not significantly different.

The measured activity of the dogs did not differ significantly between the groups. The mean of the dog+owner group was 3,809 steps per day (SD = 911), whereas that of the dog only group was 3,918 steps per day (SD = 1,857). The median number of steps made by the owners in the owner+dog group was 11,348 per day (range = 1,061 to 23,027), which was more than recommended for this trial. The daily steps were not measured in the dog only group, however in earlier studies averages have been measured around 9,500 steps per day (18), much less than the dog+owner group in this study.

The perceived responsibility for the weight loss, the habit strength, and the perceived self-efficacy of the owner for them self and their dog were measured before and after participating in the pilot for both the dog+owner group and the dog only group (**Table 5**). The owner's perceived responsibility, increased significantly for both the weight of the owner, and for the weight of the dog. There was no significant difference between groups, so the results were pooled together to increase sample size. The perceived habit strength regarding unhealthy feeding and exercise behaviors, decreased significantly in the habits related to the dogs, however, the habits in relation to the owner's diet

TABLE 5 | The mean of perceived responsibility, strength of habit, and self-efficacy of (all participants combined), dog-centered trial.

	Day 0 (SD)	Day 42 (SD)	*p*-value
Responsibility—dog	5.4 (1.4)	6.2 (1.2)	0.017
Responsibility—owner	6.1 (0.8)	6.9 (0.3)	0.036
Strength habit—dog	3.2 (1.8)	2.5 (1.6)	0.040
Strength habit—owner	3.1 (1.4)	2.8 (1.3)	0.398 (NS)
Self-efficacy—dog	4.3 (1.9)	5.7 (1.1)	0.022
Self-efficacy—owner	3.8 (1.8)	5.3 (1.5)	0.056 (NS)

The mean and standard deviation (SD) of perceived responsibility, strength of habit and self-efficacy of all participants in the active weight loss trial in dogs (n = 13) at the start and end of the trial on a scale of 1 to 7, where 1 is "totally disagree," 4 is "neutral," and 7 is "totally agree." All answers were included regardless of group allocation, as there was no significant difference between groups in any measured parameter. NS, not significant; dog, as perceived in relation to the pet; owner, as perceived in relation to the owner.

did not decrease significantly. The perceived self-efficacy of the owner for the weight loss of their dog, increased significantly. The difference in perceived self-efficacy for the owner's weight was almost significant ($p = 0.056$). When asked about the perceived effects of the study for the dog, owners in the dog+owner group scored themselves consistently worse than the owners in the do only group, however these differences were not significant. When asked whether they kept to the guidelines provided, the mean for the dog+owner group was 5.0 (SD = 2.4) compared to 6.2 (SD = 1.0) in the dog only group ($p = 0.317$). Although the question "This program had a beneficial effect on the health of my dog" differed only slightly in favor of the dog only group with a mean of 5.7 (SD = 2.3) compared to 5.5 (SD = 2.5) in the dog+owner group ($p = 0.908$), the question. "My dog has become healthier in the recent period" gave a mean of 6.7 (SD = 0.5) in the dog only group compared to 5.3 (SD = 2.3) in the dog+owner group ($p = 0.188$). However, when asked about the perceived effects on their own health "This program has motivated me to lose weight," the difference was in favor of the dog+owner group with a mean of 5.6 (SD = 1.3) compared to 3.8 (SD = 1.8) in the dog only group ($p = 0.07$).

DISCUSSION

This study aimed to explore the mutual effects of a weight loss program for both dogs and owners on each other. Based on power analysis at least 48 dog-owner pairs should have been included in each of the 4 groups. In the two conducted trials, the groups finally consisted of 29 vs. 31 pairs, and 7 vs. 6 pairs, so the trials were underpowered. The explanations can be found in unsuccessful recruitment. It was a challenge to get overweight people to a veterinary teaching hospital for treatment of their own overweight. Admitting to be overweight, and finally taking the step to do something about it is already a barrier, especially men experience barriers to start a weight loss program, which explains the lower number of men as participants (19). Surprisingly, even less people wanted their dogs to be enrolled in the weight loss trial. Unfortunately, a lot of pet owners do not recognize their dog being overweight, and if they do, they often do not consider it a condition that needs treatment (20, 21).

In this study, a mean percentage weight loss of 2.4% or 2.4 kg in 55 days (8 weeks) was found when combining both groups in the active weight loss trial in dog owners. This percentage is higher than can be expected based on previous studies evaluating the effects of diet and exercise instructions on weight loss in people. A meta-analysis determined an average of 5% (95% CI, 3.6–6.5) loss of body weight in 52 weeks (22). On the other hand, a 52 weeks is very different from an 8 weeks trial, as it also implies a higher demand for maintaining a healthy life style for a longer period of time. Another study evaluated weight loss through self-help vs. through commercial weight loss strategies found a median amount of weight loss of 2.7 kg (range = 1.5–4 kg) in a 13 weeks period (23). In the dog-centered trial, the dog owners lost on average 2.5 kg in the dog+owner group, which was similar to the amount of weight loss in the human-centered trial, whereas dog owners in the dog only group lost an average of 0.5 kg. The dogs lost on average 8.0% in the dog+owner group and 8.3% in the dog only group, respectively, in an 8 week period (i.e., 1% per week) in the dog-centered trial, and 3.7% in the owner+dog group, and 1.2% in the owner only group, respectively, in the human-centered trial (i.e., 0.3% per week). The 1% per week is a bit higher compared to levels reported in other active weight loss trials in dogs. A cohort study evaluating the success of controlled weight loss programs for obese dogs reported an average of 0.7% weight loss per week in the dogs that completed the weight loss trials (8). The 0.3% per week in the active weight loss trial in dog owners, is similar to a trial demonstrating 15% weight loss in dogs when their owners underwent a weight loss program in 52 weeks (i.e., 0.3% per week) (15).

No significant differences in weight loss parameters between the groups in both studies were found. This might be due to mutual benefits, which narrows the potential differences between groups, but it can also be due to various limiting factors. First and foremost, the group size for these trials was too small according to the power calculations to observe a significant difference between groups. It is also possible that the study's set-up did not create a big enough contrast between the intervention- and control group protocols. This was supported by the same observed effect in primary- and secondary outcomes in both groups. Furthermore, recall bias and social desirability bias most likely played a role in answering the questionnaires. A questionnaire was the only option to find out whether participants experienced peer support, but participants were aware of the research questions which may have caused socially desirable answers. Also, if dogs had a healthy weight, some questions concerning support and eating/exercise were skipped by some participants. Sometimes participants answered 1 (totally disagree/never), or sometimes 4 (neutral) to note "no difference because my dog is on healthy weight." Even though this was adjusted for in data analysis by only taking into account the answers from owners with an overweight dog (which decreased sample size for that particular question), it may have interfered with resulting means. The translation of the Likert scores from 5-point to a 7-point scale after could have influenced the results of the questionnaires. As all measurements were performed by one researcher who was aware of group allocation and all available baseline data, it is possible that information bias have influenced the measurements. Most measurements were

performed using weighing scales and, as such, are not affected. Selection may have played a role as well, as only participants who signed up for a weight loss program were observed. These people were highly motivated to work on their physique, which may have resulted in more weight loss. Also the baseline measurement of activity could have been biased, as it was only based on 1 day of activity just after the first visit to the clinic, and the participants were probably aware that they had to demonstrate an increase in activity over time. Sixty of the 87 participants finished trials (69%). In the human-centered trial, participants canceled their 8 weeks follow-up appointment mainly because results were not as they expected. This suggests we might have overestimated weight loss parameters. Lastly, the duration of the experiment may have played a role in interpreting the results. Six to eight weeks may not have been long enough to see any effects of the dog's peer support. Especially when attempting to compare to literature, 6–8 weeks follow-up is limited. On the other hand, the shorter time frame could have ensured a greater commitment on the part of the owners, which might not have happened if the program ran for 52 weeks.

No peer support from friends/family was found to affect the results of this trial, even though literature is adamant that peer support from family/friends affects weight loss. This is possibly due to the different types of peer support that can be distinguished. Literature shows controversy in dyadic support (14), but also demonstrated that group support can result in twice as much weight loss (24, 25). This study used dyadic support, and did not find a relationship between support from friends/family or dogs and weight loss results. However, support from dogs and friends/family did not differ significantly. Therefore, future studies should have a longer follow up period to allow for better comparison with literature.

Dogs appear to benefit from their weight losing owner, as the amount of dogs with a healthy BCS increased from 53.3 to 73.3% (both groups in the human-centered trial combined). This difference was statistically significant, also in the owner only group that did not receive weight loss instructions for their dog. The same is true for the benefit of the weight loss program for the dogs, as their owners also lost weight, especially when they also got some tools or guidelines (0.4% of their body weight and 1.9% of their body weight, respectively). Despite the fact that most participants mentioned to perceive peer support from dogs as well as from family/friends, this support did not significantly impact weight loss. The participants mentioned to perceive more support from their dogs compared to family/friends, however, the only significant motivator was the fact that they were participating in a research project. This is probably caused by the fact that both participants and researchers will be faced with the results (fear of embarrassment), because some participants wanted to achieve more weight loss than the others (competiveness), and because participants took participating in research very seriously (responsibility). This is confirmed by the high percentage of drop-outs (31%), amongst whom the most common reason for not returning to clinics to finish trials was the fact that weight loss had not occurred as expected. However, the support from participating in a research project does not explain any potential difference between this study's result and those found in literature, as this same support was present in all other weight loss studies. Perhaps the pedometers, which were mentioned as a form of support by some, were not included in other studies and may have contributed to the results in this study. The number of steps increased during the trial in all groups, and it appeared that setting a goal for more steps and measuring this with pedometers was effective. Furthermore, dog owners preferred to increase the number of steps (at least partly) together with their dogs. Another option is that participants benefited from peer support by the dog (and friends/family), but it was simply not found in this study due to various limitations, which is supported by the fact that we found no support from friends/family although it is known that other studies found this type of support. Considering we found friends/family and dogs supported similarly, it would be interesting to continue research in this field to confirm that dogs can also provide peer support.

The findings from the evaluation questionnaire hint toward a perceived effect of peer support by the dog on the overall health of the owner. This effect was expected due to the increased perceived social support of the dog (the weight loss partner) (13, 26) and the increased physical activity (12, 27). The perceived responsibility increased for both the owner and the dog during the trials, which could provide increased motivation for a long term weight loss plan. The perceived habit strength regarding unhealthy feeding and exercise behaviors in relation to both dogs and themselves decreased during the trials. The habit strength for giving treats and table scraps is a major underlying cause of overweight and obesity in dogs (28, 29), so this change in habit strength could contribute to the shift to a healthy lifestyle for the pet. There was also a significant increase in self-efficacy for the dog and a tendency to increased self-efficacy for the owner, which can improve the overall weight loss in overweight owners (30).

Although peer support from the dog did not significantly contribute to weight loss using current data (nor did peer support from friends/family), dogs and owners significantly lost weight during the 6 or 8 week intervention period. Support from participating in research contributed significantly to weight loss, and dogs seem to benefit from their weight-losing owner and vice versa. This is demonstrated by weight loss in dogs and dog owners in all of the groups, and by the change in habit strength and perceived self-efficacy. Considering the prevalence of obesity in humans and dogs alike, these study results show a promising option to tackling both issues at the same time. Further research with a larger group and longer intervention duration/follow-up is required for a more accurate outcome and comparison to weight-loss programmes in literature.

CONCLUSION

Active weight loss in either dog owner or dog, seemed to lead to passive weight loss in the other, especially when some tools or guidelines were provided. These findings support mutual benefits of weight loss programs for dogs and dog owners, and support future weight loss programs to be a One Health approach.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

Ethical review and approval was not required for the study on human participants in accordance with the local legislation and institutional requirements. The patients/participants provided their written informed consent to participate in this study. Ethical review and approval was not required for the animal study because this study included dogs that had to lose weight or had to maintain weight. If weight loss was needed, treatment was similar to admitted patients in the hospital, and closely monitored. Therefore, no ethical approval was needed under local legislation. Written informed consent was obtained from the owners for the participation of their animals in this study.

AUTHOR CONTRIBUTIONS

JN conducted the human-centered trial, TM conducted the dog-centered trial. MN, EM, FK, and DR were involved in study design and data analysis. RC was the supervisor of the trials and also involved in study design and data analysis. All authors were involved in drafting and adjusting the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2021.653920/full#supplementary-material

Appendices A, B are the questionnaires that were used, translated in English.

REFERENCES

1. Hales CM, Carroll MD, Fryar CD, Ogden CL. *Prevalence of Obesity and Severe Obesity Among Adults: United States, 2017-2018*. National Center for Health Statistics Data Brief No. 360 (2020).
2. Pet Obesity Prevention. *Figures on the Prevalence of Dog Overweight 2018*. (2020). Available online at: https://petobesityprevention.org/ (accessed April 10, 2020).
3. National Statistics. *Statistics on Obesity, Physical Activity and Diet, England* (2019).
4. Read C. The growth of pet obesity. *Vet Rec.* (2019) 185:1–3. doi: 10.1136/vr.l6498
5. Nijland ML, Stam F, Seidell JC. Overweight in dogs, but not in cats, is related to overweight in their owners. *Public Health Nutrition.* (2010) 13:102–6. doi: 10.1017/S136898000999022X
6. Manios Y, Kourlaba G, Grammatikaki E, Androutsos O, Moschonis G, Roma-Giannikou E, et al. Development of a diet-lifestyle quality index for young children and its relation to obesity: the Preschoolers Diet-Lifestyle Index. *Public Health Nutr.* (2010) 13:2000–9. doi: 10.1017/S1368980010000698
7. Mason MR, Ickes MJ, Campbell MS, Bollinger LM. An incentivized, workplace physical activity intervention preferentially increases daily steps in inactive employees. *Am J Health Promotion.* (2018) 32:638–45. doi: 10.1177/0890117117723803
8. German AJ, Titcomb JM, Holden SL, Queau Y, Morris PJ, Biourge V. Cohort study of the success of controlled weight loss programs for obese dogs. *J Vet Intern Med.* (2015) 29:1547–55. doi: 10.1111/jvim.13629
9. Wakshlag JJ, Struble AM, Warren BS, Maley M, Panasevich MR, Cummings KJ, et al. Evaluation of dietary energy intake and physical activity in dogs undergoing a controlled weight-loss program. *J Am Vet Med Assoc.* (2012) 240:413–19. doi: 10.2460/javma.240.4.413
10. Cutt H, Giles-Corti B, Knuiman M, Timperio A, Bull F. Understanding dog owners' increased levels of physical activity: results from RESIDE. *Am J Public Health.* (2008) 98:66–9. doi: 10.2105/AJPH.2006.103499
11. Giles-Corti B, Donovan RJ. Relative influences of individual, social environmental, and physical environmental correlates of walking. *Am J Public Health.* (2003) 93:1583–89. doi: 10.2105/AJPH.93.9.1583
12. Johnson RA, Meadows RL. Dog-walking: motivation for adherence to a walking program. *Clin Nurs Res.* (2010) 7:22. doi: 10.1177/1054773810373122
13. Schulz C, König H-H, Hajek A. Differences in self-esteem between cat owners, dog owners, and individuals without pets. *Front Vet Sci.* (2020) 7:552. doi: 10.3389/fvets.2020.00552
14. Ramchand R, Ahluwalia S, Xenakis L, Apaydin E, Raaen L, Grimm G, et al. A systematic review of peer-supported interventions for health promotion and disease prevention. *Prev Med.* (2017) 101:156–70. doi: 10.1016/j.ypmed.2017.06.008
15. Kushner RF, Blatner DJ, Jewell DE, Rudloff K. The PPET Study: people and pets exercising together. *Obesity.* (2006) 14:1762–70. doi: 10.1038/oby.2006.203
16. Corrêa CR, Formolo NPS, Dezanetti T, Speretta GFF, Nunes EA. Relative fat mass is a better tool to diagnose high adiposity when compared to body mass index in young male adults: a cross-section study. *Clin Nutr ESPEN.* (2021) 41:225–33. doi: 10.1016/j.clnesp.2020.12.009
17. Laflamme DP. Development and validation of a body condition score system for dogs; a clinical tool. *Canine Pract.* (1997) 22:10–5.
18. Bohannon RW. Number of pedometer-assessed steps taken per day by adults: a descriptive meta-analysis. *Phys Ther.* (2007) 87:1642. doi: 10.2522/ptj.20060037
19. Elliott M, Gillison F, Barnett J. Exploring the influences on men's engagement with weight loss services: a qualitative study. *BMC Public Health.* (2020) 20:249. doi: 10.1186/s12889-020-8252-5
20. Eastland-Jones RC, German AJ, Holden SL, Biourge V, Pickavance LC. Owner misperception of canine body condition persists despite use of a body condition score chart. *J Nutr Sci.* (2014) 3:e45. doi: 10.1017/jns.2014.25
21. Holmes K, Morris P, Abdulla Z, Hackett R, Rawlings J. Risk factors associated with excess body weight in dogs in the UK. *J Anim Physiol Anim Nutr.* (2007) 91:166–7. doi: 10.1111/j.1439-0396.2007.00680_9.x
22. Barte J, Veldwijk J, Teixeira P, Sacks F, Bemelmans W. Differences in weight loss across different BMI classes: a meta-analysis of the effect of interventions with diet and exercise. *Int J Behav Med.* (2014) 21:784–93. doi: 10.1007/s12529-013-9355-5
23. Heshka S, Anderson JW, Atkinson RL, Greenway FL, Hill JO, Phinney SD, et al. Weight loss with self-help compared with a structured

commercial program: a randomized trial. *JAMA*. (2003) 289:1792–8. doi: 10.1001/jama.289.14.1792
24. Cherrington A, Willig A, Agne A, Fowler M, Dutton G, Scarinci IC, et al. Development of a theory-based, peer support intervention to promote weight loss among Latina immigrants. *BMC Obes.* (2015) 2:17. doi: 10.1186/s40608-015-0047-3
25. Imanaka M, Ando M, Kitamura T, Kawamura T. Effectiveness of web-based self-disclosure peer-to-peer support for weight loss: randomized controlled trial. *J Med Internet Res.* (2013) 15:7. doi: 10.2196/jmir.2405
26. Gorin A, Phelan S, Tate D, Sherwood N, Jeffery R, Wing R. Involving support partners in obesity treatment. *J Consult Clin Psychol.* (2005) 73:341. doi: 10.1037/0022-006X.73.2.341
27. Hoerster KD, Mayer JA, Sallis JF, Pizzi N, Talley S, Pichon LC, et al. Dog walking: its association with physical activity guideline adherence and its correlates. *Prev Med.* (2011) 52:33–8. doi: 10.1016/j.ypmed.2010.10.011
28. Bland IM, Guthrie-Jones A, Taylor RD, Hill J. Dog obesity: owner attitudes and behaviour. *Prev Vet Med.* (2009) 92:333–40. doi: 10.1016/j.prevetmed.2009.08.016
29. Kienzle E, Bergler R, Mandernach A. A comparison of the feeding behavior and the human-animal relationship in owners of normal and obese dogs. *J Nutr.* (1998) 128:2779S−82S. doi: 10.1093/jn/128.12.2779S
30. Shin H, Shin J, Liu PY, Dutton GR, Abood DA, Ilich JZ, et al. Self-efficacy improves weight loss in overweight/obese postmenopausal women during a 6-month weight loss intervention. *Nutr Res.* (2011) 31:822–8. doi: 10.1016/j.nutres.2011.09.022

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White and Red Sorghum as Primary Carbohydrate Sources in Extruded Diets of Felines

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The research objectives were to evaluate the effect of dietary supplementation of white (**WSH**) and red (**RSH**) sorghum grains on gastrointestinal health of felines through the determination of apparent total tract macronutrient digestibility (**ATTD**), fecal characteristics, fermentative end-products, and microbiota, compared with a traditional corn-based diet. We hypothesize that inclusion of RSH and WSH, respectively, would be well-accepted by cats, and the RSH and WSH diets would be comparable to corn when added as the main carbohydrate source in extruded diets. Three diets containing 30% corn, 30% WSH, or 30% RSH were formulated to meet or exceed the AAFCO (2018) nutrient profiles for cats during growth. Nine male cats (0.8 ± 0.00 yr) were randomly assigned to one of the three dietary treatments using a triplicated 3 × 3 Latin square design. Experimental periods consisted of 14 d (10 d of diet adaption and 4 d of total and fresh fecal collections). The ATTD of dry matter (**DM**) did not differ amongst treatments, organic matter was greatest ($P < 0.05$) for both sorghum diets (86.4%) and lowest for the corn diet (84.2%), crude protein was comparable among diets ranging from 84.5 to 86.6%, acid hydrolyzed fat was high among diets varying between 91.4 and 92.8%, and total dietary fiber was greatest ($P < 0.05$) for the WSH diet (56.0%) with the corn diet being lowest (44.7%). Digestible energy was greatest ($P < 0.05$) for the WSH diet (4.66 kcal/g) and lowest for the corn diet (4.54 kcal/g), with the RSH diet being intermediate (4.64; $P > 0.05$). Fecal pH (6.3–6.5) and most fecal metabolites did not differ among diets except for phenol/indole concentrations that were significantly lower ($P < 0.05$) in cats fed the RSH diet (1.5 μmole/g DM) than for cats fed the corn diet (2.1 μmole/g DM). *Bacteroidetes*, *Firmicutes*, *Fusobacteria*, and *Proteobacteria* were the major phyla observed in the microbiota of feces of cats fed the three experimental diets, with no differences seen amongst all treatments. Data indicate that dietary supplementation of these varieties of WSH and RSH as carbohydrate sources were well-tolerated by the cat.

Keywords: carbohydrate, felines, gut health, microbiota, sorghum, nutrient digestibility, ancient grain

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INTRODUCTION

In recent years, there has been a trend for the companion animal sector to emulate human food market trends (1). As such, human interest in ancient grains replacing modern carbohydrate sources has reached the pet food market, and there has been increased focus on corn and wheat-free diets in pet foods. Due to the fact that sorghum (*Sorghum bicolor)* is considered an ancient grain (2),

and the United States is the largest producer of this crop (3), sorghums can be an effective substitute for more traditional carbohydrate sources (i.e., rice and corn) in companion animal nutrition.

In some regions, rice (a common carbohydrate source in pet foods) can be more costly than sorghum (4). Since carbohydrates can be included in diets at concentrations of 30–60% on a DM basis (5), formulation costs can be substantial. In the U.S., sorghum has been used as an alternative ingredient in gluten-free products to replace wheat for human consumption (6). The chemical composition of the sorghum grain on a dry matter basis (**DMB**) is similar among different varieties, being comprised mainly of starch (∼75%), protein (∼12%), lipids (∼4%), crude fiber (∼3%), and ash [∼ 2%; (7)]. The phytate and tannin concentrations in sorghum, however, can potentially lead to inconsistent utilization and digestibility of this cereal grain, which has been documented when sorghum based-diets were fed to broilers (8). In contrast, others (4, 5, 9) found their varieties of sorghum to be an acceptable carbohydrate source for canines and felines. Based on these previous studies, diets containing 55.2 and 59.3% sorghum, respectively, resulted in no detrimental effects on nutrient digestibility or fecal score, and resulted in a lower post-prandial insulin response compared with dogs fed diets containing similar amounts of rice (52.1 and 45.66%, respectively) or corn [53.5%; (10, 11)]. Additionally, the sorghum diet resulted in a lower blood glucose concentration compared with a corn diet, but with a similar insulin response for both (9).

Currently, little information is available regarding the effects of different varieties of sorghum as carbohydrate sources in pet foods and their impact on fecal fermentative end-products and microbiota of cats. Thus, the objective of this research was to evaluate the effect of dietary supplementation of red or white sorghum varieties in extruded diets on the gastrointestinal health of cats by determining apparent total tract macronutrient digestibility (**ATTD**), fecal characteristics and fermentative end-products, and fecal microbiota when compared with a corn-based extruded diet. Our hypothesis was that these varieties of red sorghum and white sorghum would be well-accepted by cats, and the red sorghum (**RSH**) and white sorghum (**WSH**) diets would be comparable to corn when added as the main carbohydrate source in extruded diets for cats.

MATERIALS AND METHODS

Animals and Experimental Design

All animal procedures were approved by the University of Illinois Institutional Animal Care and Use committee (Protocol No. 17241). Nine, intact, male domestic shorthaired cats (average age: 0.8 ± 0.00 yr; average weight: 4.5 ± 0.23 kg) were used in a triplicated 3 × 3 Latin square design. Each period consisted of 10 d of diet adaptation and 4 d of total fecal collection. Cats were housed in a temperature-controlled room in the Edward R. Madigan Laboratory at the University of Illinois at Urbana-Champaign. The room was kept on a 14 h light/10 h dark schedule. Cats were group-housed except during meal times and for the duration of fecal collection periods. Cats were randomly assigned to one of the three experimental diets and were fed to maintain body weight (**BW**) and current body condition score of 5 using a 9-point scale, which were measured weekly during each experimental period (12). Water was available *ad libitum* and feeding was done twice daily at 800 and 1,500. Cats had access to their assigned food for 2 h (800–1,000 and 1,500–1,700) when food refusals, if present, were collected and recorded. During the collection phase, cats were housed individually in cages (73.7 cm long × 152.4 cm wide), given the same access to food and water, and had visual contact with the majority of cats in the room. During collection phases, cats were allowed access to litter-free collection boxes. The morning following the 4 d of total fecal and urine collections, blood draws were conducted for complete blood count (**CBC**) and serum metabolite analyses to assess health of the cats.

Diets

Three diets containing 30% corn, 30% **WSH**, or 30% **RSH** were formulated to meet or exceed the Association of American Feed Control Officials [**AAFCO**; (13)] nutrient profiles for cats during growth. The diets were manufactured at the Grain Science and Industry Bioprocessing and Industrial Value-Added Products Innovation Center (BIVAP) at Kansas State University, Manhattan using a pilot-scale screw extruder (X-20; Wenger Manufacturing, Sabetha, KS, USA). All three experimental diets had similar ingredient composition except for the carbohydrate source (**Table 1**). Feed refusals were recorded after each meal throughout the study. Metabolizable energy requirements (100 kcal/kg $BW^{0.67}$) were used to calculate food allowance for each individual cat to maintain BW and ideal body condition score. When necessary, food intake was adjusted accordingly.

Sample Collection

Throughout the 4 d of total fecal collection, all feces were collected, weighed, and scored using the following 5-point scale: 1= hard, dry pellets; small hard mass; 2 = hard formed, remains firm and soft; 3 = soft, formed and moist stool, retains shape; 4 = soft, unformed stool; assumes shape of container; 5 = watery, liquid that can be poured. All individual fecal samples identified by cat and period were stored in a −20°C freezer until chemical analyses were conducted and **ATTD** of macronutrients was determined.

One fresh fecal sample from each cat was collected within 15 min of defecation and analyzed for dry matter (**DM**), phenols and indoles, short-chain fatty acids (**SCFA**), branched-chain fatty acids (**BCFA**), and ammonia. The pH, fecal score, and total sample weight also were determined. Dry matter was measured by drying ∼2 g of feces in duplicate in a 105°C forced air oven until all moisture was removed (48 h). Approximately 2 g of feces in duplicate were stored in plastic tubes covered in Parafilm and frozen at −20°C for subsequent indole and phenol analyses. Four grams of sample were stored in Nalgene bottles containing 4 mL of 2N hydrochloric acid and frozen at −20°C to determine SCFA, BCFA, and ammonia concentrations. Fecal samples allocated for microbiota were stored in 2 mL cryovials and stored at −80°C until analysis.

TABLE 1 | Ingredient composition of treatments containing different carbohydrate sources for adult cats.

Ingredient, % as-fed basis	Treatments[a]		
	Corn	WSH	RSH
Corn	30.00	–	–
White sorghum[b]	–	30.00	–
Red sorghum[b]	–	–	30.00
Poultry by-product meal	56.83	56.83	56.83
Corn gluten meal	6.09	6.09	6.09
Beet pulp	4.06	4.06	4.06
Sodium chloride	0.91	0.91	0.91
Potassium chloride	0.71	0.71	0.71
Choline chloride	0.51	0.51	0.51
Mineral premix[c]	0.30	0.30	0.30
Vitamin premix[c]	0.30	0.30	0.30
Myco-curb[d]	0.20	0.20	0.20
Naturox plus[d]	0.08	0.08	0.08

[a] *WSH, white sorghum; RSH, red sorghum.*

[b] *United Sorghum Checkoff Program.*

[c] *Minerals provided per kg diet: 17.4 mg manganese ($MnSO_4$), 284.3 mg iron ($FeSO_4$), 17.2 mg copper ($CuSO_4$), 2.2 mg cobalt ($CoSO_4$), 166.3 mg zinc ($ZnSO_4$), 7.5 mg iodine (KI), and 0.2 mg selenium (Na_2SeO_3). Vitamins provided per kg diet: 11,000 IU vitamin A Acetate; 900 IU vitamin D3; 57.5 IU vitamin E Acetate; 0.6 mg vitamin K; 7.6 mg thiamine HCl; 11.9 mg riboflavin; 18.5 mg pantothenic acid; 93.2 mg niacin; 6.6 mg pyridoxine HCl; 12.4 mg biotin; 1,142.1 μg folic acid; 164.9 μg vitamin B12, 0.1% mannitol.*

[d] *Myco-Curb = mold inhibitor (Kemin; Des Moines, IA); Naturox Plus = mixed-tocopherol antioxidant (Kemin; Des Moines, IA).*

After overnight fasting, 4 mL of blood were collected via jugular venipuncture from each cat at the end of each experimental period. All cats were sedated prior to collection using 0.9 ml/kg of a mix of Dexmedetomidine (0.062 mg/mL), Ketamine (62.4 mg/mL), and Butorphanol (2.5 mg/mL). Sedation was reversed by giving 0.1 mL of Atipemezole (5 mg/mL) to each cat. Blood samples from each cat were collected in BD Vacutainer serum separator tubes and EDTA tubes (Becton, Dickinson and Company, Franklin Lakes, NJ) that were used for serum metabolite and complete blood count analyses, respectively. These analyses were conducted by the Clinical Pathology Laboratory at the University of Illinois College of Veterinary Medicine (Urbana, IL).

Chemical Analyses

Fecal samples from each cat and period were pooled and dried in a 57°C oven before grinding in a Wiley mill (model 4, Thomas Scientific, Swedesboro, NJ) with a 10 mesh (2 mm) screen size and used for subsequent analyses. Dry matter, organic matter (**OM**), and ash were determined for the diets and feces using the Association of Official Analytical Chemists (AOAC) procedure [(14); methods 934.01 and 942.05]. Acid-hydrolyzed fat (**AHF**) of the diet and feces were conducted following methods of the American Association of Cereal Chemists [(15); method 30–14] and Budde et al. (16). Crude protein (**CP**) analysis was conducted by measuring total nitrogen using a LECO TruMac (Leco Corporation, St. Joseph, MI; model 630-300-300) and following the Official Method of AOAC International [(14); method 992.15]. Gross energy (**GE**) of diets and feces were measured using a Parr 6200 calorimeter (Parr Instrument Company, Moline, IL). Total dietary fiber (**TDF**) was analyzed according to Prosky et al. (17) and the Official Method of AOAC International [(14); Methods 985.29 and 991.43].

Short-chain fatty acids and BCFA concentrations were analyzed using gas chromatography with a glass 6'x1/4" ODx4mmID column and 10%SP1200/1%H_3PO_4 on 80/100 Chrom-WAW, Supleco packing and following the methods of Erwin et al. (18), Supleco Inc. (19), and Goodall and Byers (20). Gas chromatography also was used to measure phenols and indoles as cited in Flickinger et al. (21). Fecal ammonia concentrations were determined using gas chromatography according to Chaney and Marbach (22).

DNA Extraction, Amplification, Sequencing, and Bioinformatics

Total DNA from fresh fecal samples was extracted using Mo-Bio PowerSoil kits (MO BIO Laboratories, Inc., Carlsbad, CA) and DNA concentration was quantified using a Qubit® 3.0 Fluorometer (Life technologies, Grand Island, NY). Amplification of the 16S rRNA gene was completed using a Fluidigm Access Array (Fluidigm Corporation, South San Francisco, CA) in combination with Roche High Fidelity Fast Start Kit (Roche, Indianapolis, IN). The primers 515F (5′-GTGCCAGCMGCCGCGGTAA-3′) and 806R (5′-GGACTACHVGGGTWTCTAAT-3′) that target a 252 bp-fragment of V4 region was used for amplification [primers synthesized by IDT Corp., Coralville, IA; (23)]. Fluidigm specific primer forward (CS1) and reverse (CS2) tags was added according to the Fluidigm protocol. Fragment Analyzer (Advanced Analytics, Ames, IA) was used to confirm the quality of amplicons' regions and sizes. A DNA pool was generated by combining equimolar amounts of the amplicons from each sample. The pooled samples were sized selected on a 2% agarose E-gel (Life technologies, Grand Island, NY) and extracted using Qiagen gel purification kit (Qiagen, Valencia, CA). Cleaned size-selected pooled products were run on an Agilent Bioanalyzer to confirm appropriate profile and average size. Illumina sequencing was performed on a MiSeq using v3 reagents (Illumina Inc., San Diego, CA) at the W. M. Keck Center for Biotechnology at the University of Illinois. Fluidigm tags were removed using FASTX-Toolkit (version 0.0.14), and sequences were analyzed using QIIME 2.0, version 2020.6 (24) and DADA2 [version 1.14; (25)]. High quality (quality value $\geq$ 20) sequence data derived from the sequencing process were demultiplexed. Sequences then were clustered into operational taxonomic units (OTU) using opened-reference OTU picking against the SILVA 138 reference OTU database with a 97% similarity threshold (26). Singletons (OTUs that are observed fewer than two times) and OTUs that had <0.01% of the total observation were discarded. A total of 949,169 reads were obtained, with an average of 58,392 reads (range = 31,047–44,759) per sample. The dataset was rarified to 36,130 reads for analysis of diversity and species

TABLE 2 | Analyzed chemical composition of raw grains (DMB)[a].

Proximate analysis	Ingredient[a]		
	Raw corn	Raw WSH	Raw RSH
Dry matter (%)	88.4	88.3	89.6
Organic matter (%)	98.6	98.5	98.3
Ash (%)	1.4	1.5	1.7
Crude protein (%)	9.1	9.4	11.5
Acid hydrolyzed fat (%)	4.9	3.6	4.3
Gross energy (kcal/g)	4.39	4.38	4.45
Total dietary fiber (%)	13.9	12.5	15.9
Insoluble fiber (%)	11.0	9.4	13.1
Soluble fiber (%)	2.8	3.1	2.8
Starch (%)	62.7	66.4	59.2
Trypsin inhibitor (TIU/g)	751.0	n/d	514
Phytic acid (%)	1.0	1.1	1.1
Protein dispersibility index (%)[b]	45.8	16.9	14.6
Tannins (%)	0.1	0.1	0.2

[a]*DMB, dry matter basis; WSH, white sorghum; RSH, red sorghum.*
[b]*Protein dispersibility index is a means of comparing the solubility of protein in water, with a greater value indicating more solubility.*

richness. Principal coordinates analysis (**PCoA**) was performed, using both weighted and unweighted unique fraction metric (**UniFrac**) distances that measures the phylogenetic distance between sets of taxa in a phylogenetic tree as the fraction of the branch length of the tree, on the 97% OTU composition and abundance matrix (27).

Statistical Analysis

Data were analyzed using SAS (SAS Institute, INC., version 9.4, Cary, NC) using MIXED model procedures. The statistical model included the fixed effect of diet and the random effect of animal with each cat being the experimental unit. Data normality (based on residuals) was checked using the UNIVARIATE procedure of SAS. All treatment least-squares means were compared with each other and Tukey adjustment was used to control for the Type 1 experiment-wise error. P-values < 0.05 were considered statistically different, Standard errors of the mean (**SEM**) were reported as determined from the MIXED models procedure of SAS.

RESULTS

Composition of Raw Grains

Overall, the proximate analyses of the raw corn, WSH, and RSH were comparable (**Table 2**). Dry matter content ranged from 88.3 to 89.6%, OM ranged from 98.3 to 98.6%, CP ranged from 9.1 to 11.5%, AHF ranged from 3.6 to 4.9%, and GE ranged from 4.38 to 4.45 kcal/g. The TDF concentration for the raw corn, WSH, and RSH ranged from 12.5 to 15.9%. The insoluble fiber concentration varied from 9.4 to 13.1%, whereas the soluble fiber concentration fluctuated from 2.8 to 3.1%. The primary difference among grain sources was that the RSH contained more CP, TDF, and insoluble fiber than the corn and WSH.

TABLE 3 | Analyzed chemical composition of diets containing different carbohydrate sources for cats.

Item	Treatments[a]		
	Corn	WSH	RSH
Dry matter, %	94.2	94.1	94.2
		% DM[b] basis	
Organic matter	89.7	89.9	89.5
Ash	10.3	10.1	10.6
Acid hydrolyzed fat	18.0	18.9	18.1
Crude protein	45.0	44.7	44.2
Total dietary fiber	13.3	14.2	13.6
Soluble dietary fiber	4.1	5.7	4.7
Insoluble dietary fiber	9.2	8.5	8.9
Gross energy, kcal/g	5.34	5.36	5.32
Amino acids, % as-fed basis	**Treatments[a]**		
	Corn	**WSH**	**RSH**
Essential			
Arginine	2.7	2.7	2.5
Histidine	0.9	0.9	0.9
Isoleucine	1.8	1.8	1.7
Leucine	3.5	3.6	3.5
Lysine	2.5	2.5	2.4
Methionine	0.8	0.8	0.8
Phenylalanine	1.9	1.9	1.8
Threonine	1.6	1.6	1.6
Tryptophan	0.4	0.4	0.4
Valine	2.1	2.1	2.0
Taurine	0.4	0.4	0.4
Non-essential			
Alanine	2.9	2.9	2.9
Aspartate	3.4	3.4	3.2
Cysteine	0.5	0.5	0.5
Glutamate	5.7	5.9	5.7
Glycine	3.6	3.4	3.4
Proline	2.8	2.7	2.7
Serine	1.6	1.6	1.5
Tyrosine	1.4	1.5	1.4

[a]*WSH, white sorghum; RSH, red sorghum.*
[b]*DM, dry matter.*

Food Intake, Apparent Total Tract Digestibility of Macronutrients, and Fecal Characteristics

All three diets were formulated targeting a similar nutrient profile and to be isonitrogenous and isocaloric (**Table 1**). Analyzed chemical composition of the experimental diets revealed that all diets had similar nutrient composition (**Table 3**).

Daily food intake (DMB), fecal output g/d (as-is), and fecal score did not differ among treatments (**Table 4**). However, fecal output (g/d; DMB) for cats fed the RSH diet was lower (P

TABLE 4 | Body weight, food intake, fecal characteristics, and total tract apparent macronutrient digestibility of cats fed diets containing different carbohydrate sources.

Item	Treatments[a]			SEM[b]
	Corn	WSH	RSH	
Body weight (kg)	4.7	4.7	4.7	0.17
Food intake, g/d (DMB)	74.3	73.9	74.1	0.92
Fecal output, g/d (as-is)	44.5	40.6	40.7	2.11
Fecal output, g/d (DMB)	16.1[c]	14.0[cd]	14.0[c]	0.62
Fecal score	2.4	2.5	2.3	0.09
Digestibility, %				
Dry matter	78.3	81.0	81.1	0.88
		% DM[a] basis		
Organic matter	84.2[d]	86.4[c]	86.4[c]	0.67
Acid hydrolyzed fat	91.4	92.8	92.8	0.48
Crude protein	84.5	86.0	86.6	0.73
Total dietary fiber	44.7[d]	56.0[c]	53.2[c]	2.25
Digestible energy, kcal/g	4.54[d]	4.66[c]	4.64[cd]	0.03

[a]*DMB, dry matter basis, DM, dry matter; WSH, white sorghum; RSH, red sorghum.*
[b]*Standard error of the mean.*
[cd]*Means within a row with no common superscript letter are different ($P < 0.05$).*

TABLE 5 | Fecal fermentative-end products for cats fed diets containing different carbohydrate sources.

Item, μmole/g DM[a]	Treatments[b]			SEM[c]
	Corn	WSH	RSH	
Fecal pH	6.5	6.3	6.3	0.13
Ammonia	114.6	127.4	129.1	8.46
Total phenols/indoles	2.1[e]	1.9[ef]	1.5[f]	0.14
Phenols[d]	ND	ND	ND	–
Indoles	2.1[e]	1.9[ef]	1.5[f]	0.13
Total short-chain fatty acids	364.0	359.3	404.0	35.32
Acetate	236.6	231.6	263.4	25.31
Propionate	89.8	82.9	94.8	8.22
Butyrate	37.7	44.8	45.8	3.56
Total branched-chain fatty acids	25.8	27.9	30.1	2.35
Isobutyrate	5.9	5.8	6.5	0.44
Isovalerate	8.3	8.6	9.2	0.68
Valerate	11.6	13.5	14.4	1.62

[a]*DM, dry matter, all values except fecal pH expressed as μmole/g DM.*
[b]*WSH, white sorghum; RSH, red sorghum.*
[c]*Standard error of the mean.*
[d]*Not detected.*
[ef]*Means within a row with no common superscript letter are different ($P < 0.05$).*

< 0.05) than for the corn diet, with the WSH diet being intermediate ($P > 0.05$; **Table 4**). The ATTD of DM, CP, and AHF were not affected by treatment. Organic matter and TDF digestibility for cats fed WSH and RSH diets were not different from one another, but were greater ($P < 0.05$) than for the corn diet. Digestible energy of the WSH diet was higher ($P < 0.05$) than for corn, with RSH being intermediate ($P > 0.05$).

Fecal pH and concentrations of ammonia (μmole/g DMB) did not differ among treatments, while phenol concentrations were not detected in sufficient quantities among treatments (**Table 5**). Similarly, fecal concentrations of SCFA and BCFA did not differ among treatments. However, total phenols/indoles and indoles alone were higher ($P < 0.05$) for the corn treatment compared with the RSH treatment, the WSH diet was intermediate ($P > 0.05$; **Table 5**).

Serum metabolite profiles of cats fed diets containing WSH, RSH, or corn were mostly within reference ranges for healthy cats and did not differ among treatments, except for calcium which was highest ($P < 0.05$) for WSH and lowest for RSH, corn was intermediate, but still was within reference range (**Table 6**). Metabolites in excess of the reference range (i.e., glucose and phosphorus) might be due to the administration of the sedative, and were not affected by treatment. Likewise, CBC results were normal among all cats and did not differ among dietary treatments (data not shown).

TABLE 6 | Fasted serum metabolite profiles for cats fed diets containing different carbohydrate sources.

Item	Reference range	Treatments[a]			SEM[b]
		Corn	WSH	RSH	
Creatinine, mg/dL	0.4–1.6	1.41	1.50	1.50	0.069
Blood urea nitrogen, mg/dL	18–38	23.56	24.44	24.11	0.809
Total protein, g/dL	5.8–8.0	6.24	6.36	6.23	0.091
Albumin, g/dL	2.8–4.1	3.34	3.38	3.37	0.045
Globulin, g/dL	2.6–5.1	2.90	2.98	2.87	0.082
Albumin/globulin, g/dL	0.6–1.1	1.16	1.13	1.18	0.042
Calcium, mg/dL	8.8–10.2	9.30[cd]	9.44[c]	9.24[d]	0.075
Phosphorus, mg/dL	3.2–5.3	5.81	5.90	5.83	0.149
Sodium, mmol/L	145–157	149.00	149.22	149.56	0.332
Potassium, mmol/L	3.6–5.3	4.32	4.32	4.27	0.085
Sodium/potassium ratio	28–36	34.56	34.67	35.22	0.719
Chloride, mmol/L	109–126	115.33	115.22	115.56	0.440
Glucose, mg/dL	60–122	143.00	133.78	146.67	10.448
Alkaline phosphatase total, U/L	10–85	52.78	57.00	53.56	3.770
Alanine aminotransferase, U/L	14–71	47.44	49.22	49.56	4.036
Gamma-glutamyl transferase, U/L	0–3	0.33	0.00	0.22	0.128
Total bilirubin, mg/dL	0.0–0.3	0.11	0.11	0.11	0.011
Creatine kinase, U/L	10–250	233.11	311.22	321.67	37.279
Cholesterol total, mg/dL	66–160	129.44	134.56	117.67	10.286
Triglycerides, mg/dL	21–166	38.33	40.22	37.00	3.720
Bicarbonate (TCO_2), mmol/L	12–21	19.33	19.11	19.33	0.311
Anion gap	10–27	18.56	19.22	18.89	0.539

[a]*WSH, white sorghum; RSH, red sorghum.*
[b]*Standard error of the mean.*
[cd]*Means within a row with no common superscript letter are different ($P < 0.05$).*

Fecal Microbial Populations

Beta-diversity based on unweighted (**Figure 1A**) and weighted (**Figure 1B**) UniFrac distances and alpha-diversity did not differ in feces of cats fed corn, WSH, or RSH (**Figure 2**). A total of six bacterial phyla were observed with Firmicutes, Bacteroidetes,

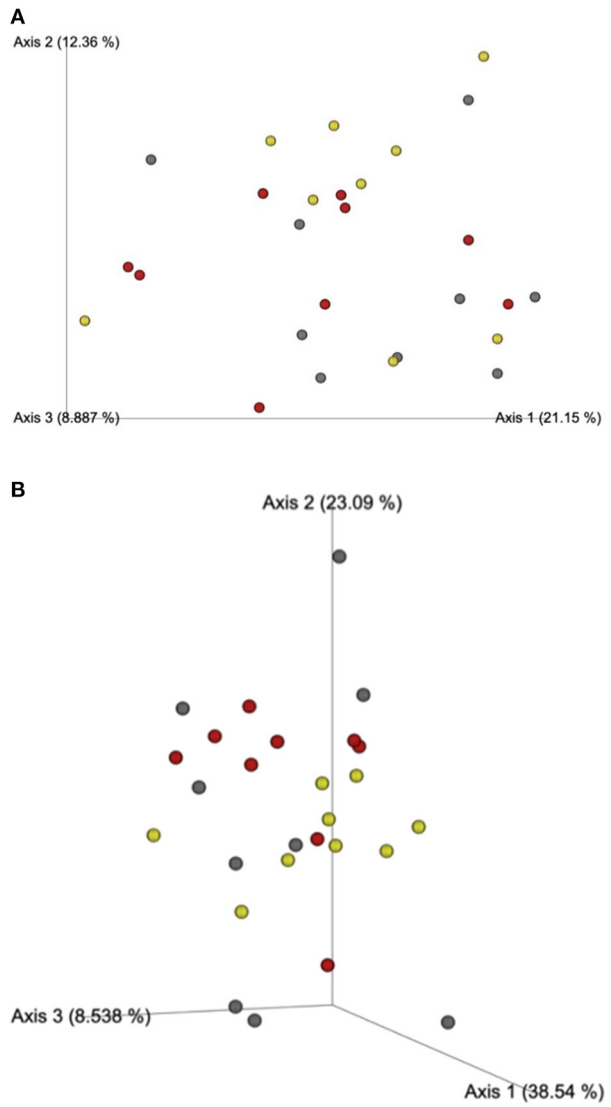

FIGURE 1 | (A) Fecal microbial β-diversity based on unweighted* UNIFRAC analysis of cats fed diets containing different carbohydrate sources. Yellow dots = corn diet (control), red dots = red sorghum diet, gray dots = white sorghum diet. *Unweighted considers the presence or absence of observed taxa incorporating phylogenetic distances, with each dot representing a cat. **(B)** Fecal microbial β-diversity based on weighted* UNIFRAC analysis for cats fed diets containing different carbohydrate sources. *Weighted considers the proportion of observed taxa incorporating phylogenetic distances (the relative relatedness of microbial community), with each dot representing a cat.

Fusobacteria, and Proteobacteria being the most predominant phyla, comprising more than 90% of all sequences (**Figure 3**).

Twenty-one bacterial families were observed, accounting for ∼96% of all sequences. The most predominant taxa were *Prevotellaceae* (Bacteroidetes), *Lachnospiraceae* (Firmicutes), *Ruminococcaceae* (Firmicutes), *Peptostreptococcaceae* (Firmicutes), *Bacteroidaceae* (Bacteroidetes), *Erysiplotrichaceae* (Firmicutes), and *Fusobacteriaceae* (Fusobacteria), accounting for ∼70% of all sequences (**Figure 4**). Only *Ruminococcaceae* (Firmicutes) was different ($P < 0.05$) amongst the three dietary treatments (**Figure 5**). Cats fed the corn (9.3%) and RSH (8.9%) diets had greater ($P < 0.05$) relative abundance of *Ruminococcaceae* compared with cats fed the WSH diet (7.5%).

DISCUSSION

Grain Composition

The compositions of the raw grains were comparable and agree well with previous literature (7, 28–31). The current study corn and sorghum values were in strong agreement with the values determined by the Bazolli et al. (32) study who determined values on a DMB for their maize and sorghum of 88.3 and 88.8% DM, 1.2 and 1.4% ash, 9.1 and 10.2% CP, 6.3 and 3.8% fat, and 11.2 and 12.6% TDF, respectively.

Minor differences were determined amongst the three grains. Of the three grains, the raw RSH was ∼2 percentage units greater in CP than either the raw corn or the raw WSH. Additionally, raw RSH was ∼2–3% units greater in TDF than either of the other two grains. The increase in CP for RSH was expected as RSH traditionally has more CP compared with white varieties. The current study CP values for RSH and WSH agree with previous research that reported values of 13.4 and 9.8% for red and white sorghum, respectively (33). The TDF concentrations of the RSH and WSH were analyzed in the current study while Selle et al. (33) measured crude fiber content. While a direct comparison among these 2 types of fiber methods is inappropriate, both the current study and Selle et al. (33) reported greater fiber content for red sorghum compared with white. The greater TDF concentration for RSH compared with the other two grains was expected due to the more comprehensive characterization of the fiber content of RSH varieties.

Apparent Total Tract Digestibility

The sorghum diets in the current study had greater ATTD values compared with de-Oliveira et al. (9) study which determined ATTD values for their sorghum diet when fed to cats of 76.3% for DM, 80.0% for OM, 80.6% for CP, and 83.3% for AHF. The ATTD of the corn diet in the current study was comparable to the values determined in the de-Oliveira et al. (9) study for their corn diet values of 78.5% for DM, 82.5% for OM, 83.2% for CP. The current study determined higher ATTD of TDF for the corn and both sorghum diets compared with de-Oliveira et al. (9). Contrary to current study, de-Oliviera et al. found that the TDF digestibility was not different than a corn diet (9).

Information about feline digestibility of energy and nutrients is scarce, for that reason comparisons of other feline diets incorporating different carbohydrate sources are warranted. The current study found comparable results to Thiess et al. (34) that fed a high carbohydrate diet (40.3%) to male cats with ATTD values of 84.5% for DM, 86.0% for OM, 82.0% for CP, and 95.3% for crude fat. The carbohydrate source in Thiess et al. (34) was steamed polenta, and its ATTD agreed well with the ATTD of the corn diet used in the current study, as well as both sorghum diets. While the fat extraction method varied from the current

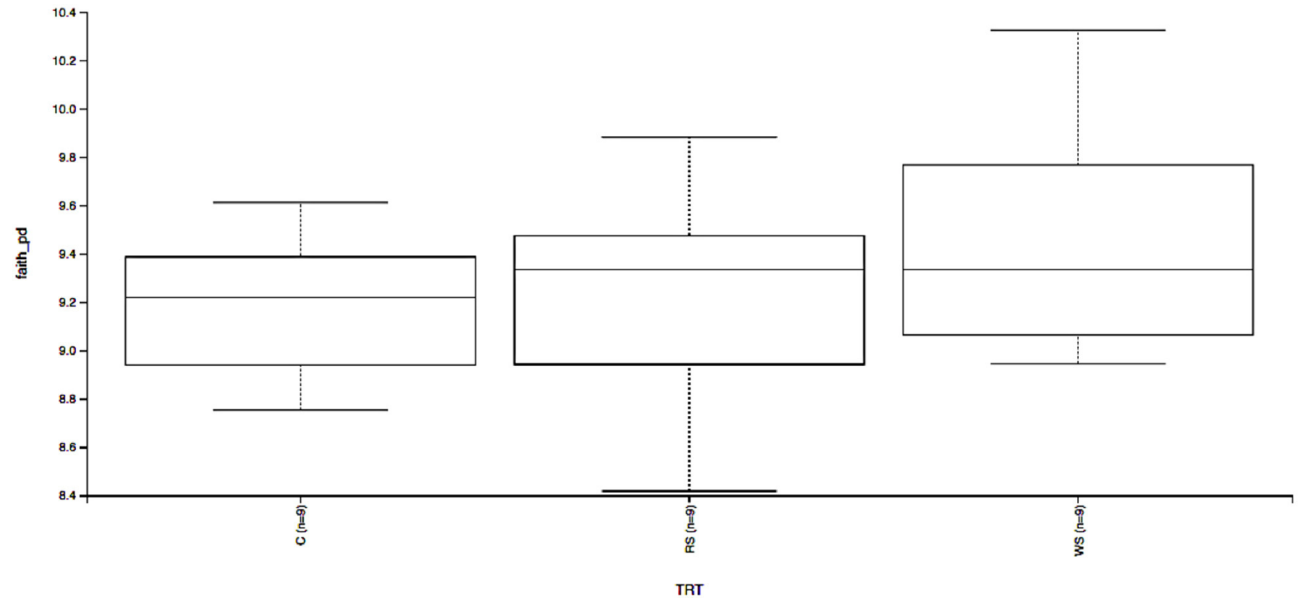

FIGURE 2 | Fecal microbial α-diversity* based on Faith's phylogenetic diversity of cats fed diets containing different carbohydrate sources. C = corn diet (control), RS = red sorghum diet, WS = white sorghum diet, TRT = treatment, Y-axis is Faith's phylogenetic diversity, X-axis left to right C, RS, WS ($n = 9$). * α-diversity measures species richness within sample, and whole trees indicate it was not affected by treatment ($P > 0.05$).

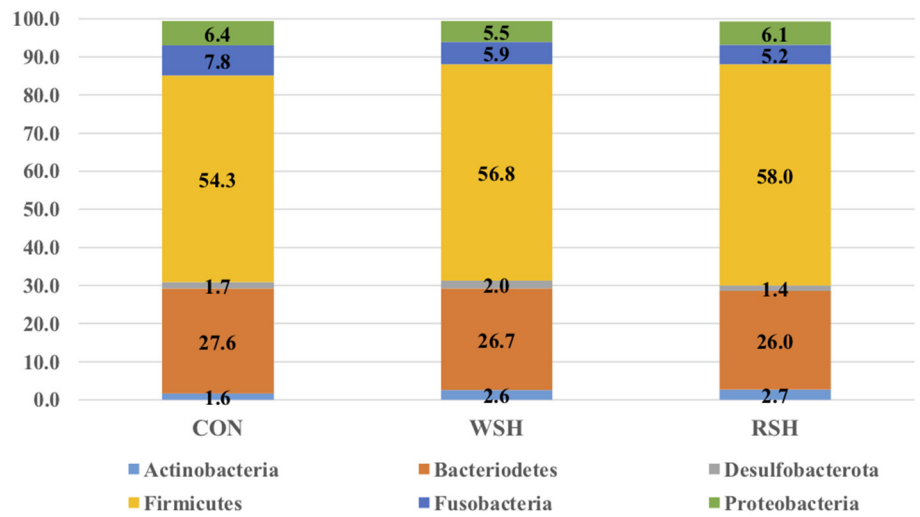

FIGURE 3 | Fecal microbial relative abundance (%, total sequences) of predominant phyla of cats fed diets containing different carbohydrate sources. CON = corn diet (control), WSH = white sorghum diet, RSH = red sorghum diet.

study, it is still applicable to showing that high fat (>90%) ATTD is observed in feline nutrition.

Asaro et al. (35) determined the ATTD in three different commercial diets with ingredients that had different glycemic responses. The medium glycemic response diet contained corn and whole grain sorghum as the carbohydrate sources. The ATTD of the diet was similar to the current study diets with values of 86.2% for DM, 89.5% for OM, 87.3% for CP, and 95.4% for fat. The 8-percentage unit increase in DM ATTD compared with the corn diet in the current study could be attributed to the inclusion of whole grain sorghum in the medium glycemic response diet, as the current study saw a significant increase in OM ATTD for both sorghum diets compared with corn. Asaro et al. (35) obtained a digestible energy (**DE**) concentration (4.75 kcal/g) that was similar to the current study values.

Barry et al. (36) fed a diet consisting of 27.8% brewer's rice and found ATTD values that were similar to the current study (81.4% for DM, 84.4% for OM, 84.2% for CP, and 95.7% for

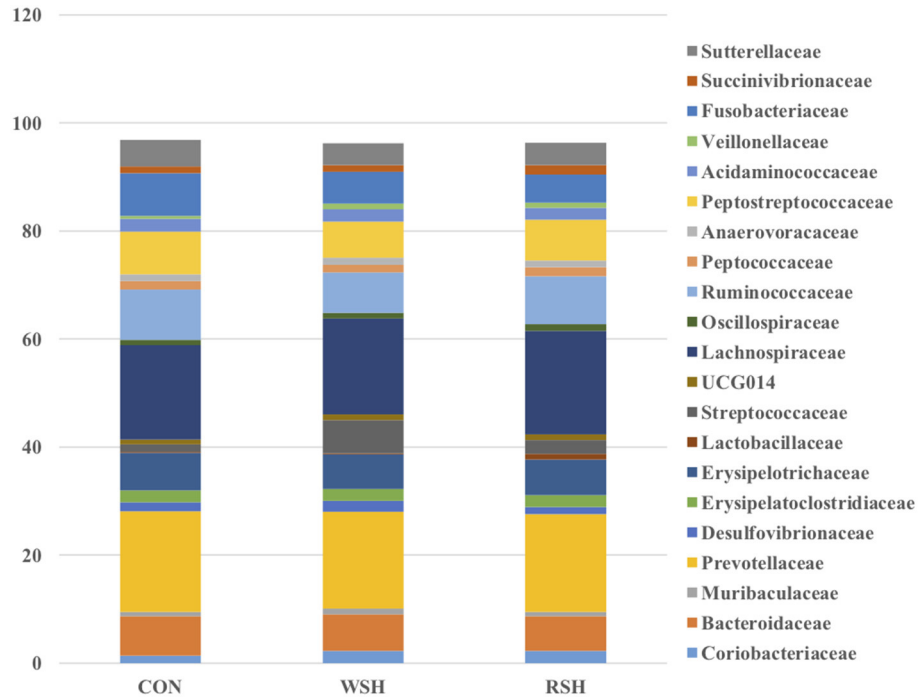

FIGURE 4 | Family fecal microbial relative abundance (%, total sequences) of cats fed diets containing different carbohydrate sources. CON = corn diet (control), WSH = white sorghum diet, RSH = red sorghum diet.

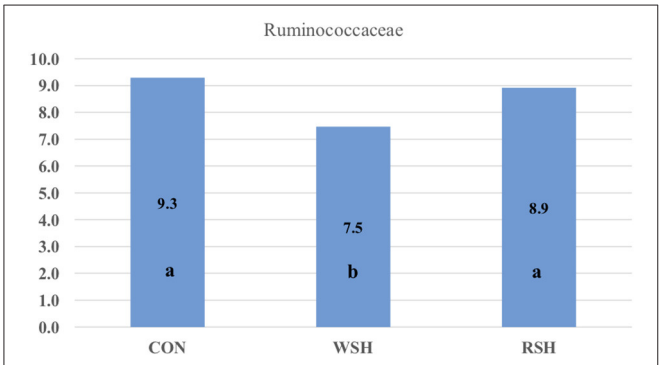

FIGURE 5 | Significant bacterial family group in feces of cats fed diets containing different carbohydrate sources. CON = corn diet (control), WSH = white sorghum diet, RSH = red sorghum diet. [ab]Bars containing no common letters **(a,b)** represent statistical difference ($P < 0.05$).

AHF). The ammonia level of 110.0 μmole/g DM agreed with the current study values regardless of dietary treatment. Both the pH (6.7) and fecal score (2.7) were elevated compared with the current study. Additionally, total SCFA values were smaller 246.7 μmole/g DM compared with the current study values (36). This could be due to the lower fiber content of brewer's rice and greater ATTD compared with corn and sorghum. The total BCFA concentration (31.3 μmole/g DM) from Barry et al. (36) agreed with the current study with the strongest agreement being observed with the WSH diet.

Fecal Microbial Populations

The gastrointestinal microbiome, which is becoming recognized as a metabolically active organ, of cats has been increasingly recognized as being associated with pet health (37). The gastrointestinal tract of animals and humans is the primary microbial habitat and has a profound impact on their host (38). Previously, most microbiome research centered on the characterization of altered composition in diseased states; however, mounting evidence of dietary components impacting allergies, oral health, diabetes, and weight management through gut microbiome is becoming clearer (37). The age, sex, breed, amount, and form of food, health status, and composition of diet can all influence the gastrointestinal microbiome (38, 39).

The gastrointestinal microbiota of cats has been reported as similar to dogs (40), the dominant microbial phyla in both consisting of *Bacteroidetes*, *Firmicutes*, *Fusobacteria*, *Proteobacteria,* and *Actinobacteria* (38, 41). However, others have reported that the feline microbiota is more varied and diverse in composition, and this variation can be attested to adaption of the microbiome to different diets [omnivorous for canine, carnivorous for feline; (37)]. Kanakupt et al. (42) determined *Bacteroidetes* (36.1%), *Firmicutes* (36.3%), *Proteobacteria* (12.4%), and *Actinobacteria* (7.7%) to be dominate phyla in their feline fecal samples in adult cats fed an extruded diet containing rice and corn as the main carbohydrate sources. At 42 weeks of age (similar aged cats were used in the current study), the relative abundance of major phyla for felines consisted of 11.8% *Actinobacteria*, 34.7% *Bacteroidetes*, 36.1% *Firmicutes*, and 11.4% *Proteobacteria* (43). The most predominant phyla

for 16-week-old cats fed a moderate carbohydrate (40.4% dried potato product) and moderate protein (30.8% chicken meal) extruded diet was *Firmicutes* [71.0%; (44)]. The predominant phyla reported by Kanakupt et al. (42), Deusch et al. (43), and Hooda et al. (44) are in agreement with the current study, *Bacteriodetes* (26.0−27.6%), *Firmicutes* (54.3−58.0%), and *Proteobacteria* (5.5−6.4%). However, the current study had greater levels of *Fusobacteria* (5.2−7.8%) compared with *Actinobacteria* (1.6 – 2.7%).

Ritchie et al. (45) found the major bacterial families in the feces consisted of *Bacteroidaceae*, *Porphyromonadaceae*, *Prevotellaceae*, and *Rikenellaceae* in cats fed an extruded commercial diet. *Bacteroidaceae*, *Eubacteriaceae*, *Clostridiaceae*, *Streptococcacceae*, and *Lactobacillaceae* have also been reported as dominant families in feline fecal samples by Itoh et al. (46) and Rochus et al. (47). In the present study, *Bacteroidaceae* and *Prevotellaceae* also were predominant families among cats fed corn, WSH, or RSH. However, other predominant families *Lachnospiraceae*, *Peptostreptococcaceae*, *Erysiplotrichaceae*, and *Fusobacteriaceae* were also prevalent and at a higher relative abundance compared with previous research. The current study relative abundance of *Ruminococcaceae* (7.5−9.3%) was in agreement with Hooda et al. (44) who determined a relative abundance of 7.16%. The increase ($P < 0.05$) in relative abundance for *Ruminococcaceae* for cats fed the corn and RSH diets compared with the WSH diet could be due to greater fiber and carbohydrate substrates reaching the hindgut of cats fed those diets. *Ruminococcaceae* are able to process a wide range of substrates from glucose and cellobiose to cellulose and hemicellulose (48). In humans, increase in fecal concentrations of SCFA-producing bacteria have been reported in response to increased intake of dietary fiber (49–51).

The differences between the current study and previous studies evaluating fecal microbiota of cats might be attributed to differences in diets being fed, breed of cats used, stage of life, environmental factors, and sequencing methods and bioinformatics analysis used. Overall, the relative abundance of fecal microbial taxonomic populations of cats fed corn, WSH, and RSH was not altered, which agrees with minor changes observed in fecal concentration of metabolites derived from microbial fermentation in the feline hindgut.

Serum Biochemical Differences

The significant differences observed in the fasted serum calcium profile (**Table 6**). While there were significant differences among treatments, all fasted serum calcium concentrations were within the reference range and likely had no impact on the physiology and health of these animals.

CONCLUSIONS

The data demonstrate that diets formulated with up to 30% of WSH or RSH are well-tolerated, had greater ATTD of OM and TDF, and resulting in no signs of gastrointestinal discomfort or intolerance when fed to cats. Overall, coefficients of digestibility for the macronutrients analyzed were high and comparable to extruded commercial feline diets. Fecal concentrations of SCFA (e.g., acetate, propionate, and butyrate) indicate that WSH and RSH diets resulted in comparable saccharolytic hindgut fermentation and fecal microbiota as the corn diet, whereas red sorghum variety also resulted in lower fecal indole concentration.

DATA AVAILABILITY STATEMENT

The data presented in the study are deposited in the Illinois Data Bank repository, accession number doi: 10.13012/B2IDB-2580847_V1.

ETHICS STATEMENT

The animal study was reviewed and approved by University of Illinois Institutional Animal Care and Use Committee.

AUTHOR CONTRIBUTIONS

MRCG designed the experiment. PvS and FH performed the laboratory analyses. PvS and MRCG performed the statistical analyses. BRS and SLR-Z performed bioinformatic analysis for fecal microbial analysis. PvS wrote the manuscript. All authors revised and provided intellectual input on this manuscript.

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REFERENCES

1. Aldrich G. *Rendered Products in Pet Food: Essential Rendering All About the Animal By-Products Industry*. Arlington, VA: National Renderers Association, Kirby Lithographic Co., Inc. (2006). p. 159–78. Available online at: www.rendereres.org (accessed November 15, 2018).
2. Longin CFH, Würschum T. Back to the future–tapping into ancient grains for food diversity. *Trends Plant Sci.* (2016) 21:731–7. doi: 10.1016/j.tplants.2016.05.005
3. United States Department of Agriculture. *Foreign Agriculture Service*. (2018). Available online at: https://apps.fas.usda.gov/psdonline/circulars/grain.pdf (accessed December 12, 2018).
4. Twomey LN, Pethick DW, Rowe JB, Choct M, Pluske JR, Brown W, et al. The use of sorghum and corn as alternative to rice in dog foods. *J Nutr.* (2002) 132:1704S−5S. doi: 10.1093/jn/132.6.1704S
5. Fortes CMLS, Carciofi AC, Sakomura NK, Kawauchi IM, Vasconcellos RS. Digestibility and metabolizable energy of some carbohydrate sources for dogs. *Anim Feed Sci Technol.* (2010) 156:121–5. doi: 10.1016/j.anifeedsci.2010.01.009

6. Fenster C. White food sorghum in the American diet. In: *U.S. Grains Council 43rd Board of Delegates Meeting*. Minneapolis, MN (2003).
7. Hwang KT, Cuppett SL, Weller CL, Hanna MA. Properties, composition, and analysis of grain sorghum wax. *J Amer Oil Chemists Soc.* (2002) 79:521–7. doi: 10.1007/s11746-002-0515-5
8. Selle PH, Cadogan DJ, Li X, Bryden WL. Implications of sorghum in broiler chicken nutrition. *Anim Feed Sci and Technol.* (2010) 156:57–74. doi: 10.1016/j.anifeedsci.2010.01.004
9. De-Oliveira LD, Carciofi AC, Oliveira MCC, Vasconcellos RS, Bazolli RS, Pereira GT, et al. Effects of six carbohydrate sources on diet digestibility and postprandial glucose and insulin responses in cats. *J Anim Sci*. (2008) 86:2237–46. doi: 10.2527/jas.2007-0354
10. Twomey LN, Pluske JR, Rowe JB, Choct M, Brown W, Pethick DW. The replacement value of sorghum and maize with or without supplemental enzymes for rice in extruded dog foods. *Anim Feed Sci Tech*. (2003) 108:61–9. doi: 10.1016/S0377-8401(03)00168-8
11. Carciofi AC, Takakura FS, De-Oliveira LD, Teshima E, Jeremias JT, Brunetto MA, et al. Effects of six carbohydrate sources on dog diet digestibility and post-prandial glucose and insulin response. *Anim Physiol Anim Nutr*. (2008) 92:326–36. doi: 10.1111/j.1439-0396.2007.00794.x
12. Laflamme D. Development and validation of a body condition score system for cats: a clinical tool. *Feline Pract*. (1997) 25:13–8.
13. Association of American Feed Control Officials (AAFCO). *Official Publication*. Champaign, IL: Association of American Feed Control Officials, Inc. (2018).
14. Association of Official Analytical Chemists (AOAC). (2006). *Official methods of analysis*. 17th ed. Gaithersburg, MD: Assoc. Off Anal Chem.
15. American Association of Cereal Chemists (AACC). (1983). *Approved methods*. 8th ed. St. Paul, MN: AACC.
16. Budde EF. The determination of fat in baked biscuit type of dog foods. *J Assoc Off Agric Chem.* (1952) 35:799–805. doi: 10.1093/jaoac/35.3.799
17. Prosky L, Asp NG, Schweizer TF, Devries JW, Furda I. Determination of insoluble and soluble dietary fiber in foods and food products: collaborative study. *J Assoc Off Anal Chem.* (1992) 75:360–7. doi: 10.1093/jaoac/75.2.360
18. Erwin ES, Marco GJ, Emery EM. Volatile fatty acid analysis of blood and rumen fluid by gas chromatography. *J Dairy Sci*. (1961) 44:1768–71. doi: 10.3168/jds.S0022-0302(61)89956-6
19. Supleco, Inc. *GC separation of VFAC2-C25. Bull. No. 7498*. Bellefonte, PA: Supleco, Inc. (1975).
20. Goodall SR, Byers FM. Automated micro method for enzymatic L(+) and D(-) lactic acid determinations in biological fluids containing cellular extracts. *Anal Biochem*. (1978) 89:80–9. doi: 10.1016/0003-2697(78)90728-5
21. Flickinger EA, Schreijen EMWC, Patil AR, Hussein HS, Grieshop CM, Merchen NR, et al. Nutrient digestibilities, microbial populations, and protein catabolites as affected by fructan supplementation of dog diets. *J Anim Sci*. (2003) 81:2008–18. doi: 10.2527/2003.8182008x
22. Chaney AL, Marbach EP. Modified reagents for determination of urea and ammonia. *Clin Chem*. (1962) 8:130–2. doi: 10.1093/clinchem/8.2.130
23. Caporaso JG, Lauber CL, Walters WA, Berg-Lyons D, Huntley J, Fierer N, et al. Ultra-high–throughput microbial community analysis on the Illumina HiSeq and MiSeq platforms. *ISME J*. (2012) 6(8):1621–4. doi: 10.1038/ismej.2012.8
24. Caporaso JG, Kuczynski J, Stombaugh J, Bittinger K, Bushman FD, Costello EK, et al. QIIME allows analysis of high-throughput community sequencing data. *Nat Methods*. (2010) 7:335. doi: 10.1038/nmeth.f.303
25. Callahan BJ, McMurdie PJ, Rosen MJ, Han AW, Johnson AJA, Holmes SP. DADA2: High-resolution sample inference from Illumina amplicon data. *Nat Methods*. (2016) 13:581–3. doi: 10.1038/nmeth.3869
26. Quast C, Pruesse E, Yilmaz P, Gerken J, Schweer T, Yarza P, et al. The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. *Nucleic Acids Res*. (2013) 41:D590–6. doi: 10.1093/nar/gks1219
27. Lozupone C, Knight R. UniFrac: a new phylogenetic method for comparing microbial communities. *Appl Environ Microbiol*. (2005) 71:8228–35. doi: 10.1128/AEM.71.12.8228-8235.2005
28. Bednar GE, Pati AR, Murray SM, Grieshop CM, Merchen NR, Fahey Jr GC. Starch and fiber fractions in selected food and feed ingredients affect their small intestinal digestibility and ferment-ability and their large bowel fermentability *in vitro* in a canine model. *J Nutr*. (2001) 131:276–86. doi: 10.1093/jn/131.2.276
29. Murray SM, Flickinger EA, Patil AR, Merchen NR, Brent JL, Fahey Jr GC. *In vitro* fermentation characteristics of native and processed cereal grains and potato starch using ileal chyme from dogs. *J Anim Sci*. (2001) 79:435–44. doi: 10.2527/2001.792435x
30. NRC. *Nutrient Requirements of Swine*. 11th ed. Washington, DC: National Academy Press (2012).
31. Beloshapka AN, Buff PR, Fahey Jr GC, Swanson KS. Com-positional analysis of whole grains, processed grains, grain co-products, and other carbohydrate sources with applicability to pet animal nutrition. *Foods*. (2016) 5:23. doi: 10.3390/foods5020023
32. Bazolli RS, Vasconcellos RS, De-Oliveira LD, Sá FC, Pereira GT, Carciofi AC. Effect of the particle size of maize, rice, and sorghum in extruded diets for dogs on starch gelatinization, digestibility, and the fecal concentration of fermentation products. *J Anim Sci*. (2015) 93:2956–66. doi: 10.2527/jas.2014-8409
33. Selle PH, Liu SY, Khoddami A, Cai J, Cowieson AJ. Steam-pelleting temperatures and grain variety of finely ground, sorghum-based broiler diets. 1. Influence on growth performance, relative gizzard weights, nutrient utilisation, starch and nitrogen digestibility. *Anim Prod Sci*. (2014) 54:339–46. doi: 10.1071/AN13080
34. Thiess S, Becskei C, Tomsa K, Lutz TA, Wanner M. Effects of high carbohydrate and high fat diet on plasma metabolite levels and on iv glucose tolerance test in intact and neutered mate cats. *J Feline Med Surg*. (2004) 6:207–18. doi: 10.1016/j.jfms.2003.09.006
35. Asaro NJ, Guevara MA, Berendt K, Zijlstra R, Shoveller AK. Digestibility is similar between commercial diets that provide ingredients with different perceived glycemic responses and the inaccuracy of using the modified Atwater calculation to calculate metabolizable energy. *Vet Sci.* (2017) 4:54–65. doi: 10.3390/vetsci4040054
36. Barry KA, Middelbos IS, Vester Boler BM, Dowd SE, Suchodolski JS, Henrissat B, et al. Effects of dietary fiber on the feline gastrointestinal metagenome. *J Proteome Res*. (2012) 11: 5924–33. doi: 10.1021/pr3006809
37. Wernimont SM, Radosevich J, Jackson MI, Ephraim E, Badri DV, MacLeay JM, et al. The effects of nutrition on the gastrointestinal microbiome of cats and dogs: impact on health and disease. *Front Microbiol*. (2020) 11:1266. doi: 10.3389/fmicb.2020.01266
38. Deng P, Swanson KS. Gut microbiota of humans, dogs and cats: current knowledge and future opportunities and challenges. *Br J Nutr*. (2015) 113:S6–17. doi: 10.1017/S0007114514002943
39. Hang I, Rinttila T, Zentek J, Kettunen A, Alaja S, Apajalahti J, et al. Effect of high contents of dietary animal-derived protein or carbohydrates on canine faecal microbiota. *BMC Vet Res*. (2012) 8:90. doi: 10.1186/1746-6148-8-90
40. Hoffmann AR, Proctor LM, Surette MG, Suchodolski JS. The microbiome: the trillions of microorganisms that maintain health and cause disease in humans and companion animals. *Vet Psychol*. (2016) 53: 10–21. doi: 10.1177/0300985815595517
41. Handl S, Dowd SE, Garcia-Mazcorro JF, Steiner JM, Suchodolski JS. Massive parallel 16S rRNA gene pyrosequencing reveals highly diverse fecal bacterial and fungal communities in healthy dogs and cats. *FEMS Microbiol Ecol*. (2011) 76:301–10. doi: 10.1111/j.1574-6941.2011.01058.x
42. Kanakupt K, Vester Boler BM, Dunsford BR, Fahey Jr GC. Effects of short-chain fructooligosaccharides and galactooligosaccharides, individually and in combination, on nutrient digestibility, fecal fermentative metabolite concentrations, and large bowel microbial ecology of healthy adult cats. *J Anim Sci*. (2011) 89:1376–84. doi: 10.2527/jas.2010-3201
43. Deusch O, O'Flynn C, Colyer A, Swanson KS, Allaway D, Morris P. A longitudinal study of the feline faecal microbiome identifies changes into early adult-hood irrespective of sexual development. *PLoS ONE*. (2015) 10:e0144881. doi: 10.1371/journal.pone.0144881
44. Hooda S, Vester Boler BM, Kerr KR, Dowd SE, Swanson KS. The gut microbiome of kittens is affected by dietary protein: carbohydrate ratio and associated with blood metabolite and hormone concentrations. *Br J Nutr*. (2013) 109:1637–46 doi: 10.1017/S0007114512003479
45. Ritchie LE, Steiner JM, Suchodolski JS. Assessment of microbial diversity along the feline intestinal tract using 16S rRNA gene analysis. *FEMS Microbiol Ecol*. (2008) 66:590–8. doi: 10.1111/j.1574-6941.2008.00609.x

46. Itoh K, Mitsuoka T, Maejima K. Comparison of faecal flora of cats based on different housing conditions with special reference to Bifidobacterium. *Lab Anim*. (1984) 18:280–4. doi: 10.1258/002367784780958303
47. Rochus K, Janssens GP, Hesta M. Dietary fibre and the importance of the gut microbiota in feline nutrition: a review. *Nutr Res Rev*. (2014) 27:295–307. doi: 10.1017/S0954422414000213
48. Biddle A, Stewart L, Blanchard J, Leschine S. Untangling the genetic basis of fibrolytic specialization by Lachnospiraceae and Ruminococcaceae in diverse gut communities. *Diversity*. (2013) 5:627–40. doi: 10.3390/d5030627
49. Vanegas SM, Meydani M, Barnett JB, Goldin B, Kane A, Rasmussen H, et al. Substituting whole grains for refined grains in a 6-wk randomized trial has a modest effect on gut microbiota and immune and inflammatory markers of healthy adults. *Am J Clin Nutr*. (2017) 105:635–50. doi: 10.3945/ajcn.116.146928
50. Costabile A, Klinder A, Fava F, Napolitano A, Fogliano V, Leonard C, et al. Whole-grain wheat breakfast cereal has a prebiotic effect on the human gut microbiota: a double-blind, placebo-controlled, crossover study. *Br J Nutr*. (2007) 99:110–20. doi: 10.1017/S0007114507793923
51. Costabile A, Bergillos-Meca T, Rasinkangas P, Korpela K, de Vos WM, Gibson GR. Effects of soluble corn fiber alone or in synbiotic combination with *Lactobacillus rhamnosus* GG and the pilus-deficient derivative GG-PB12 on fecal microbiota, metabolism, and markers of immune function: a randomized, double-blind, placebo-controlled, crossover study in healthy elderly (saimes study). *Front Immunol*. (2017) 8:1443. doi: 10.3389/fimmu.2017.01443

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Clinical Findings in Healthy Dogs Fed With Diets Characterized by Different Carbohydrates Sources

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In recent years, pet owners have become more interested in the ingredients, and quality of pet-food, and several studies have demonstrated that feed management could affect healthy status. Recently, some authors indicated that commercial diets formulated without cereals, or using unconventional protein, and starch sources, can cause a reduction in taurine levels in both whole blood, and plasma. Nevertheless, the specific mechanism by means of which nutritional factors determine this reduction is not completely clear. Thirty neutered half-breed dogs were recruited at a kennel in the province of Naples (Italy) to investigate the influence of carbohydrates sources, and dietary density of nutrients on healthy status of dogs in terms of blood count, and biochemical parameters. The dogs were housed in the kennel and divided into three distinct groups. Three iso-energy, and iso-nitrogen commercial kibble diets (named GF1, GF2, and CB) with different protein, and carbohydrates contents, and carbohydrates sources were chosen for the trial. The chemical composition and amino acid profile of each of the three tested diets were analyzed. Moreover, blood samples of each dog were collected to evaluate the hematological and biochemical profiles. The taurine level was determined both on plasma and whole blood. The effect of the diets was analyzed statistically, and all tested diets were compared to the control one. There were significant differences between the three tested diets as regards their chemical composition. The concentrations of all amino acids seem to reflect protein content diets. The hematological profile resulted within the ranges considered physiological for the canine species for all subjects. Compared to the control diet, the three tested diets showed significant differences in blood count for MCHC and platelets. The biochemical profile showed significant differences between the diets, particularly their AST, fructosamine, lipase, and triglycerides values. The diets did not affect the blood and plasma taurine levels. They resulted in higher than optimal reserve levels. Preliminary results showed that the sources of carbohydrates and use of balanced diets affected only some biochemical parameters and did not alter the levels of taurine in healthy adult dogs.

Keywords: blood count, biochemical profile, taurine, pea, spelt, oats, pumpkin, amino acids

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INTRODUCTION

In all developed countries, the companion animal population has gradually increased over the last 20 years, particularly in great urban centers (1). In Italy, about 39% of the population live with a cat and/or a dog (2). As a result, pet owners have been giving more attention to animal welfare and have become increasingly interested in the characteristics and production processes of commercial diets (3). The pet industry has produced several diets with particular ingredients or nutrients, suggested by specific claims, which could indicate beneficial effects, for example, grain-free diets which were formulated using tubers, and legumes as starch sources. Each carbohydrate source has a unique nutritional composition that could affect both the production process and use of nutrients (4). For instance, legume grains (pea and lentil) are rich in soluble dietary fiber (SDF, mean value: 26.9% of total dietary fiber, TDF), and proteins (CP, mean value: 23.87% DM) compared to cereals grains (e.g., corn and rice) (CP: 10.72% DM, SDF: 10.00% TDF) (5–7). Moreover, the amino acid profile of different protein sources varied significantly, legume protein is less rich in essential amino acids (e.g., taurine, L-carnitine) compared to protein sources of animal origin (6, 8, 9).

In 2018, the Food and Drug Administration (10) published a report on the possible link between grain-free diets and dilated cardiomyopathy related to taurine deficiency. Cardiomyopathy is characterized by a dilation of the left ventricle or both ventricles, in association with impairment of ventricular contractions (11) and it causes cardiac dysfunction (12). Moreover, some studies have indicated a correlation between taurine reduction due to the administration of grain-free diets and dilated cardiomyopathy in dogs (13–15).

Nevertheless, the results are controversial in the literature. A recent study conducted (16) on 86 Golden Retrievers observed significantly higher taurine values in whole blood in dogs fed a diet containing cereal compared to dogs fed grain-free diets. Significant differences in plasma taurine levels, however, were not observed.

On the contrary, Donadelli et al. (17) demonstrated significant increases in plasma taurine and whole blood taurine levels in Golden Retrievers fed grain-free diets for 26 weeks. Similarly, Pezzali et al. (18) found that grain-free diets had no effect on taurine levels. A recent review (19) highlighted some limits as far as current literature is concerned as regards identifying the specific nutritional causes of taurine deficiency and, consequently, dilatative myocardiopathy development in the dog.

The purpose of this study was to evaluate if the administration of three diets (two grain-free: GF1, and GF2 vs. one cereal-based: CB), over a medium-term period (5 weeks), formulated with different carbohydrate sources and amounts could affect blood profile, and biochemical parameters with particular regard to taurine levels in healthy dogs. We hypothesized that different carbohydrates sources could influence the healthy status of adult dogs.

MATERIALS AND METHODS

Animals and Diets

The nutritional double-blind trial was performed at a private kennel located in the province of Naples (Italy). At the beginning of the trial, a veterinarian clinically examined 50 adult dogs and performed, hematological, biochemical, and parasitological tests (20) to exclude subjects with signs of pathologies. Subsequently, 30 adults, neutered, half-breed dogs (age 4 ± 1.20 years, weight 20.79 ± 6.38 kg, BCS 3.96 ± 0.95 on five point scale) were recruited. Each dog was housed in an individual box of 8 m^2 (2 × 4) consisting of a closed rest portion (2 × 2), and an open common walking area for five adjacent boxes. Before the beginning of the study, and during the first days of each adaptation period, all dogs were submitted to copromicroscopic analysis for intestinal nematodes (*Trichuris*, *Toxocara*, *Toxascaris,* and *Ancylostomidae*), cardiopulmonary nematodes (*Angiostrongylus* and *Capillaria*), Cestode (*Dipylidium* and other *Taeniidae*), and Protozoa (*Giardia* and *Cystoisospora*). If they were found to be positive, they were immediately treated with specific deworming drugs.

After enrollment, the dogs were divided into three distinct groups (blue, red, and black), homogeneous for sex, age, weight, and BCS. For the experimentation, three commercial dry diets (Farmina-pet food, Nola, Italy) named GF1, GF2, and CB, respectively, were chosen and administered alternately to experimental groups following a Latin square scheme (3 diets × 3 groups); each experimental period had a total duration of 50 days (15 days of adaptation and 35 days of trial). Each diet was administered in a ratio of 110 kcal/$kg^{0.75}$ of metabolizable energy (EM) (6).

The diets were characterized by similar energy densities (3,995 ± 4.73 kcal/kg), formulated mainly with the same protein source (chicken), but consisting of different carbohydrates sources (cereal grain vs. legume or tubers).

The ingredients of each diet were the following:

- Diet CTR: rice, beet pulp, poultry, and turkey meals, fat and oil, and minerals;
- Diet GF1: boneless chicken, dehydrated chicken protein, sweet potato, chicken fat, dried eggs, herring, dehydrated herring protein, fish oil (from herring), pea fiber, and dried carrot;
- Diet GF2: boneless chicken, dehydrated chicken protein, pea starch, chicken fat, dried pumpkin, dried eggs, herring, dehydrated herring protein, fish oil (from herring), pea fiber, and dried carrot;
- Diet CB: boneless chicken, dehydrated chicken protein, spelt, oats, chicken fat, dried eggs, herring, dehydrated herring protein, dried beet pulp, fish oil (from herring), and dried carrot.

Diets Chemical Composition and Amino Acid Profile

An aliquot of 500 g for each diet was analyzed by means of near-infrared spectroscopy (NIRS DS 2005F, FOSS, Hilleroed,

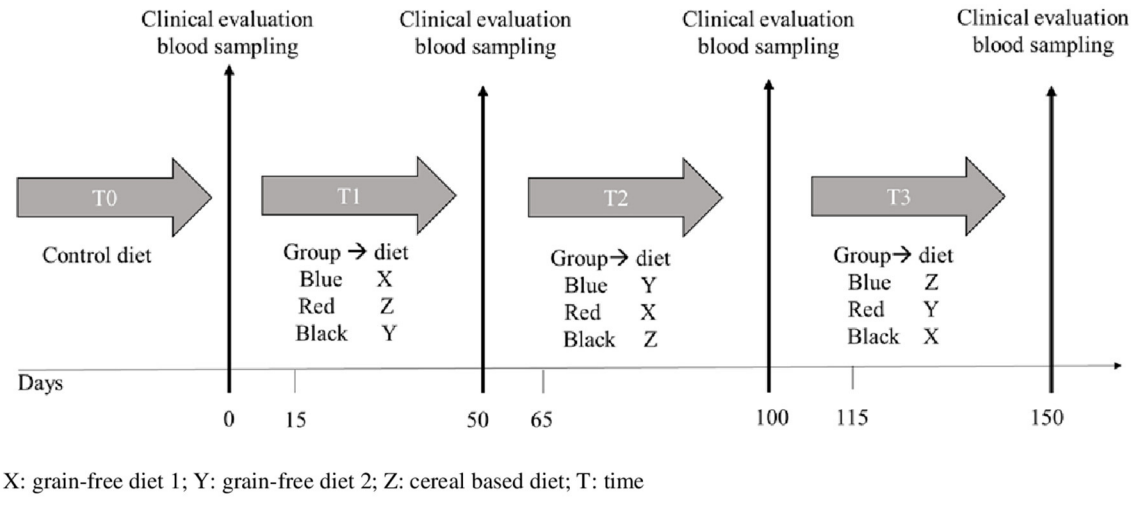

FIGURE 1 | Study timeline.

Denmark) to determine its chemical composition (21, 22). Total dietary fiber, and proportion soluble, and insoluble fractions were determined according to (23, 24). The diets were analyzed also to determine its amino acid profile using high-performance liquid chromatography (HPLC, Agilent Technologies 1290, California, CA, United States) according to Spitze et al. (25).

Clinical Examination, Weight Checks, and Sampling

At the beginning of each experimental period, the dogs were subjected to physical examination, weighed, and their body condition score (BCS) was evaluated. At recruitment and the end of each nutritional phases (**Figure 1**), fasted dogs were clinically evaluated and ∼10 mL of blood was collected in three tubes:

- two with EDTA, one for determination of the blood count, and one for the dosage of plasmatic taurine;
- one for whole blood with separator gel from which to obtain the serum for determination of the biochemical profile.

The whole blood samples intended for the evaluation of the blood count were refrigerated, and quickly transported to the clinical analysis laboratory of the Department of Veterinary Medicine and Animal Production of the Federico II University of Naples. Each blood sample was analyzed using an impedance device to carry out an instrumental count (HeCo 5 Vet C, Real Time Diagnostic Systems; San Giovanni a Valdarno, Italy) after slow and constant mixing for 20 min.

At the kennel, to obtain the serum, the gel separator tubes were left at room temperature for about 15 min until the clot formed, then the samples were centrifuged for 10 min at a speed of 1,500 × g. The serum was stored at −80°C and subsequently sent on dry ice to a reference laboratory (Kornwestheim, Germany) where the following parameters were determined using a Beckman biochemical analyzer (Beckman Coulter AU5400; Olympus America, Melville, NY, USA): Globulin; Total protein (TP); Albumin (Alb); Alkaline phosphatase (AP); Glutamic Pyruvic Transaminase (GPT); Alanine Transaminase (ALT); Gamma-glutamyl transferase (GGT); Aspartate Transferase (AST); Glutamate dehydrogenase (GLDH); Fructosamine (Fr); Glucose (GLU); α-amylase; Lipase (LP); Sodium (Na); Potassium (K); Calcium (Ca); Chloride (Cl); Phosphorus (P); Magnesium (Mg); Cholesterol (Col); Triglycerides (Tri); Creatinine (Crea); BUN, Creatine kinase (CK).

At the end of each phase from five dogs per group blood were sampled to determine taurine on whole blood; after sampling blood was collected in a tube containing lithium heparin. Subsequently, it was gently mixed and stored at a temperature of −80°C up to the shipment on dry ice to the Amino Acid Laboratory (University of California, Davis, CA, USA). Whole blood taurine concentrations were determined using a Beckman 6,300 amino acid analyzer (Beckman Industries, Palo Alto, CA, USA).

Taurine in plasma was determined by reversed phase chromatography hyphenated to a triple quadrupole mass spectrometer (LC-MS/MS Sciex API4000QTRAP plus Agilent HPLC and CTC PAL autosampler, Santa Clara, CA, USA) (26).

Statistical Analysis

The effects of the diets were analyzed by one-way analysis of variance (ANOVA). Tukey's HSD test was used when significant differences were observed.

To compare each diet with the control one, the Dunnett test was used, which allows multiple comparisons. All statistical analyses were performed using the software JMP 14 (SAS Institute, NC, USA).

RESULTS

Table 1 shows the chemical composition of the diets used during the trial. All statistical analyses were referred comparing the diets. GF1 showed the highest value in protein, and

TABLE 1 | Diets chemical composition (g/kg as is) and energy content (ME kcal/kg as is).

Nutritional characteristics	CTR*	GF1	GF2	CB	RMSE
Crude Protein	240	363^{A}	316^{B}	317^{B}	19.1
Total fat	100	185	193	194	51.5
Crude Fiber	38.0	23.0	23.7	24.7	9.41
Ash	99.0	65.0^{a}	60.0^{b}	60.3^{b}	20.3
Metabolizable Energy*	3,200	3,999	3,990	3,997	140

**Chemical composition of control diet as reported in the label; GF1, grain-free diet 1; GF2, grain-free diet 2; CB, cereal based diet; along the row the capital letters indicate differences for $P < 0.01$ and $P < 0.001$; lowercase indicated differences for $P < 0.05$. RMSE, root mean square error. The statistical comparison was performed between GF1, GF2, and CB diets. *ME, calculated according to the predictive equation indicated by NRC 2006 (6).*

TABLE 2 | Amino acids profile of diets used during the trial.

	GF1	GF2	CB	GF1	GF2	CB	FEDIAF*
	% as is			g/1,000 kcal ME			
Protein	36.3	31.6	31.7	97.19	85.25	84.91	45.00
Alanine	2.19	1.99	1.90	5.86	5.37	5.09	NA
Arginine	2.11	1.88	1.80	5.65	5.07	4.82	1.30
Aspartic acids	2.83	2.80	2.26	7.58	7.55	6.05	NA
Glutamic acids	4.29	4.17	4.54	11.5	11.3	12.2	NA
Glycine	3.24	2.67	2.86	8.67	7.20	7.66	NA
Histidine	0.76	0.72	0.68	2.03	1.94	1.82	0.58
Hydroxyproline	1.01	0.71	0.95	2.70	1.92	2.54	NA
Isoleucine	1.13	1.12	0.97	3.03	3.02	2.60	1.15
Leucine	2.32	2.20	2.07	6.21	5.94	5.54	2.05
Lysine	2.08	2.11	1.65	5.57	5.69	4.42	0.25
Phenylalanine	1.30	1.22	1.18	3.48	3.29	3.16	1.35
Proline	2.20	1.77	2.21	5.89	4.78	5.92	NA
Serine	1.43	1.32	1.29	3.83	3.56	3.46	NA
Threonine	1.27	1.22	1.08	3.40	3.29	2.89	1.30
Tyrosine	0.87	0.84	0.77	2.33	2.27	2.06	NA
Valine	1.54	1.48	1.35	4.12	3.99	3.62	1.48
Cystine	0.42	0.37	0.43	1.12	1.00	1.15	NA
Methionine	1.10	1.00	0.93	2.95	2.70	2.49	1.00
Tryptophane	0.33	0.33	0.32	0.88	0.89	0.86	0.43
Taurine	0.22	0.23	0.18	0.59	0.62	0.48	NA

**Nutritional values and FEDIAF (2020) (27) recommendation based on a metabolizable energy requirement of 110 kcal ME/kg0.75; GF1, grain-free diet 1; GF2, grain-free diet 2; CB, cereal based diet; NA, not applicable.*

ash ($P < 0.01$ and $P < 0.05$, respectively). The energy nutrients (protein, carbohydrates, and lipids) of GF1, GF2, and CB diets were equally proportioned. Moreover, the three diets tested resulted in higher protein and energy levels than the CTR one, which showed higher carbohydrates content. Total dietary fiber (TDF) content of GF1 was significantly ($P < 0.05$) lower than CB one (data not reported 76.78 vs. 83.73 vs. 94.30 g/kg as is, for GF1, GF2, and CB, respectively). Moreover, all diets showed higher incidence of soluble fiber than insoluble fiber (60.65; 58.61; and 63.15% TDF, respectively).

Table 2 shows the amino acid concentrations of the three diets. In all cases, the most present amino acids were glutamic acid, glycine, alanine, arginine, aspartic acid, and lysine. The concentrations of all amino acids seem to reflect protein content of the diets.

The results of the blood count are reported in **Table 3**. All recorded values fall into the relative physiological range for canine species. The analysis of variance did not show differences between the diets. Nevertheless, the Dunnett test indicated significant ($P < 0.01$) differences between CTR diet and each tested one for MCHC, which resulted in 1 g/dL lower in CTR than GF1, GF2, and CB. Moreover, platelet values resulted significantly ($P < 0.05$) higher for CTR compared to that of CB diet (328 vs. 287 K/uL, respectively).

TABLE 3 | Blood profile of dogs in function of administered diet.

		Diets					CTR vs.		
Items	**Units**	**CTR**	**GF1**	**GF2**	**CB**	**RMSE**	**GF1**	**GF2**	**CB**
RBC	M/uL	6.88	7.00	6.95	6.76	0.64	NS	NS	NS
WBC	K/uL	12.8	11.4	12.6	13.00	2.42	NS	NS	NS
Hgb	g/dL	16.4	15.8	16.3	16.2	1.77	NS	NS	NS
Hct	%	48.1	47.9	47.3	47.3	4.57	NS	NS	NS
MCV	fL	69.2	68.4	68.0	68.7	2.80	NS	NS	NS
MCH	Pg	23.8	24.0	23.9	24.1	1.16	NS	NS	NS
MCHC	g/dL	34.4	35.1	35.1	35.1	0.70	**	**	**
Plt	K/uL	328	298	306	287	42.0	NS	NS	*

*CTR, control; GF1, grain-free diet 1; GF2, grain-free diet 2; CB, cereal based diet; RBC, red blood cells; WBC, white blood cells; Hgb, hemoglobin; Hct, hematocrit; MCV, medium corpuscular volume; MCH, medium corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; Plt, platelets; *, **, $P < 0.05$ and $P < 0.01$, respectively; NS, not significant; RMSE, root mean square error.*

TABLE 4 | Biochemical profile of dogs in function of diets.

		Diets					CTR vs.		
Items	**Units**	**CTR**	**GF1**	**GF2**	**CB**	**RMSE**	**GF1**	**GF2**	**CB**
Gl	g/L	37.6	35.8	34.7	36.0	5.47	NS	NS	NS
PT	g/L	66.9	67.1	64.9	65.2	6.25	NS	NS	NS
Alb	g/L	29.4	28.9	30.3	29.6	1.62	NS	NS	NS
AP	U/L	38.2	30.5	31.3	30.7	11.8	NS	NS	NS
Crea	μmol/L	80.7	72.6	74.2	76.0	12.1	NS	NS	NS
BUN	mmol/L	6.81	5.74	6.11	6.57	1.46	NS	NS	NS
CK	U/L	113	106	103	102	27.0	NS	NS	NS
ALT	U/L	39.3	39.1	38.5	39.3	8.44	NS	NS	NS
GGT	U/L	3.11	3.04	2.96	3.19	0.97	NS	NS	NS
AST	U/L	40.0^{A}	33.9^{AB}	30.5^{B}	31.2^{B}	7.61	*	***	*
GLDH	U/L	3.05	3.08	3.12	3.42	1.29	NS	NS	NS
Fr	μmol/L	204^{A}	187^{B}	186^{B}	188^{B}	9.93	***	***	***
Glu	mmol/L	5.03	4.79	4.94	4.95	0.64	NS	NS	NS
α-amylase	U/L	781	763	759	797	109	NS	NS	NS
LP	U/L	133^{A}	67^{B}	65^{B}	69^{B}	15.9	***	***	***
Chol	mmol/L	4.48	4.93	4.78	4.99	0.86	NS	NS	NS
Tri	mmol/L	0.88^{A}	0.59^{B}	0.58^{B}	0.56^{B}	0.17	***	***	***

*CTR, control diet; GF1, grain-free diet 1; GF2, grain-free diet 2; CB, cereal based diet; Gl, Globulin; PT, Total protein; Alb, Albumin: AP, Alkaline phosphatase; Crea, Creatinine; CK, Creatine kinase; ALT, Alanine Transaminase; GGT, Gamma-glutamyl transferase; AST, Aspartate Transferase; GLDH, Glutamate dehydrogenase; Fr, Fructosamine; Glu, Glucose; LP, Lipase; chol, Cholesterol; Tri, Triglyceride along the row the capital letters indicate differences to $P < 0.01$ and $P < 0.001$; NS, not significant, *, ***: $p < 0.05$, $p < 0.001$, respectively; RMSE, root mean square error.*

Table 4 shows biochemical values. The nutritional treatment affected only a few parameters. The analyses of variance evidenced that the dogs showed significantly higher ($P < 0.01$) values as regards AST, fructosamine, lipase, and triglycerides when fed CTR diets. The Dunnett test evidenced the differences between CTR diets vs. each tested diet more clearly, in particular for triglycerides which were about double when CTR diet was administered.

Table 5 shows the mineral profile. All parameters are within the physiological ranges for healthy dogs. With both statistical analyses, significant differences were observed for potassium, phosphorus, and magnesium which were always significantly higher in CTR than in GF1, GF2, and CB diets.

In **Table 6**, whole blood and plasma taurine levels are shown. In both cases, taurine levels were not significantly affected by the administered diet, even if the CTR diet showed the lowest value of both parameters.

DISCUSSION

All diets showed nutritional characteristics able to satisfy the nutritional requirement of adult dogs located in a kennel

TABLE 5 | Mineral profile (mmol/L) of dogs in function of diets and comparison of each diet to control one.

	Diets					CTR vs.		
Items	**CTR**	**GF1**	**GF2**	**CB**	**RMSE**	**GF1**	**GF2**	**CB**
Na	147	147	146	146	2.40	NS	NS	NS
K	4.92^{A}	4.55^{B}	4.41^{B}	4.59^{B}	0.40	*	***	*
Ca	2.43	2.45	2.44	2.44	0.14	NS	NS	NS
Cl	111	111	110	111	2.71	NS	NS	NS
P	1.33^{A}	1.37^{A}	1.24^{AB}	1.18^{B}	0.18	NS	NS	*
Mg	0.90^{A}	0.81^{B}	0.80^{B}	0.82^{B}	0.08	***	***	**

*CTR, control diet; GF1, grain-free diet 1; GF2, grain-free diet 2; CB, cereal based diet; Na, Sodium; K, Potassium; Ca, Calcium; Cl, Chloride; P, Phosphorus; Mg, Magnesium; along the rows the capital letters indicate differences to $p < 0.01$ and $p < 0.001$; NS, not significant, *, **, ***, $p < 0.05$, $p < 0.01$, $p < 0.001$, respectively; RMSE, root mean square error.*

TABLE 6 | Blood ($n = 60$) and plasma ($n = 120$) taurine levels of dogs in function of diets.

		Diets					CTR vs.		
Taurine	**Units**	**CTR**	**GF1**	**GF2**	**CB**	**RMSE**	**GF1**	**GF2**	**CB**
Whole blood	μmol/l	288	316	316	318	51.3	NS	NS	NS
Plasma	μmol/l	101	128	127	125	32.8	NS	NS	NS

CTR, control diet; GF1, grain-free diet 1; GF2, grain-free diet 2; CB, cereal based diet; NS, not significant; RMSE, root mean square error.

(6). In particular, the amount of crude protein, total fat, and metabolizable energy fall into the levels recommended by FEDIAF 2020 (27). During the experimental period, we did not find refusals probably due to the high palatability of all diets. Considering that no significant differences were observed as regards live weight, and body condition score (final live weight 21.59 ± 5.70 kg; BCS 3.97 ± 0.81 on a five-point scale) we can assert that the amount of feed administered was correctly calculated in all groups (28).

The amount of essential amino acids (g/1,000 kcal) in the three tested diets were about double the minimum levels recommended by FEDIAF (27) for an adult dog which has metabolized energy requirement equal to 110 kcal/kg$^{0.75}$. All experimental diets are able to fully satisfy even the aforementioned nutritional requirements for a dog with a lower energy necessity (95 kcal/kg$^{0.75}$), also considering an apparent availability of 70% (29).

There were no significant changes observed in the hematological profiles within the diets. All dogs showed the blood count values within the ranges considered physiological. The only exceptions were recorded for two factors, MCHC (CTR vs. GF1, GF2, and CB, $P < 0.01$) and platelets (CTR vs. CB, $P < 0.05$). Actually, from our study, whether these findings are coincidental or just a trend cannot be established, nor it is possible to give a definitive explanation. It is essential to underline that, even if there were significant differences, values for MCHC and platelets were both within ranges. Considering the absence of clinical signs or clinicopathological alterations and the negativity as regards the mainly canine vector-borne diseases, it seems likely that the results were due to any pathological cause. There is very little data available concerning the influence of diet on hematological parameters (30, 31) that could clarify the differences that resulted from our trial. However, it is interesting to note that in the study by Anturaniemi et al. (31), higher erythrocyte counts, and hemoglobin levels occurred in dogs fed with a high protein diet when as compared to those fed on the lowest protein diet. In the present study, the increased values of MCHC in dogs fed with diet which was richer in proteins than the control may corroborate the same trend, also considering that dietary protein may play a role in maintaining appropriate red blood cell indices (32). Clearly, further study is needed to better determine which dietary factor is responsible.

Some pre-analytical and analytical bias cannot be excluded, even if points sampling procedures and processing were standardized at all times (33, 34). Moreover, no specific trend for platelets in dogs or diets during the trial was observed. Furthermore, platelets numbers could vary in different physiological conditions, above all due to the presence of platelet clumps, often caused by sampling procedures and collection (35).

In addition, the biochemical profile also falls into the physiological range for canine species. The differences related to fructosamine, lipase, and triglycerides obtained by the Dunnett test seem to indicate a dietary effect on carbohydrates and lipid metabolism. In particular, the redaction of these parameters observed when the dogs were fed GF1, GF2, and CB diets could be related to the different proportions of carbohydrates, lipids and proteins in these diets compared to CTR. The sources of carbohydrates used in the formulation of the control diet showed a higher content of nitrogen free extractives than the others (NFE: 42.3 vs. 30.83; 34.83, and 34.60% as is, respectively, for the CTR, GF1, GF2, and CB diets), while the fat and protein contents are significantly lower in the control diet than those used for the trial. The reduction of AST observed with all diets than the control could be indicative of lower hepatic stress.

Aspartate aminotransferase is an enzyme that is found mainly in the liver and heart and, in lower concentrations, in the kidneys and muscles and low levels of AST are indicative of good health, while when the liver or muscle cells are damaged, the enzyme is released into the blood in higher quantities. Although elevated serum levels of AST could be considered a sign of a hepatic injury or disease, concomitant with other variations of hematological parameters (e.g., lower ALT values) and other clinical signs (36). In our case, clinical signs of hepatic injury were not observed, and all parameters could be considered physiological. Nevertheless, higher metabolic activity in the liver could be indicated by the higher AST values registered with CTR, and GF1 diets (37).

Another important aspect may have also concerned the carbohydrate sources used. While the CTR diet was formulated with rice (source of starch) and beet pulp, which mainly provides insoluble fiber, in the other three diets carbohydrates sources such as pea starch, spelt and oats, which are characterized by low glycemic index, and carrot, squash and pea fiber as sources of dietary fiber were used (38, 39). These ingredients guarantee a greater intake of soluble dietary fiber, able to modulate the post-prandial glycemic response. Moreover, these different dietary components guarantee the maintenance of the balance of saprophytic bacterial populations of the large intestine as it is fermented here, thereby producing short-chain fatty acids (40), in particular butyrate, which is considered the main energy source for erythrocytes and colonocytes. Dietary fiber has been indicated as a nutritional factor able to modify lipid absorption reducing, directly and indirectly, bile acid reabsorption (41). The decreasing of triglycerides observed with diets GF1, GF2, and CB could be due to limited absorption of triglycerides in the small intestine (41, 42). The reduced lipid absorption was confirmed by the significant reduction of pancreatic lipase production. Indeed, Stock-Damge' et al. (43), administering a diet supplemented with 5 g/d of wheat bran for 4 weeks, observed significantly higher ($P < 0.05$) pancreatic secretion and lower ($P < 0.05$) lipase concentration.

The significantly higher serum concentration of potassium, phosphorus, and magnesium registered when the dogs fed control diet could be related to the higher concentration of phosphorus in this diet (12 vs. 8 mg/kg). On the other hand, the higher value of phosphorus level in serum of dog fed GF1 diet could be related to the higher bioavailability of this element that mainly derived from animal sources in this diet. Moreover, CTR and GF1 diets were characterized by the higher Ca:P ratio (1.50; 1.25; 1.12 and 1.12, CTR; GF1; GF2; CB diet, respectively) (6).

Although no statistically significant differences were observed, there was an increased value in both parameters compared to the initial values of 288.27 and 101 nmol/l, recorded with the control diet. Another interesting aspect is that in all cases the taurine levels were higher than the optimal reserve levels indicated by FEDIAF (27) (>40 nmol/l in plasma and >200 nmol/l in whole blood) and by University researchers California (44) (>70 nmol/ml in plasma and >250 nmol/ml in whole blood), regardless of the dilatative cardiomyopathy risk in adult dogs.

The literature concerning the effect of diet on taurine concentration in blood and serum is controversial. Delaney et al. (45) observed that the whole blood taurine concentration was lower in dogs fed whole grain rice, rice bran, or barley. Freid et al. (46) in a retrospective study on dogs affected by dilatative myopathies, observed that dogs fed a non-traditional diet (grain-free contained novel ingredients such as peas or lentils as the main component) showed status improvement after their diet was changed.

Donadelli et al. (17) did not observe a reduction in plasma amino acids and taurine status when Labrador Retrievers were fed with a commercial grain-free diet after 26 weeks.

In our study, conducted on healthy dogs, increased taurine levels in whole blood and plasma were observed after 5 weeks of the administration of three diets. The control diet (used before the trial) could be defined as a traditional diet (grain inclusive with rice and beet pulp). While the three diets tested showed particular nutritional characteristics, and carbohydrates ingredients: GF1 (grain-free with sweet potato, pea fiber, dried carrot); GF2 (grain-free with pea starch, dried pumpkin, pea fiber, dried carrot); and CB (grain inclusive with spelt, oats, dried beet pulp, dried carrot). It seems possible to affirm that the relative proportions of the nutrients in the diets rather than the use of novel ingredients could affect taurine level. Indeed, all tested diets are characterized by the use of high-quality protein sources (dehydrated and fresh chicken, herring, and eggs), and high levels of protein inclusion allow protein and amino acids requirements to be satisfied and, consequently, the taurine status.

Moreover, the relative lower root means square error of taurine in whole blood compared to plasma one confirms the previous observation (45) that taurine has greater stability in whole blood.

CONCLUSION

Our preliminary results showed that only a few hematological parameters were affected when balanced diets were administered to healthy dogs. The sources of carbohydrates (starch and dietary fiber) and the appropriate equilibrium between energy nutrients (e.g., protein, fat, and starch) could modify the indicators of lipid, and carbohydrate metabolism (AST, fructosamine, lipase, triglycerides) and improve liver function.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

All the procedures used in the study have been approved by the Ethics Committee for the care and use of animals of the University of Naples Federico II in accordance with local and national regulations and guidelines (Legislative Decree 26 of 04/03/2014).

AUTHOR CONTRIBUTIONS

MC: conceptualization. AV, GM, and IB: formal analysis. IB and AV: methodology and data curation. SC and AV: statistical analysis. MG and GM: clinical visitation. MG and AV: writing—original draft. SC and MC: writing—review and editing. MC and MG: supervision. All authors contributed to the article and approved the submitted version.

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REFERENCES

1. Wong PWC, Yu RWM, Ngai JTK. Companion animal ownership and human well-being in a metropolis—the case of Hong Kong. *Int J Environ Res Public Health*. (2019) 16:1729. doi: 10.3390/ijerph16101729
2. ASSALCO. *REPORT ON THE FEEDING AND CARE OF PETS. Pets: Rights and Responsible Ownership. The Italian Perspective*. ASSALCO (2019).
3. Vinassa M, Vergnano D, Valle E, Giribaldi M, Nery J, Prola L, et al. Profiling Italian cat and dog owners' perceptions of pet food quality traits. *BMC Vet Res.* (2020) 16:131. doi: 10.1186/s12917-020-02357-9
4. Pezzali GJ, Aldrich CG. Effect of ancient grains and grain-free carbohydrate sources on extrusion parameters and nutrient utilization by dogs. *J Anim Sci.* (2019) 97:3758-67. doi: 10.1093/jas/skz237
5. Carciofi AC, Takakura FS, de-Oliveira LD, Teshima E, Jeremias JT, Brunetto MA, et al. Effects of six carbohydrate sources on dog diet digestibility and post-prandial glucose and insulin response. *J Anim Physiol Anim Nutr.* (2008) 92:326-36. doi: 10.1111/j.1439-0396.2007.00794.x
6. NRC. Nutrient Requirements of Dogs and Cats. Washington, DC: National Academy Press (2006).
7. Singh N. Pulses: an overview. *J Food Sci Technol.* (2017) 54:853–7. doi: 10.1007/s13197-017-2537-4
8. Hall C, Hillen C, Robinson JG. Composition, nutritional value, and health benefits of pulses. *Cereal Chem.* (2017) 94:11–31. doi: 10.1094/CCHEM-03-16-0069-FI
9. Mansilla WD, Marinangeli CPF., Ekenstedt KJ, Larsen JA, Aldrich G, Columbus DA, et al. Special topic: the association between pulse ingredients and canine dilated cardiomyopathy: addressing the knowledge gaps before establishing causation. *J Anim Sci.* (2019) 97:983–97. doi: 10.1093/jas/sky488
10. Food and Drug administration (FDA). *Investigation into Potential Link between Certain Diets and Canine Dilated Cardiomyopathy*. Available online at: https://www.fda.gov/animal-veterinary/outbreaks-and-advisories/fda-investigation-potential-link-between-certain-diets-and-canine-dilated-cardiomyopathy (accessed January 27, 2021).
11. Dukes-McEwan J, Borgarelli M, Tidholm A, Vollmar AC, Häggström J. Proposed guidelines for the diagnosis of canine idiopathic dilated cardiomyopathy. *J Vet Cardiol.* (2003) 5:7-19. doi: 10.1016/S1760-2734(06)70047-9
12. Petrič AD, Tomsič K. Diagnostic methods of cardiomyopathy in dogs - old and new perspectives and methods. *Slov Vet Res.* (2008) 45:5-14.
13. Adin DTC, De Francesco B, Keene S, Tou K, Meurs C, Atkins B, et al. Echocardiographic phenotype of canine dilated cardiomyopathy differs based on diet type. *J Vet Card.* (2019) 21:1–9. doi: 10.1016/j.jvc.2018.11.002
14. Kaplan JL, Stern JA, Fascetti AJ, Larsen JA, Skolnik H, Peddle GD, et al. Correction: taurine deficiency and dilated cardiomyopathy in Golden Retrievers fed commercial diets. *PLoS One.* (2018) 13:1-19. doi: 10.1371/journal.pone.0210233
15. Freeman LM, Stern JA, Fries R, Adin DB, Rush JE. Diet-associated dilated cardiomyopathy in dogs: what do we know? *J Am Vet Med Assoc.* (2018) 253:1390–4. doi: 10.2460/javma.253.11.1390
16. Ontiveros ES, Whelchel BD, Yu J, Kaplan JL, Sharpe AN, Fousse SL, et al. Development of plasma and whole blood taurine reference ranges and identification of dietary features associated with taurine deficiency and dilated cardiomyopathy in Golden Retrievers: a prospective, observational study. *PLoS One.* (2020) 15:e0233206. doi: 10.1371/journal.pone.0233206
17. Donadelli RA, Pezzali GJ, Oba PM, Swanson KS, Coon C, Verney J, et al. Commercial grain-free diet does not decrease plasma amino acids and taurine status but increases bile acid excretion when fed to Labrador Retrievers. *Transl Anim Sci.* (2020) 4:1-12. doi: 10.1093/tas/txaa141
18. Pezzali JG, Acuff HL, Henry W, AlexanderC, Swanson KS, Aldrich CG. Effects of different carbohydrate sources on taurine status in healthy Beagle dogs. *J Anim Sci.* (2020) 98:1–9. doi: 10.1093/jas/skaa010
19. McCauley SR, Clark SD, Quest BW, Streeter RM, Oxford EM. Review of canine dilated cardiomyopathy in the wake of diet-associated concerns. *J Anim Sci.* (2020) 98:1–20. doi: 10.1093/jas/skaa155
20. Gizzarelli M, Foglia Manzillo V, Ciuca L, Morgoglione ME, El Houda Ben Fayala N, Cringoli G, et al. Simultaneous detection of parasitic vector borne diseases: a robust cross-sectional survey in hunting, stray and sheep dogs in a mediterranean area. *Front Vet Sci.* (2019) 6:288. doi: 10.3389/fvets.2019.00288
21. Hervera M, Castrillo C, Albanell E, Baucells MD. Use of near-infrared spectroscopy to predict energy content of commercial dog food. *J Anim Sci.* (2012) 90:4401-7. doi: 10.2527/jas.2012-5106
22. Goi A, Manuelian CL, Currò S, Marchi M. Prediction of mineral composition in commercial extruded dry dog food by near-infrared reflectance spectroscopy animals. *Animals (Basel).* (2019) 9:640. doi: 10.3390/ani9090640
23. Lee SC, Prosky L. International survey on dietary fiber definition, analysis and references materials. *J AOAC Int.* (1995) 79:22-36. doi: 10.1093/jaoac/78.1.22
24. Prosky L, Asp GN, Scheweizer TF, de Vries JW, Fuurda I. Determination of insoluble, soluble, and total dietary fiber in foods and food products: interlaboratory study. *J of AOAC.* (1988) 71:1017-23. doi: 10.1093/jaoac/71.5.1017
25. Spitze AR, Wong DL, Rogers QR, Fascetti AJ. Taurine concentrations in animal feed ingredients; cooking influences taurine content. *J Anim Physiol Anim Nutr.* (2003) 87:251–62. doi: 10.1046/j.1439-0396.2003.00434.x
26. McMahon GP, O'Kennedy R, Kelly MT. High-performance liquid chromatographic determination of taurine in human plasma using pre-column extraction and derivatization. *J Pharm Biomed Anal.* (1996) 14:1287-94. doi: 10.1016/0731-7085(95)01697-X
27. FEDIAF. *Nutritional Guidelines for Complete and Complementary Pet Foods for Dogs and Cats*. Bruxelles: European Pet Food Industry Federation (2020).
28. Debraekeleer J, Gross KL, Zicker SC. Feeding young adult dogs: before middle age. In: *Small Clinical Nutrition*. 5th ed. Topeka, KS (2010). pp. 257-72.
29. Hendriks WH, Bakker EJ, Bosch G. Protein and amino acid bioavailability estimates for canine foods. *J Anim Sci.* (2015) 93:4788-95. doi: 10.2527/jas.2015-9231
30. Brown WY, Vanselow BA, Redman AJ, Pluske JR. An experimental meat-free diet maintained haematological characteristics in sprint-racing sled dogs. *Br J Nutr.* (2009) 102:1318–23. doi: 10.1017/S0007114509389254
31. Anturaniemi J, Zaldívar-López S, Moore R, Kosola M, Sankari S, Barrouin-Melo SM. The effect of a raw vs dry diet on serum biochemical, hematologic, blood iron, B12, and folate levels in Staffordshire Bull Terriers. *Vet Clin Pathol.* (2020) 49:258–69. doi: 10.1111/vcp.12852
32. Ober J, Gillette RL, Angle TC, Haney P, Fletcher DJ, Wakshlag JJ. The effects of varying concentrations of dietary protein and fat on blood gas, hematologic serum chemistry, and body temperature before and after exercise in Labrador Retrievers. *Front Vet Sci.* (2016) 3:59. doi: 10.3389/fvets.2016.00059
33. https://eclinpath.com/hematology/sample-collection-heme/ (accessed January 26, 2021).

34. Jensen AL, Wenck A, Koch J, Poulsen JS. Comparison of results of haematological and clinical chemical analyses of blood samples obtained from the cephalic and external jugular veins in dogs. *Res Vet Sci.* (1994) 56:24–9. doi: 10.1016/0034-5288(94)90191-0
35. Day MJ, Mackin A, Littlewood JD. *Ematologia e medicina trasfusionale del cane e del gatto*. Torino: UTET editori (2004).
36. Fraser CM, Bergeron JA, Mays A, Aiello SE. *Merck Veterinary Manual*. 7th ed. Rahway, NJ: Merck & Co., Inc (1991).
37. Swanson KS, Kuzmuk KN, Schook LB, Fahey GC Jr. Diet affects nutrient digestibility, hematology, and serum chemistry of senior and weanling dogs. *J Anim Sci.* (2004) 82:1713–24. doi: 10.2527/2004.826 1713x
38. Musco N, Calabrò S, Tudisco R, Grossi M, Addi L, Moniello G, et al. Diet effect on short- and long-term glycaemic response in adult healthy cats. *Vet Ital.* (2017) 53:141-5. doi: 10.12834/VetIt.57.166.3
39. Lombardi P, Musco N, Calabrò S, Tudisco R, Mastellone V, Vastolo A, et al. Different carbohydrate sources affect swine performance and post-prandial glycaemic response. *Ital J Anim Sci.* (2020) 19:421-30. doi: 10.1080/1828051X.2020.17 49899
40. Calabrò S, Carciofi AC, Musco N, Tudisco R, Gomes MOS, Cutrignelli MI. Fermentation characteristics of several carbohydrates sources for dog diets using *in vitro* gas production technique. *Ital J Anim Sci.* (2013) 12:e4. doi: 10.4081/ijas.2013.e4
41. Eastwood MA. The physiological effect of dietary fiber: an update. *Annu Rev Nutr.* (1992) 12:19–35. doi: 10.1146/annurev.nu.12.070192. 000315
42. Kim M. The water-soluble extract of chicory reduces cholesterol uptake in gut-perfused rats. *Nutr Res.* (2000) 20:1017–26. doi: 10.1016/S0271-5317(00)00192-5
43. Stock-Damge' C, Bouchet P, Dentinger A, Aprahamian M, Grenier JF. Effect of dietary fiber supplementation on the secretory function of the exocrine pancreas in the dog. *Am J Clin Nutr.* (1983) 38:843–8. doi: 10.1093/ajcn/38.6.843
44. https://www.vetmed.ucdavis.edu/labs/amino-acidlaboratory (accessed January 26, 2021).
45. Delaney SJ, Kass PH, Rogers QR, Fascetti AJ. Plasma and whole blood taurine in normal dogs of varying size fed commercially prepared food. *J Anim Physiol Anim Nutr.* (2003) 87:236–44. doi: 10.1046/j.1439-0396.2003.00433.x
46. Freid KJ, Freeman LM, Rush JE, Cunningham SM, Davis MS, Karlin ET, et al. Retrospective study of dilated cardiomyopathy in dogs. *J Vet Int Med.* (2020) 35:58-67. doi: 10.1111/jvim.15972

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Supplemental Fiber Affects Body Temperature and Fecal Metabolites but Not Respiratory Rate or Body Composition in Mid-Distance Training Sled Dogs

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Dietary fiber affects canine physiology in many ways, such as increasing colonic absorption of water and improving gut health, both of which may positively impact exercise performance. The objectives of this study were to investigate the effects of increased dietary soluble fiber and incremental training on respiratory rate (RR), internal body temperature (BT), body composition, and fecal metabolites in mid-distance training sled dogs. Fourteen dogs (12 Siberian and 2 Alaskan Huskies) were blocked by age, sex, and body weight (BW) and then randomly allocated into one of two diet groups. Seven dogs were fed a dry extruded control diet (Ctl) with an insoluble:soluble fiber ratio of 4:1 (0.74% soluble fiber on a dry-matter basis), and seven dogs were fed a dry extruded treatment diet (Trt) with an insoluble:soluble fiber ratio of 3:1 (2.12% soluble fiber on a dry-matter basis). Fecal samples were taken once a week. All dogs underwent 9 weeks of incremental exercise conditioning where the running distance was designed to increase each week. Every 3 weeks, external telemetry equipment was used to non-invasively measure and record RR and internal BT at resting, working, and post-exercise recovery states. Body composition was measured on weeks −1 and 9 using quantitative magnetic resonance. Body composition, RR, BT, and fecal metabolites were analyzed using a mixed model with dog as a random effect and week and diet group as fixed effects. Dogs on Trt had lower working and post-exercise BT than Ctl ($P < 0.05$). In addition, Trt dogs had lower recovery BT at weeks 2 and 5 than Ctl dogs ($P < 0.05$). Treatment dogs had greater fecal short-chain fatty acid concentrations than Ctl ($P < 0.05$). Diet had no effect on RR or body composition ($P > 0.10$), but exercise resulted in an overall 7% increase in lean and 3.5% decrease in fat mass ($P < 0.05$). These data suggest that increasing dietary soluble fiber may positively influence BT and gut health; however, it has no effect on RR or body composition. Soluble fiber did not negatively impact any measures of overall health and performance and should be considered for use in performance dogs.

Keywords: physiology, canine, exercise, dietary fiber, nutrition

INTRODUCTION

Exercise has the capacity to affect whole-body physiology; however, high-intensity endurance training, such as that experienced by sled dogs, can lead to gastrointestinal (GI) disturbances, heat stress, and possible dehydration (1, 2). Nutritional solutions, such as increased dietary soluble fiber inclusion, may support exercise performance through mitigation of these deleterious effects (3–6).

Dietary fiber can be divided into two categories based on its solubility in water [i.e., soluble and insoluble fiber; (7, 8)]. Soluble fibers typically have an increased extent of fermentation by GI microbes yielding short-chain fatty acids [SCFAs: mainly acetate, propionate, and butyrate; (7)]. SCFAs play a variety of physiological roles, including serving as an energy source for epithelial cells, regulating epithelial barrier integrity, supporting the immune system, and modulating inflammatory responses (9–11). In addition, SCFAs increase water absorption in the GI tract (12). As sled dogs primarily thermoregulate via respiratory evaporation (i.e., panting), water loss by way of salivation increases considerably during exercise leading to signs of hypertonic dehydration (13). Hypertonic dehydration can affect a variety of physiological outcomes, including lowering respiratory rate (RR) and increasing internal body temperature (BT) in dogs (14–16). As a BT exceeding 42°C can have serious physiological consequences in dogs (17), the ability to dissipate heat during exercise is key to minimizing deleterious outcomes and maintaining performance. Therefore, as SCFAs can increase water absorption in the colon of dogs, providing a diet with optimized soluble fiber may prevent dehydration and aid in heat dissipation that could influence RR and BT during exercise (18).

Soluble fiber may also impact body composition of actively training sled dogs. For example, sedentary humans who increased their soluble fiber intake had lower body weights (BWs), body mass index, and fat mass (FM) than those ingesting maltodextrin as a control (19). FM and lean body mass (LBM) are common measurements of body composition and have been reported to change with exercise (13, 20, 21). However, the effects of increased dietary soluble fiber on body composition in an exercising dog model have not yet been directly studied.

Therefore, the objective of this study was to investigate the effects of an increased soluble fiber diet and an incremental training regimen on the outcomes of RR, BT, body composition, and fecal metabolites in mid-distance training huskies. We hypothesized that increased soluble fiber supplementation would decrease exercise-induced internal BT and FM, increase LBM and fecal SCFAs, and have no effect on RR in actively training sled dogs.

MATERIALS AND METHODS

Animals and Housing

The study was approved by the University of Guelph's Animal Care Committee (Animal Use Protocol #4008). Twelve client-owned domestic Siberian Huskies (8 females: 8 intact; 4 males: 1 intact and 3 neutered) and 2 Alaskan Huskies (2 neutered males) with an average age of 3.75 ± 2.7 years (mean ± SD) and BW of 21.54 ± 2.83 kg were used in the study. Dogs resided and trained at an off-site facility (Rajenn Siberian Huskies, Ayr, ON) that had been previously visited and approved by the University of Guelph's Animal Care Services. During the study, dogs were group-housed in free-run, outdoor kennels (3.5–80 m^2) containing anywhere from 2 to 10 dogs each.

Diets and Study Design

Dogs were blocked for age, sex, and BW before being randomly allocated into one of two diet groups: control (Ctl; $n = 7$; 3 females: 3 intact; 4 males: 1 intact and 3 neutered) or treatment (Trt; $n = 7$; 5 females: 5 intact; 2 males: 2 neutered). For 2 weeks prior to the study period, all dogs were acclimated to a dry extruded Ctl diet [Champion Petfoods Ltd., Morinville, AB; for ingredient and nutrient composition refer to (22)] that met or exceeded all National Research Council (23) and Association of American Feed Control Officials (24) nutrient recommendations for adult dogs at maintenance. During both the acclimation and study periods, dogs were consistently fed once daily at 15:00 h. Feed allowance was first determined using historical feeding records and the calculated metabolizable energy (ME) content of the diet. Body weight was measured at baseline and each week after, and diet intake was adjusted to maintain baseline BW. Dogs in the Ctl group were fed the Ctl diet with an insoluble:soluble fiber ratio of 4:1 [dry-matter basis: 4,074 kcal/kg ME, 94% dry matter (on an as-fed basis), 47% crude protein, 25% fat, and 0.74% soluble fiber], while Trt dogs were fed a dry extruded diet with an insoluble:soluble fiber ratio of 3:1 [dry-matter basis: 4,120 kcal/kg ME, 94% dry matter (on an as-fed basis), 47% crude protein, 26% fat, and 2.12% soluble fiber; Champion Petfoods Ltd., Morinville, AB; **Table 1**]. BioMOS®, a mannan-oligosaccharide (MOS)-derived strain of *Saccharomyces cerevisiae*, oat soluble fiber, flaxseed meal, *Yucca schidigera* extract, and chicory root were included as soluble fiber sources in the Trt diet, in place of pea starch in the Ctl diet. These ingredients were chosen and added with the objective of reaching an inclusion level previously reported to positively influence canine GI metabolites (9, 25–27), while yucca was added in the Trt diet to control fecal odor (28). Daily rations were mixed with 1 cup of water before feeding; diets were mixed for 10 min to allow for a homogenous mixture. At feeding, all dogs were tethered and individually fed to allow for monitoring of food consumption. Dogs were allowed 30 min to eat their allotted food, and any orts were weighed and recorded daily. Throughout the entirety of the study, dogs had *ad libitum* access to fresh water.

Exercise Regimen

A 9-week exercise regimen was implemented with exercise distance increasing incrementally throughout the trial period. The dogs were anticipated to run 6 km a day (4 days a week) at week 0 and reach 56 km a day (4 days a week) by week 8, but ambient conditions played a role in determining the actual daily run distance (RD; **Table 2**). Training consisted of all dogs running on a 14-dog gangline with staggered, pairwise groupings of Trt and Ctl dogs. The gangline was attached to an all-terrain vehicle with one rider who controlled the machine in its lowest gear. A pace of ∼20 km/h was maintained throughout

TABLE 1 | Nutrient content and ingredient composition[a] of the control and treatment diet on a dry-matter basis.

Nutrient contents	Trt	Ctl
Metabolizable energy, kcal/kg (calculated)[b]	4,120.15	4,074.35
DM[c], %	94.15	94.15
CP, %	47.46	47.92
EE, %	25.82	24.61
TDF, %	8.28	5.52
SDF, %	2.12	0.74
IDF, %	6.16	4.67
Ash, %	8.52	9.08

[a]*Ingredient composition: pork meal, pea starch, low ash herring meal, fresh chicken, chicken fat, dried chicken, chicken meal, fresh pork, fresh pork organ blend, flaxseed meal, chicken and turkey giblets, oat soluble fiber, spray dried pork liver, pork pal (liquid), hydrolyzed poultry protein, herring oil, pork pal (dry), sodium chloride, dried kelp, choline chloride, chicory root, enticer, alpha-tocopherol, BioMOS®, natural antioxidant (liquid), thiamine, pantothenic acid, pyridoxine, potassium chloride, taurine, Yucca schidigera extract, Sel-Plex, zinc proteinate, natural antioxidant (dry), and copper proteinate. Adapted from Templeman et al. (22); ingredients in bold were only included in the Trt diet.*

[b]*Calculated metabolizable energy based on modified Atwater values.*

[c]*DM, dry matter; CP, crude protein; EE, ether extract; TDF, total dietary fiber; SDF, soluble dietary fiber; IDF, insoluble dietary fiber.*

the training period. Running speed and distance were measured using a speedometer and odometer, respectively, on the all-terrain vehicle.

Exercise Challenges

On weeks −1, 2, 5, and 8, one off-day in the dogs' training schedule (no running) was replaced by an exercise challenge. During each exercise challenge, dogs were run for 3 km at a pace of 20 km/h in either a three- or four-dog team. The distance run during exercise challenges was based on a previous study from our laboratory (29) as well as trail access and availability. Each group contained at least one Trt and one Ctl dog. Following the 3-km run, dogs were watered immediately. During each exercise challenge, all dogs were equipped with external telemetry jackets to non-invasively record both RR and BT (emka TECHNOLOGIES, Sterling, VA, USA).

Telemetry Jackets

Collection of RR data was based on a previous study by Thornton et al. (29).

A novel pilot procedure to take non-invasive BT measurements was executed by modifying a commercial resistance temperature detector (RTD)-based skin temperature probe (emka TECHNOLOGIES, Inc.) into a hermetically sealed RTD-based rectal temperature sensor. The probes were lubricated and inserted ∼2 in. into the rectum of two dogs per group. The probes were fixed to the base of the tail using adhesive tape and vet wrap. To validate the manufacturers' calibration, each probe was individually calibrated in water baths at 0 and 40°C. A standard laboratory-grade glass thermometer was used as a reference standard. Calibration was performed just prior to placement of the sensor. Internal BT was measured every second at resting (rBT), working (wBT), and post-exercise state (post-BT). Once the challenge ceased, dogs were immediately watered and remained on the gangline for 30 min to continue gathering RR and BT data until post-RR and post-BT were obtained.

Blood Sample Collection and Analysis

Blood samples were collected and analyzed as described by Templeman et al. (22). In brief, fasting blood samples were collected on weeks −1, 2, 5, and 8 to assess standard serum veterinary diagnostic measurements and markers of nutritional and health status (**Supplementary Tables 1, 2**). Dogs were fasted for 12 h overnight, and 5-ml samples were collected by way of cephalic venipuncture with a serum Vacutainer® system (Becton, Dickinson and Company, Franklin Lakes, NJ, USA). Whole blood samples (1 ml) were kept on ice prior to being analyzed for hematological indices [e.g., complete blood cell count (CBC)] using a Siemens ADVIA 2120 hematology analyzer (Siemens Healthcare Ltd., Oakville, ON). Separate samples (4 ml) were centrifuged at 2,000 × g for 20 min at 4°C using a Beckman J6-MI centrifuge (Beckman Coulter, Indianapolis, IN). Serum aliquots were collected and analyzed for serum biochemical components using a Roche Cobas 6000 c501 biochemistry analyzer (Roche Diagnostics, Indianapolis, IN).

Body Composition Assessment

Body composition [FM, LBM, and total body water (TBW)] were measured using quantitative magnetic resonance (QMR) technology (CanCog Tech, Fergus, ON) on weeks −1 and 9. With the use of QMR technology, dogs were able to be imaged while avoiding anesthetization (30).

Fecal Collection and Analysis

During fecal collection, whole samples were collected within 15 min of being voided, and all visible contaminants (e.g., grass and hair) and portions of samples that were in contact with the ground were removed. Samples were transferred into a Whirl-pak bag (Thermo Fisher Scientific, Waltham, MA) to be homogenized. Homogenized samples were then stored in 50-ml sterile centrifuge tubes (Thermo Fisher Scientific, Waltham, MA), frozen, and kept at −20°C until further analysis. Samples were analyzed for SCFAs (acetic acid, propionic acid, and butyric acid), branched-chain fatty acids (BCFAs; isobutyric acid and isovaleric acid), lactic acid, formic acid, and monosaccharides using an Agilent HP1000 series high-performance liquid chromatography (HPLC; Agilent Technologies, Santa Clara, CA).

HPLC analysis was based on a previous study from our laboratory by Templeman et al. (22). In brief, samples were prepared by combining 0.1 g of sample with 1 ml of 0.005 N sulfuric acid (concentrated sulfuric acid in Milli-Q water; Sigma Aldrich, Oakville, ON). Samples were vortexed until the fecal sample had completely dissolved and were then centrifuged using a Fisherbrand accuSpin Micro 17 (Thermo Fisher Scientific, Waltham, MA) at 13,300 rpm for 15 min. Four hundred microliters of supernatant was then drawn into an HPLC vial, and 400 μl of 0.005 M sulfuric acid was added to achieve a 2× dilution. For the HPLC system, the mobile phase was 0.005 M

TABLE 2 | Mean daily run distance, feed intake, and BW data for all dogs from weeks −1 (exercise challenge distance) to 8, body composition data (lean body mass, fat mass, and total body water) at weeks −1 and week 8, and anticipated run distance for the proposed exercise regimen with mean temperature and humidity for each week.

	Week											*P*-value		
	−1	**0**	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**SEM**	**DG[1]**	**Wk**	**DG × Wk[2]**
RD, km/day[3]	3.0^{f}	5.8^{de}	11.2^{c}	17.0^{de}	23.8^{e}	30.1^{d}	25.4^{c}	20.4^{b}	27.0^{a}	37.1^{a}	1.08	0.99	<0.01	1.00
ARD, km/day[4]	3	6	12	18	24	30	36	42	48	54				
FI, g/d	273.4^{c}	325.9^{d}	367.5^{d}	427.3^{c}	480.6^{b}	570.9^{a}	563.9^{a}	560.4^{a}	560.4^{a}	560.4^{a}	30.9	0.44	<0.01	0.93
BW, kg	21.7^{abc}	21.3^{bc}	21.3^{bc}	21.1^{c}	21.0^{c}	21.7^{abc}	22.1^{ab}	22.1^{ab}	22.3^{a}	22.5^{a}	0.34	0.34	<0.01	0.57
LBM, %	73.1^{b}									80.1^{a}	2.80	0.97	<0.01	0.92
FM, %	16.2^{a}									12.7^{b}	1.90	0.85	<0.01	0.15
TBW, %	54.7^{b}									60.0^{a}	2.02	0.96	<0.01	0.69
T, °C	-3.8^{dc}	2.2^{a}	2.4^{a}	-2.0^{cd}	0.1^{b}	-7.8^{f}	-0.6^{bc}	2.3^{a}	-6.0^{cf}	-3.5^{d}	0.6	0.82	<0.01	1.00
H, %	81.4^{e}	95.0^{a}	89.7^{abc}	86.2^{bcde}	84.8^{cde}	89.3^{abc}	87.4^{bcd}	91.0^{ab}	80.7^{de}	81.3^{e}	1.6	0.89	<0.01	1.00

FI, feed intake; BW, body weight; LBM, lean body mass; FM, fat mass; TBW, total body water; T, ambient temperature; H, humidity.

[1]*DG, diet group; Trt, treatment (2.12% soluble fiber); Ctl, control (0.74% soluble fiber).*

[2]*Interaction effect between DG and Wk (week).*

[3]*RD, mean daily run distance determined as an average of the 4 days of each week the dogs trained.*

[4]*ARD, anticipated run distance for proposed incremental exercise regimen (distance proposed to be run 4 days/week of training).*

[a–f]*Values in a row with different superscripts differ ($P < 0.05$).*

of sulfuric acid in Milli-Q water that was then filtered through a 0.2-μm filter. The column temperature was kept at 60°C, the refractive index detector temperature was kept at 40°C, and the injection volume was 20 μl. The flow rate was 0.5 ml/min, and the cycle time was 45 min. A standard curve was developed for each SCFAs, BCFAs, and monosaccharide with the following serial dilutions: 0.25, 0.50, 1.00, 2.00, and 4.00 mmol/L. OpenLAB CDS ChemStation software was used for system control and data acquisition (Agilent Technologies, Santa Clara, CA).

Statistical Analyses

Statistical analyses were performed with Statistical Analysis System (SAS) (v. 9.4; SAS Institute Inc., Cary, NC). RR and BT data were analyzed for outliers by removing the data points that exceeded an RR <200 breaths/min and lower than 5 breaths/min. For BT, data points higher than 42°C and lower than 36°C were also considered outliers. Also, the periods during the exercise challenges when a four-dog team was stopped were removed, as these interruptions reduced the dogs' ability to maintain a wRR. These stops included instances of defecation and urination. Following data cleanup, a TRANSREG procedure was used to optimally transform the RR data. BT was calculated using the means of each 1-min interval from the start of each exercise challenge until 30 min post-exercise. Variances in RR, BT, fecal metabolites, fasted blood analytes (CBC and serum biochemistry), feed intake, BW, and body composition data were analyzed using PROC GLIMMIX of SAS (v. 9.4; SAS Institute Inc., Cary, NC). Dog was treated as a random effect; and activity level (resting, working, and post-exercise), week, and diet group (DG; Trt, or Ctl) were treated as fixed effects. Activity level was analyzed against diet group and week, as well as week against diet group. RR and fecal metabolite means were compared using the Tukey honestly significant difference (HSD), whereas BT means were compared using a Fishers least significant difference (LSD). Statistical significance was declared at $P \leq 0.05$ and trends at $0.05 < P \leq 0.10$.

RESULTS

During data analysis, 18% (27/153) of the RR observations and 31% (433/1,378) of BT observations were removed due to either software malfunctions, which caused missing data, or movement of rectal probes during the challenge run. Two dogs were removed from the trial (one on week 2, Ctl; one on week 4, Ctl) due to exercise-related injuries; all data collected from these dogs until their removal were included in the results. Due to receiver limitations, only eight dogs were monitored for BT throughout the study (3 males; Trt = 1, Ctl = 2, 5 females; Trt = 3, Ctl = 2).

Mean Daily Food Intake, Body Weight, and Body Composition

No differences were observed between mean daily feed intake, BW, and RD with diet ($P > 0.10$), but all variables differed by week ($P < 0.05$; **Table 2**). Feed intake was greatest at weeks 4–8 ($P < 0.05$; **Table 2**) with no differences between weeks 4 and 8 ($P > 0.10$; **Table 2**). Body weight decreased from baseline at weeks 2 and 3 ($P < 0.05$); however, BW did not differ from week −1 to weeks 0–1 ($P > 0.10$) and weeks 4–8 ($P > 0.10$; **Table 2**).

Body composition (FM, LBM, and TBW) at weeks −1 and 9 did not differ between diet groups ($P > 0.10$); however, when data were pooled to evaluate the effects of exercise, all variables differed by week ($P < 0.05$; **Table 2**). LBM increased by 7%, TBW increased by 5.3%, and FM decreased by 3.5% from week −1 to 9 ($P < 0.05$; **Table 2**).

TABLE 3 | Mean internal body temperature (°C) at resting, working, and post-exercise state for control (0.74% soluble fiber on a dry-matter basis) and treatment groups (2.12% soluble fiber on a dry-matter basis) in sled dogs running at a pace of 20 km/h for 3 km.

		Exercise challenge				SEM (Wk)	Mean	SEM (mean)	*P*-value		
		Week −1	Week 2	Week 5	Week 8				DG[1]	Wk	DG × Wk[2]
rBT[3], °C	Trt	38.4	38.7	38.7	38.7	1.53	38.6	0.16	0.556	0.830	0.992
	Ctl	38.6	38.9	38.9	38.8	0.39	38.8	0.17			
wBT, °C	Trt	39.7[#]	39.2	39.7	39.3[#]	0.20	39.4*	0.14	0.006	0.159	0.108
	Ctl	39.8	39.9	39.8	39.6	0.19	39.8*	0.13			
post-BT, °C	Trt	39.6[b]	38.9*	39.3[ab*]	39.1[ab]	0.23	39.2*	0.13	0.009	0.055	0.012
	Ctl	39.3[b]	39.7[ab*]	40.1[a*]	39.3[b]	0.24	39.6*	0.13			

[1]*DG, diet group; Trt, treatment dogs; Ctl, control dogs; SEM, standard error of the mean, n = 3 for rBT (Trt: n = 2; Ctl: n = 1, weeks −1 and 2), n = 7 for rBT (Trt: n = 4; Ctl: n = 3, week 5), and n = 4 for rBT (Trt: n = 3; Ctl: n = 1, week 8); n = 5 for wBT (Trt: n = 2; Ctl: n = 3, week −1), n = 7 for wBT (Trt: n = 3; Ctl: n = 4, week 2), n = 7 for wBT (Trt: n = 4; Ctl: n = 3, week 5), and n = 6 for wBT (Trt: n = 3; Ctl: n = 3, week 8); n = 5 for post-BT (Trt: n = 2,; Ctl: n = 3, week −1), n = 7 for post-BT (Trt: n = 3: Ctl: n = 4, week 2), n = 7 for post-BT (Trt: n = 4: Ctl: n = 3, week 5), and n = 6 for post-BT (Trt: n = 3: Ctl: n = 3, week 8).*

[2]*Interaction effect between DG and Wk (week).*

[3]*rBT, resting body temperature; wBT, working body temperature; post-BT, post-exercise body temperature.*

[a,b]*Values in a row with different superscripts differ (P < 0.05; within treatment).*

[#]*Mean values for body temperature tended to differ from week −1 within the same row (P < 0.10).*

**Values for body temperature different between diet groups within the same column (P < 0.05).*

TABLE 4 | Mean respiratory rate (breaths per minute) at rest, work, and post-exercise during weeks −1, 2, 5, and 8 for control (0.74% soluble fiber on a dry-matter basis) and treatment groups (2.12% soluble fiber on a dry-matter basis) in sled dogs running at a pace of 20 km/h for 3 km.

		Exercise challenge				SEM (Wk)	Mean[a]	SEM (mean)	*P*-value		
		Wk −1	Wk 2	Wk 5	Wk 8				Wk	DG	DG × Wk[b]
rRR[c], bf[d]	Trt	18	16	17	18	1.53	17	0.60	0.481	0.262	0.790
	Ctl	19	18	17	18	1.71	18	0.67			
wRR, bf	Trt	173	171	170	172	2.89	172	2.69	0.934	0.143	0.354
	Ctl	165	166	166	165	3.39	165	2.96			
post-RR, bf	Trt	22	28	26	26	2.43	26	1.93	0.723	0.809	0.382
	Ctl	25	25	24	25	3.18	25	2.06			

[a]*Trt, treatment dogs; Ctl, control dogs; SEM, standard error of the mean, n = 11 for rRR (week −1), n = 13 for rRR (week 2), and n = 10 for rRR (weeks 5 and 8); n = 13 for wRR (week −1), n = 11 for wRR (week 2), and n = 10 for wRR (weeks 5 and 8); n = 9 for post-RR (weeks −1, 2, and 5), and n = 11 for post-RR (week 8).*

[b]*Interaction effect between DG and Wk (week).*

[c]*rRR, resting respiratory rate; wRR, working respiratory rate; post-RR, post-exercise respiratory rate.*

[d]*bf, breathing frequency (breaths per minute).*

Body Temperature

No differences in rBT were observed between dietary treatments ($P > 0.10$; **Table 3**); however, Trt dogs presented with a lower mean wBT and post-BT than Ctl ($P < 0.05$, **Table 3**). Post-exercise BT was lower at weeks 2 and 5 for Trt vs. Ctl ($P < 0.05$; **Table 3**). No other differences were observed between DG; therefore, internal BT was pooled to evaluate the effect of exercise. No differences were observed between rBT over weeks ($P > 0.10$). Working BT tended to be lower at week 8 compared with week −1 ($P < 0.10$). Post-exercise BT was greater at week 5 compared with weeks 2 and 8 ($P < 0.05$) but was not different than week −1 ($P > 0.10$).

Respiratory Rate

No differences in rRR, wRR, and post-RR were observed between dietary treatments ($P > 0.10$; **Table 4**); therefore, these data were pooled to evaluate the effect of exercise. No differences in activity level (rRR, wRR, and post-RR) were observed between weeks throughout the study duration ($P > 0.10$; **Table 4**).

Fecal Short-Chain Fatty Acids, Branched-Chain Fatty Acids, Lactic Acid, and Monosaccharides

Overall, dogs receiving the Trt diet had greater concentrations of fecal propionic acid ($P < 0.05$) and butyric acid ($P < 0.05$; **Table 5**) than Ctl. Concentrations of fecal acetic acid tended to be greater in Trt compared with Ctl ($P = 0.06$; **Table 5**). Treatment dogs had greater fecal isobutyric acid concentrations than Ctl ($P < 0.05$; **Table 5**). In addition, Trt dogs had greater fecal concentrations of lactose, glucose, xylose, and arabinose than Ctl ($P < 0.05$; **Table 5**). Diet had no effect on fecal formic acid, isovaleric acid, or lactic acid ($P > 0.10$; **Table 5**); therefore, all remaining data were pooled for effect of exercise. For all dogs, fecal propionic acid concentrations were greater during

TABLE 5 | Fecal concentrations of short-chain fatty acids, branched-chain fatty acids, lactic acid, and monosaccharides of sled dogs being fed a diet containing a soluble fiber content of 0.74% (Ctl) or 2.12% (Trt) throughout 9 weeks of incremental exercise conditioning.

	Week								SEM[1]	Diet		SEM	*P*-value		
	−1	0	1	2	3	4	5	6	7	8		Trt	Ctl		Wk	DG	DG × Wk[2]
Acetic acid[3]	75.10^{c}	86.22^{bc}	89.28^{bac}	95.23^{ba}	102.00^{ba}	98.30^{ba}	95.75^{ba}	96.52^{ba}	102.83^{ba}	108.21^{a}	5.12	100.53	89.36	5.41	<0.01	0.06	0.29
Propionic acid	20.04^{b}	30.59^{a}	29.52^{a}	31.26^{a}	29.46^{a}	28.93^{a}	20.34^{a}	32.18^{a}	34.34^{a}	36.48^{a}	3.03	35.55*	25.08	1.51	<0.01	<0.01	0.02
Butyric acid	18.40^{bcd}	18.09^{abcd}	15.66^{d}	17.94^{cd}	22.54^{ab}	22.54^{ab}	23.28^{ab}	22.37^{abc}	25.22^{a}	25.54^{a}	2.00	22.76*	19.56	0.89	<0.01	<0.01	0.58
Formic acid[4]	2.22	2.20	2.26	2.22	2.22	2.23	2.23	2.20	n/a[5]	2.22	0.04	2.22	2.22	0.02	0.30	0.81	0.25
Isobutyric acid	17.15^{a}	12.91^{b}	12.66^{b}	11.92^{b}	19.71^{a}	19.99^{a}	19.39^{a}	21.14^{a}	21.85^{a}	20.64^{a}	1.45	18.58*	16.90	0.69	<0.01	0.02	0.15
Isovaleric acid	5.49	5.35	5.49	5.69	5.51	5.88	5.91	5.68	6.61	5.71	0.49	5.66	5.81	0.32	0.11	0.65	0.26
Lactic acid	2.19^{ab}	2.17^{ab}	2.20^{ab}	2.23^{ab}	2.23^{a}	2.23^{ab}	2.25^{a}	2.19^{ab}	2.14^{b}	2.16^{ab}	0.03	2.21	2.19	0.02	<0.01	0.52	0.70
Lactose	4.63^{b}	5.11^{ab}	5.69^{ab}	6.08^{ab}	5.99^{ab}	6.57^{ab}	7.22^{a}	6.94^{ab}	7.70^{a}	8.08^{a}	0.75	7.07*	5.73	0.51	0.01	0.01	0.28
Glucose	1.96^{ab}	2.01^{a}	1.96^{ab}	1.91^{b}	1.93^{ab}	1.92^{ab}	2.02^{ab}	n/a	n/a	1.96^{ab}	0.04	1.99*	1.93	0.02	0.02	0.01	0.02
Xylose	4.28	3.56	3.26	3.24	3.30	3.54	4.24	3.15	3.25	2.83	0.79	4.25*	2.68	0.28	0.22	<0.01	0.09
Arabinose	5.28	5.22	4.67	3.97	3.68	3.61	6.49	4.01	4.63	3.67^{a}	0.94	5.03*	4.03	0.39	0.07	0.01	0.08

[1]*Standard error of the mean.*

[2]*Interaction effect between DG and Wk (week).*

[3]*Data presented in micromoles per gram (μmol/g) on a dry-matter basis; SCFA: acetic acid + propionic acid + butyric acid; BCFA: isobutyric acid + isovaleric acid; monosaccharides: lactose, xylose, arabinose.*

[4]*Data presented in nanomoles per gram (nmol/g) on a dry-matter basis; formic acid; lactic acid; glucose.*

abcd[5]*Values in a row (within week) with different superscripts differ ($P \leq 0.05$).*

**Asterisk indicates difference between diet groups ($P \leq 0.05$).*

N/A Insufficient data.

weeks 0–8 than week −1 ($P < 0.05$; **Table 5**). Fecal butyric acid concentrations were greater during weeks 7 and 8 compared with week −1 ($P < 0.05$; **Table 5**). Fecal acetic acid concentrations were greater during weeks 2–8 compared with week −1 and greater at week 8 compared with week 0 ($P < 0.05$; **Table 5**). Week had no effect on fecal formic acid concentrations ($P > 0.10$; **Table 5**). Fecal isobutyric acid was greater at week −1 compared with weeks 0–2 ($P < 0.05$; **Table 5**) but did not differ thereafter ($P > 0.10$; **Table 5**). Week had no effect on fecal isovaleric acid concentrations ($P > 0.10$; **Table 5**). Fecal lactic acid concentration was greater during weeks 3 and 5 compared with week 7 ($P < 0.05$), but no other weeks differed ($P > 0.10$; **Table 5**). Fecal lactose concentrations were greater in week −1 than weeks 5, 7, and 8 ($P < 0.05$; **Table 5**). Fecal glucose concentrations were greater in week 0 compared with week 2 ($P < 0.05$; **Table 5**). Week had no effect on fecal xylose and arabinose concentrations ($P > 0.10$; **Table 5**).

Complete Blood Count and Serum Biochemistry

Treatment dogs had greater concentrations of white blood cells, leukocytes (**Supplementary Table 1**), and alanine aminotransferase (ALT; $P < 0.05$; **Supplementary Table 2**) and lower concentrations of creatinine ($P < 0.05$; **Supplementary Table 2**) than Ctl dogs; however, all mean CBC and serum biochemistry values were within standard reference range for dogs of both diet groups (as determined by the Animal Health Laboratory, University of Guelph, Guelph, ON). Except for five dogs that presented with high eosinophils at weeks −1 and 2, all dogs remained healthy throughout the study period (**Supplementary Table 1**).

DISCUSSION

This study was the first to evaluate the effects of increased dietary soluble fiber on internal BT, RR, body composition, and fecal metabolites in mid-distance training sled dogs. The data presented herein indicate that for actively training sled dogs, 9 weeks of an increased soluble fiber diet may have contributed to a decrease in the working and recovery state internal BT. In addition, dogs receiving the soluble fiber-supplemented diet had greater fecal concentrations of butyric and propionic acid than had Ctl dogs; however, increased soluble fiber had no effect on RR or body composition. Participation in 9 weeks of incremental exercise resulted in an overall increase in LBM and decrease in FM.

Body Temperature

Given that dogs primarily dissipate heat through evaporative panting (31), water lost by way of salivation increases considerably during exercise (32), contributing to an increased risk of dehydration, which, in turn, may negatively impact thermoregulatory capabilities (32). For example, sedentary dogs dehydrated by way of water restriction or induced hypertonic dehydration have been reported to have elevated BT when compared to hydrated dogs (14, 16, 33). As the Trt dogs in the current study presented with an overall reduction in internal

temperature at both a working and post-exercise state when compared to the Ctl dogs, this suggests that supplementation of soluble fiber so as to achieve an inclusion level of 2.12% may have contributed to a more efficient regulation of internal BT during exercise. For example, exercising horses fed diets high in soluble fiber were reported to have greater TBW and lower exercising BT than horses fed insoluble fiber (34); however, it should be noted that unlike dogs, horses dissipate heat primarily through active sweating (35). As the diet in the current study had no impact on changes in TBW content, and since no other measurements of hydration status were reported, we cannot definitely say that increased dietary soluble fiber improved hydration status; we can only make inferences based on changes in BT. In addition, as only eight dogs were evaluated for changes in BT, the authors acknowledge this as a limitation. Therefore, future research is warranted to investigate hydration status, exercise, and internal BT using a greater sample size of actively training dogs to support these pilot data.

Independent of diet, conditioning has been reported to reduce exercising BT in both humans (36, 37) and dogs (38). In the current study, wBT at week 8 tended to decrease from baseline, suggesting a possible improvement in the thermoregulatory ability as training progressed. As exercise training leads to an increased cardiac output with increased blood volumes directed toward respiratory muscles (39), the improved regulation of BT is thought to be due to the increased blood flow to areas of heat exchange supporting thermoregulation in dogs (40). Therefore, as previous studies report improved cardiorespiratory capacity with exercise (41, 42), the likelihood of such an event occurring in the current study is high. However, as only RR was measured in the current study, with no other cardiorespiratory variables, the ability to relate thermoregulation to improved exercise conditioning in exercising dogs requires further investigation. However, as exercise usually takes place in colder environments, this reduction in BT could also be attributed to ambient temperature. However, as both BT and ambient temperature were highly variable throughout the current training period, future research should be conducted to investigate the association between ambient temperature and internal BT in dogs exercising in cold climates.

Respiratory Rate

Hydration status has been reported to influence RR, as dehydrated dogs have lower RR (14) in an attempt to conserve body water content while panting (15, 33). Although the current study reported no changes in RR following an increased soluble fiber diet, the level of dehydration during exercise may not have been great enough to influence RR. For example, a previous study from our laboratory reported that sled dogs running 5 km daily led to signs of hypertonic dehydration (13), whereas the dogs in the current study were running just over 3 km daily. This suggests that unlike BT, RR may not be affected by slight changes in hydration. Future research is warranted to investigate changes in hydration status with a 2.12% soluble fiber diet in relation to RR in both a sedentary and exercising dog model.

For all dogs, 9 weeks of endurance exercise training had no effect on RR during any given activity level. Our laboratory previously reported that 12 weeks of aerobic exercise training resulted in a decreased RR at both a resting and post-exercise state with changes starting at week 5 (29). As the dogs in the current study were running 25.4 km/day during week 5 and the previous study's RD was 37.2 km/day at week 5, this 11.8 km/day difference could be behind the lack of exercise effects. In addition, the current study began descaling the training regimen at week 5, whereas Thornton et al. (29) began descaling at week 7. As a result, the changes in duration could have influenced RR, as our lab previously reported that RR may be sensitive to a training regimen and requires a continuous incremental training regimen to elicit changes (29).

Body Composition

Participation in regular aerobic exercise positively affects body composition resulting in reductions in FM and increases in LBM in humans (20, 43). In dogs, 12 weeks of endurance training resulted in increased LBM of 11% and decreased FM of 4.5% (22). The current study reported an increase in LBM of 7% and FM decrease of 3.5%, with diet having no effect on changes in body composition. As previous studies examined the effects of fiber supplementation on body composition utilizing overweight, sedentary subjects (19), the effects of exercise in the current study may have been greater than those of increased soluble fiber. For example, as BW was maintained and feed intake was adjusted to maintain energy requirements for increased exercise, the changes seen in LBM and reduction in FM can likely be attributed to the exercise regimen itself.

Fecal Metabolites

Soluble fiber is known to increase microbial fermentation and production of SCFAs, which can have a variety of physiological effects. For example, SCFAs can stimulate an increase in water absorption, as these major anions have been reported to be responsible for osmotic water absorption in the colon of the dogs (12). As Trt dogs in the current study had greater concentrations of both propionic and butyric acid than had Ctl dogs, there is an increased likelihood of greater water retention in the dogs consuming the higher soluble fiber diet. As such, this could lead to possible secondary effects in the dogs, resulting in lower internal BT, which could in turn help with exercise performance. Additionally, as exercise duration increased over time, the dogs' energy requirements also increased, leading to greater feed intake and subsequently protein intake. Fecal isobutyric acid concentration was greater in Trt dogs and has been reported in dogs consuming greater levels of dietary protein (44). However, as protein contents of both diets were similar and overall feed intake did not differ between treatment groups, it is possible that the inclusion of soluble fiber reduced the digestibility of protein, thereby increasing its availability for fermentation in the colon (45). Together, because SCFAs, BCFAs, and other fecal metabolites increased over time, it is likely that the environment of the hindgut microbiota was altered. As sled dogs are at risk of impaired gut health (i.e., increased gut permeability) leading to instances of diarrhea and other deleterious outcomes, provision of soluble fiber can support inflammatory responses and reduce exercise-induced diarrhea (46, 47). As diarrhea is the leading cause of exercise discontinuation in exercising sled dogs (1, 2), minimizing pathogenic microbes could have beneficial outcomes

related to performance. For example, sled dogs running in a 400-km race in Norway presenting with low levels of dysbiosis-associated bacteria (i.e., *Fusobacterium*, *Clostridium hiranonis*, and *Blautia*) prior to a race performed better than dogs with a greater level of these bacteria (48). As such, the fecal metabolite data in the current study suggest a shift toward an improved gut microbial environment. However, due to restrictions of laboratory analysis during 2020, the effects of both exercise and dietary soluble fiber on the gut microbiota were not able to be assessed. Therefore, future research should be directed toward evaluating microbial diversity and stool quality during exercise and nutritional interventions in training sled dogs.

CONCLUSION

The findings in the current study suggest that the addition of 1.38% soluble fiber to achieve an insoluble:soluble fiber ratio of 3:1 may have contributed to a reduction in internal BT at both working and recovery post-exercise states and resulted in greater fecal SCFA concentrations. Supplemental soluble fiber had no effect on RR or body composition; however, a 9-week training regimen resulted in increased LBM and decreased FM. Future research is warranted to investigate how a diet with a soluble fiber inclusion of 2.12% influences hydration status, and various cardiorespiratory measurements (i.e., heart rate and VO_2max) in exercising dogs. Overall, these results can be used to improve training regimens and diets that may influence exercise physiology, health, and performance of sled dogs.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author/s.

ETHICS STATEMENT

The animal study was reviewed and approved by University of Guelph's Animal Care Committee. Written informed consent was obtained from the owners for the participation of their animals in this study.

AUTHOR CONTRIBUTIONS

ET, JT, KS, and AS designed the experiment. ET, ER, and JT conducted the research. ET and LB analyzed the data. AS had primary responsibility for the final content. All authors read and approved the final manuscript. All authors contributed to the writing of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2021.639335/full#supplementary-material

REFERENCES

1. McKenzie E, Riehl J, Banse H, Kass PH, Nelson S, Marks SL. Prevalence of diarrhea and enteropathogens in racing sled dogs. *J Vet Int Med*. (2010) 24:97–103. doi: 10.1111/j.1939-1676.2009.0418.x
2. Gagné J, Wakshlag J, Simpson K, Dowd S, Latchman S, Brown D, et al. Effects of a synbiotic on fecal quality, short-chain fatty acid concentrations, and the microbiome of healthy sled dogs. *BMC Vet Res*. (2013) 9:246. doi: 10.1186/1746-6148-9-246
3. Gibson GR, Wang X. Regulatory effects of bifidobacterial on the growth of other colonic bacteria. *J Appl Bacteriol*. (1994) 77:412–20. doi: 10.1111/j.1365-2672.1994.tb03443.x
4. Araya-Kojima T, Yaeshima T, Ishibashi N, Shimamura S, Hayasawa H. Inhibitory effects of Bifidobacterium longum BB536 on harmful intestinal bacteria. *Bifidobacteria Microflora*. (1995) 14:59–66. doi: 10.12938/bifidus1982.14.2_59
5. Wu I-C, Chang H-Y, Hsu C-C, Chiu Y-F, Yu S-H, Tsai Y-F, et al. Association between dietary fiber intake and physical performance in older adults: a nationwide study in Taiwan. *PLoS ONE*. (2013) 8:e80209. doi: 10.1371/journal.pone.0080209
6. Okamoto T, Morino K, Ugi S, Nakagawa F, Lemecha M, Ida S, et al. Microbiome potentiates endurance exercise through intestinal acetate production. *Am J Physiol*. (2019) 316:E956–66. doi: 10.1152/ajpendo.00510.2018
7. Howarth N, Saltzman E, Roberts S. Dietary fiber and weight regulation. *Nutr Rev*. (2001) 59:129–39. doi: 10.1111/j.1753-4887.2001.tb07001.x
8. Bronkowska M, Konsediak A, Orzeł D. Assessment of the frequency of intake of selected sources of dietary fibre among persons competing in marathons. *Roczniki Panstwowego Zakladu Higieny*. (2018) 69:347–51. doi: 10.32394/rpzh.2018.0039
9. Swanson KS, Grieshop CM, Flickinger EA, Bauer LL, Healy HP, Dawson KA, et al. Supplemental fructooligosaccharides and mannanoligosaccharides influence immune function, ileal and total tract nutrient digestibilities, microbial populations and concentrations of protein catabolites in the large bowel of dogs. *J Nutr*. (2002) 132:980–9. doi: 10.1093/jn/132.5.980
10. Wong JMW, de Souza R, Kendall CWC, Emam A, Jenkins DJA. Colonic health: fermentation and short chain fatty acids. *J Clin Gastroenterol*. (2006) 40:235–43. doi: 10.1097/00004836-200603000-00015
11. Mondo E, Barone M, Soverini M, D'Amico F, Cocchi M, Petrulli C, et al. Gut microbiome structure and adrenocortical activity in dogs with aggressive and phobic behavioral disorders. *Heliyon*. (2020) 6:e03311. doi: 10.1016/j.heliyon.2020.e03311

12. Herschel DA, Argenzio RA, Southworth M, Stevens CE. Absorption of volatile fatty acid, Na, and H20 by the colon of the dog. *Am J Vet Res*. (1981) 42:1118–24.
13. Templeman J, McCarthy N, Lindinger M, Shoveller A. Changes in salivary electrolyte concentrations in mid-distance trained sled dogs during 12 weeks of incremental conditioning. *Physiol Rep*. (2020) 8:e14493. doi: 10.14814/phy2.14493
14. Kozlowski S, Greenleaf JJ, Turlejska E, Nazar K. Extracellular hyperosmolality and body temperature during physical exercise in dogs. *Am J Physiol*. (1980) 239:R180–3. doi: 10.1152/ajpregu.1980.239.1.R180
15. Horowitz M, Nadel E. Effect of plasma volume on thermoregulation in the dog. *Pflügers Archiv*. (1984) 400:211–3. doi: 10.1007/BF00585045
16. Baker M, Turlejska E. Thermal panting in dehydrated dogs- effects of plasma-volume expansion and drinking. *Pflugers Archiv Eur J Physiol*. (1989) 413:511–5. doi: 10.1007/BF00594182
17. Lewis ST, Foster RC. Effect of heat on canines and felines. *ISU Vet*. (1976) 38:117–21.
18. Zanghi B, Robbins P, Ramos M, Otto C. Working dogs drinking a nutrient-enriched water maintain cooler body temperature and improved pulse rate recovery after exercise. *Front Vet Sci*. (2018) 5:202. doi: 10.3389/fvets.2018.00202
19. Guerin-Deremaux L, Li S, Pochat M, Wils D, Mubasher M, Reifer C, et al. Effects of NUTRIOSE® dietary fiber supplementation on body weight, body composition, energy intake, and hunger in overweight men. *Int. J. Food Sci. Nutr*. (2011) 62:628–35. doi: 10.3109/09637486.2011.569492
20. Evans P. Effects of HRT and exercise training on insulin action, glucose tolerance, and body composition in older women. *J Appl Physiol*. (2001) 90:2033–40. doi: 10.1152/jappl.2001.90.6.2033
21. Allen J, Mailing L, Niemiro G, Moore R, Cook M, White B, et al. Exercise alters gut microbiota composition and function in lean and obese humans. *Med Sci Sports Exerc*. (2018) 50:747–57. doi: 10.1249/MSS.0000000000001495
22. Templeman J, Thornton E, Cargo-Froom C, Squires E, Swanson K, Shoveller A. Effects of incremental exercise and dietary tryptophan supplementation on the amino acid metabolism, serotonin status, stool quality, fecal metabolites, and body composition of mid-distance training sled dogs. *J Anim Sci*. (2020) 98:skaa128. doi: 10.1093/jas/skaa128
23. National Research Council. *Nutrient Requirements of Dogs and Cats. 2nd rev. ed*. Washington, DC: The National Academies Press (2006).
24. Association of American Feed Control Officials. *AAFCO Manual*. West Lafayette, IN: AAFCO Inc. (2016).
25. Grieshop CM, Flickinger EA, Bruce KJ, Patil AR, Czarnecki-Maulden GL, Fahey GC. Gastrointestinal and immunological responses of senior dogs to chicory and mannan-oligosaccharides. *Archiv Anim Nutr*. (2004) 58:483–93. doi: 10.1080/00039420400019977
26. Barry KA, Hernot DC, Middelbos IS, Francis C, Dunsford B, Swanson KS, et al. Low-level fructan supplementation of dogs enhances nutrient digestion and modifies stool metabolite concentrations, but does not alter fecal microbiota populations. *J Anim Sci*. (2009) 87:3244–52. doi: 10.2527/jas.2008-1659
27. Ferreira LG, Endrighi M, Lisenko KG, Oliveira MRD, de Damasceno MR, Claudino JA, et al. Oat beta-glucan as a dietary supplement for dogs. *PLoS ONE*. (2018) 13:e0201133. doi: 10.1371/journal.pone.0201133
28. Cheeke PR. Actual and potential applications of Yucca Schidigera and Quillaja Saponaria Saponins in human and animal nutrition. In: Oleszek W, Marston A, editors. *Saponins in Food, Feedstuffs and Medicinal Plants*. Dordrecht: Springer (2000). p. 241–54. doi: 10.1007/978-94-015-9339-7_25
29. Thornton E, Templeman JR, Bower M, Cant JP, Holloway GP, Shoveller AK. Exercise but not supplemental dietary tryptophan influences heart rate and respiratory rate in sled dogs. *Vet Sci*. (2020) 7:97. doi: 10.3390/vetsci7030097
30. Mitchell AD, Rosebrough RW, Taicher GZ, Kovner I. In vivo measurement of body composition of chickens using quantitative magnetic resonance. *Poult Sci*. (2011) 90:1712–9. doi: 10.3382/ps.2010-01156
31. Sharp F, Smith D, Thompson M, Hammel H. Thermoregulatory salivation proportional to hypothalamic temperature above threshold in the dog. *Life Sci*. (1969) 8:1069–76. doi: 10.1016/0024-3205(69)90159-3
32. Hardy J, Hellon R, Sutherland K. Temperature-sensitive neurones in the dog's hypothalamus. *J Physiol*. (1964) 175:242–53. doi: 10.1113/jphysiol.1964.sp007515
33. Baker M, Doris P, Hawkins M. Effect of dehydration and hyperosmolality on thermoregulatory water losses in exercising dogs. *Am J Physiol*. (1983) 244:R516–21. doi: 10.1152/ajpregu.1983.244.4.R516
34. Spooner H, Nielsen B, Schott H. *Hydration Status of Endurance Horses as Affected by Dietary Fiber Type with and without Supplemental Fat*. ProQuest Dissertations Publishing. (2008) Retrieved from: http://search.proquest.com/docview/304575867/ (accessed November 17, 2020).
35. McConaghy F. Thermoregulation. In: Hodgson DR, Rose RJ, editors. *The Athletic Horse: Principles and Practice of Equine Sports Medicine*. Philadelphia, PA: WB Saunders (1994). p. 181.
36. Piwonka R, Robinson S, Gay V, Manalis R, Piwonka R. Preacclimatization of men to heat by training. *J Appl Physiol*. (1965) 20:379–83. doi: 10.1152/jappl.1965.20.3.379
37. Shvartz E, Magazanik A, Glick Z. Thermal responses during training in a temperate climate. *J Appl Physiol*. (1974) 36:572–6. doi: 10.1152/jappl.1974.36.5.572
38. Young M. Body temperature and heat exchange during treadmill running in dogs. *J Appl Physiol*. (1959) 14:839–43. doi: 10.1152/jappl.1959.14.5.839
39. Hales J, Dampney R. The redistribution of cardiac output in the dog during heat stress. *J Therm Biol*. (1975) 1:29–34. doi: 10.1016/0306-4565(75)90008-X
40. Robbins P, Ramos M, Zanghi B, Otto C. Environmental and physiological factors associated with stamina in dogs exercising in high ambient temperatures. *Front Vet Sci*. (2017) 4:144. doi: 10.3389/fvets.2017.00144
41. Sugawara J, Murakami H, Maeda S, Kuno S, Matsuda M. Change in post-exercise vagal reactivation with exercise training and detraining in young men. *Eur J Appl Physiol*. (2001) 85:259–63. doi: 10.1007/s004210100443
42. Huang G, Wang R, Chen P, Huang S, Donnelly J, Mehlferber J. Dose–response relationship of cardiorespiratory fitness adaptation to controlled endurance training in sedentary older adults. *Eur J Prevent Cardiol*. (2016) 23:518–29. doi: 10.1177/2047487315582322
43. Mosher P, Underwood S, Ferguson M, Arnold R. Effects of 12 wks of aerobic circuit training on aerobic capacity, muscular strength, and body composition in college-age women. *J Strength Cond Res*. (1994) 8:144–8. doi: 10.1519/00124278-199408000-00004
44. Donadelli RA, Titgemeyer EC, Aldrich CG. Organic matter disappearance and production of short- and branched-chain fatty acids from selected fiber sources used in pet foods by a canine in vitro fermentation model. *J Anim Sci*. (2019) 97:4532–9. doi: 10.1093/jas/skz302
45. Gao L, Chen L, Huang Q, Meng L, Zhong R, Liu C, et al. Effect of dietary fiber type on intestinal nutrient digestibility and hindgut fermentation of diets fed to finishing pigs. *Livestock Sci*. (2015) 174:53–8. doi: 10.1016/j.livsci.2015.01.002
46. Leib MS. Treatment of chronic idiopathic large-bowel diarrhea in dogs with a highly digestible diet and soluble fiber: a retrospective review of 37 cases. *J Vet Int Med*. (2000) 14:27–32. doi: 10.1111/j.1939-1676.2000.tb01495.x
47. Minamoto M. Fecal short-chain fatty acid concentrations and dysbiosis in dogs with chronic enteropathy. *J Vet Int Med*. (2019) 33:1608–18. doi: 10.1111/jvim.15520
48. Tysnes KR, Angell IL, Fjellanger I, Larsen SD, Softeland SR, Robertson LJ, et al. Pre- and Post-Race Intestinal microbiota in long-distance sled dogs and associations with performance. *Animals*. (2020) 10:204. doi: 10.3390/ani10020204

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ORIGINAL RESEARCH
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The Effects of 7 Days of Feeding Pulse-Based Diets on Digestibility, Glycemic Response and Taurine Levels in Domestic Dogs

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Grain-based carbohydrate sources such as rice comprise 30–50% of commercial pet foods. Some pet foods however have removed the use of grains and have instead incorporated pulses, such as peas and lentils, resulting in grain-free diets. The hypothesis was dog diets with higher levels of dietary fiber will produce a low glycemic response due to decreased rates of digestion and lowered bioavailability of all macronutrients and increased fecal bile salt excretion. This in turn was hypothesized to produce lower plasma concentrations of cysteine, methionine and taurine after 7 days of feeding each test diet in dogs. Six diets were formulated at an inclusion level of 20% available carbohydrate, using white rice flour (grain) or whole pulse flours from smooth pea, fava bean, red lentil or 2 different wrinkled pea varieties (CDC 4,140–4 or Amigold) and fed to beagles in a randomized, cross-over, blinded design. After 7 days feeding each diet, fasting blood glucose was the lowest in the lentil (3.5 ± 0.1 mmol/L) and wrinkled pea (4,140–4; 3.6 ± 0.1 mmol/L) diet periods, while peak glucose levels was lowest after feeding the lentil diet (4.4 ± 0.1 mmol/L) compared to the rice diet. Total tract apparent digestibility of all macronutrients as well as taurine differed among diets yet plasma taurine was not outside normal range. Decreased macronutrient and amino acid digestibility was associated with increasing amylose and dietary fiber content but the specific causative agent could not be determined from this study. Surprisingly, digestibility decreases were not due to increased bile salt loss in the feces since increasing dietary fiber content led to decreased fecal bile salt levels. In conclusion, although pulse-based canine diets have beneficial low glycemic properties, after only 7 days, these pulse-based diets decrease macronutrient and amino acid digestibility. This is likely related at least in part to the lower animal protein content, but on a long-term basis could put domestic dogs at risk for low taurine and dilated cardiomyopathy.

Keywords: digestibility, taurine, glycemic response, pulses, canine, fiber

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INTRODUCTION

The global pet food industry has been steadily growing and is projected to reach a value of $91 billion USD by 2022 (1). In a majority of homes with pets, pet owners feed them commercially prepared diets as they are affordable and nutritionally complete with the belief that they promote animal health (2). Pet diets in North America are formulated to meet nutritional requirements based on the standards set by the Association of American Feed Control Officials (AAFCO).

Pet owners have an active role in choosing diets to feed their pets and generally make their decisions based on: (i) true knowledge of what comprises a healthy diet, (ii) perception of nutritional requirements, (iii) human diet trends and (iv) overall opinions of the pet food industry. Importantly, owners will often change their pet's diet to match the diet consumed by the human owner (3). Pet foods are marketed strategically to owners, with trendy claims such as "organic," "natural" and "grain-free" often found with premium pet foods (4). Grain-free diets exclude the use of grains such as wheat, corn, or rice flours and instead incorporate pulses such as peas, lentils and fava beans as the major carbohydrate source (5). While pulses are common dietary ingredients for both humans and animals, they are considered to be highly nutritious primarily due to their high levels of protein, in addition to carbohydrate, fiber, vitamins and minerals (6). Pulse crops are slowly digested due to relatively high amylose, resistant starch and dietary fiber content (7, 8). This characteristic of pulses can be utilized to optimize satiety through a lowered glycemic response and glycemic index (6), a feature that is also in dogs [(9, 10), Briens et al., unpublished].

Postprandial blood glucose responses can vary based on the type of carbohydrate and the rate of digestion (11). In dogs, diets with different levels of starch cause variations in postprandial glucose responses (12). In human studies, foods with a low glycemic index, such as legumes or pulses, are slowly digested, which provides a slow and sustained release of glucose into the blood stream (11). Alternatively, foods that are quickly digested, such as white bread, have a high glycemic index, which result in rapid and high postprandial blood glucose levels (11). Similarly, in both dogs and cats, legumes result in a slow or negligible postprandial glucose response while grains result in a faster, higher postprandial blood glucose response [(9, 13), Briens et al., unpublished]. Thus, pulse-containing diets promote glucose control, insulin sensitivity, satiety, weight control and longer-term health in humans and dogs (10, 14).

While high-protein pulses can decrease the cost to produce a high protein pet food due to a lower need for animal protein, a downside to pulses is that plant protein lacks taurine. Moreover, compared to cereal grains, pulses contain limited amounts of the non-essential amino acid cysteine and reduced amounts of the essential amino acids, methionine and tryptophan (6). Cysteine and methionine are used by the dog liver and the central nervous system to synthesize taurine via the transsulfuration pathway. Thus, taurine is not considered to be an essential amino acid in dogs. While dilated cardiomyopathy is common in dogs (15), some cases are associated with low plasma taurine and can be reversed with taurine supplementation (16). Canine dilated cardiomyopathy was reported by the US Food and Drug Administration (FDA), based initially on 9 case studies, to be associated with feeding grain-free diets in July 2018. This led to a decrease in grain-free dog food sales, decreased use of pulses in pet food and losses to the pulse-growing agriculture sector. Confusion among veterinarians and pet owners as to whether grain-free diets are healthy for dogs was further exacerbated by a recent acknowledgment by the FDA in November 2020 that causes of dilated cardiomyopathy in dogs may be more complicated than just a single ingredient such as pulses and is instead likely multi-factorial. To begin to address some of these questions, this study aimed to explore whether grain-free diets lead to taurine deficiency and if this is associated with simultaneous low cysteine and methionine levels. Moreover, mechanisms by which pulses could deplete taurine, cysteine or methionine require experimental confirmation.

One possible explanation is dietary fiber which was linked to decreased taurine levels in dogs (17, 18). Fiber increases the excretion of fecal bile acids, and since taurocholate is the most common bile salt in dogs, this leads to increased fecal loss of taurine (19, 20). In addition to taurine loss, high dietary fiber decreases protein digestibility, resulting in decreased cysteine and methionine (precursors of taurine) bioavailability. Again, while the slow carbohydrate digestibility of pulses has some beneficial health effects such as low glycemic index, high dietary fiber of pulses may exacerbate the already low taurine, cysteine and methionine found in grain-free diets (20, 21). What is unclear is what component of dietary fiber is responsible for the increased fecal bile salt loss, decreased protein digestibility and decreased sulfur amino acid bioavailability. As a first exploration, this study aims to use diets with increasing levels of dietary fiber and amylose content using different pulses as well as to explore its role in these processes in dogs.

The hypothesis was that dog diets with higher levels of dietary fiber will produce a low glycemic response due to decreased rates of digestion and lowered bioavailability of all macronutrients and increased fecal bile salt excretion. This in turn is hypothesized to produce lower plasma concentrations of cysteine, methionine and taurine after 7 days of feeding each test diet in dogs. In order to investigate these hypotheses, whole and complete diets formulated to include 20% available carbohydrate using a grain (white rice flour) compared to whole pulse flours from smooth pea (CDC Inca), fava bean (CDC Snowdrop), red lentil (CDC Maxim) or 2 different wrinkled pea varieties (CDC 4,140–4 or Amigold) were fed to beagles in a randomized, cross-over, blinded design. After 7 days of feeding each diet, macronutrient digestibility and glycemic responses were examined along with plasma concentrations of cysteine, methionine, and taurine as well as fecal bile salt concentrations in beagle dogs.

EXPERIMENTAL METHODS

All procedures and handling of the dogs were conducted following protocols approved by the University of Saskatchewan's Animal Research Ethics Board according to guidelines that were

established by the Canadian Council on Animal Care (Animal Utilization Protocol #20190055).

Animals

Adult Beagle dogs ($n = 8$; 4 spayed females, 4 neutered males; 8.87 ± 0.90 kg, 2–4 years old) were obtained from certified scientific breeders (Marshal Bioresources, North Rose, NY, USA and King Fisher International, Stouffville, Ontario, Canada). Beagles were housed at the Animal Care Unit (ACU) in the Western College of Veterinary Medicine at the University of Saskatchewan, Saskatoon, SK, Canada. Beagles were group-housed during the day in a large enclosure to allow for daily socialization but were individually kenneled during feedings and overnight. Dogs were walked and socialized on a daily basis. The Beagles were also provided regular health examinations, deworming and routine vaccinations from certified veterinarians to ensure optimal health.

Diets

The test diets included one control (rice; a grain-containing diet) and five pulse-based diets [all grain-free diets: smooth pea (CDC Inca), wrinkled pea (4,140–4 variety), wrinkled pea (Amigold variety), red lentil (CDC Maxim) and fava bean (CDC Snowdrop)]. All diets were formulated at 20% available carbohydrate using locally obtained flours (flour proximate analyses shown in **Supplementary Table 1**). A non-digestible Celite marker was also incorporated at 1% for determination of total tract apparent digestibility and measured as acid-insoluble ash in proximate analyses of diet and feces (22). Diets were formulated using the software Creative Concept 5 (Creative Formulation Concepts, Pierz, MN, USA) and were structured to meet the nutritional requirements for canine adult maintenance (see **Table 1** for formulations). These requirements were based on AAFCO and the National Research Council recommendations. Feed ingredients were sourced from local and commercial sources as needed and diets were extruded into a dry kibble format using a laboratory-scale, co-rotating, twin-screw extruder (Baker Perkins Ltd, Peterborough, UK) at the University of Winnipeg (Food Science Laboratory, Winnipeg, MB, Canada). All diets were extruded under the same conditions as described in **Supplementary Table 2**. Diets were then vacuum coated with fat at the Canadian Feed Research Centre (North Battleford, SK, Canada). Samples of all diets were then sent to analytical laboratories for proximate and amino acid analysis (Central Testing Laboratory Ltd., Winnipeg, MB, Canada) according to AOAC standards (23). Dry matter was determined by oven-drying the sample and crude protein determined using the Kjeldahl method while non-fiber carbohydrate and fat were determined through acid-hydrolysis solvent extraction. Metabolizable energy (ME) content of diets was determined through calculation (see footnote to **Table 2** for equation). Fiber analyses were performed according to the AOAC 2011.25 method (Eurofins, Toronto, ON, Canada).

Dogs were fed twice daily, weighed weekly and body condition scored using a 9-point scale (24). During the pre-trial phase of at least a month, each dog was fed a standard commercial diet (Purina Proplan, Mississauga, ON Canada) and individual food portion/maintenance energy required to maintain ideal weight (body condition score of 4–5 on a 9-point scale) determined. Once on trial, isocaloric test diet portions to that determined for each dog in the pre-trial phase was calculated and used throughout the feeding trial without any further adjustment to portion size.

Glycemic Index & Digestibility Testing

To establish both glycemic responses and starch digestibility of diets as well as the effects on circulating amino acid and taurine levels, Beagles were fed each test diet for 7 days. This was done in a randomized, cross-over, blinded, repeated measures design study with a 3-day washout period on the commercial diet between each test diet and another 7-day feeding period followed by 3-day washout repeated until all diets had been tested in each dog. Total tract apparent digestibility was determined in feces collected on the sixth and seventh day of each feeding period. After collection, feces were kept at−20°C until they were dried at 65°C for 72 h. Fecal samples were then sent to an analytical laboratory to assess nutrient excretion (Central Testing Laboratory Ltd., Winnipeg, MB, Canada). In addition, another portion of dried feces was used to conduct total bile acid analyses using a commercial kit according to manufacturer instructions (Total Bile Acid Assay Kit, Cell Biolabs Inc. San Diego, California, USA) which uses a colorimetric enzyme driven reaction in which bile acids are incubated in the presence of 3-alpha hydroxysteroiddehydrogenase and thio-NADH.

Total tract apparent digestibility was calculated using the formula:

$$\textit{Apparent Digestibility} = \left\{1 - \left(\frac{\%\ \textit{Nutrient in Feces}}{\%\ \textit{Nutrient in Diet}}\right)\ x\ \left(\frac{\%\ \textit{Indicator in Diet}}{\%\ \textit{Indicator in Feces}}\right)\right\} \times 100$$

On day 7 of feeding, dogs were fasted overnight and 8.0 mL of whole blood was collected the next day from the jugular vein. While still fasted, 5.0 mL of whole blood was collected into EDTA tubes and centrifuged at 2,200 RPM to obtain plasma. Samples were stored at−80°C until assayed for plasma cysteine (measured as the total of the cysteine dimer, cystine, plus the deprotonated form of cysteine called half-cystine), methionine, and taurine at a contract analytical laboratory (Amino Acid Laboratory, University of California Davis, Davis, CA, USA). Beagles were then catheterized in the cephalic vein and fasting blood glucose was determined using an Ultra2 glucometer (OneTouch, LifeScan Canada ULC, Malvern, PA, USA). Depending on the diet fed during the week, dogs were either fed glucose (oral glucose tolerance test) providing 1 g/kg body weight of glucose after consumption of the commercial diet or fed a portion of the test diet fed that week to provide 1 g/kg available carbohydrate (1 g/kg divided by % available carbohydrate) according to

TABLE 1 | Formulation of test diets with increasing fiber and amylose content listed from left to right. All diets were formulated to include 20% available carbohydrate.

	Rice diet	Lentil diet	Smooth pea diet	Fava bean diet	Wrinkled pea diet (4,140-4)	Wrinkled pea diet (Amigold)
Flour	23.12	42.19	41.67	46.40	58.65	58.14
Chicken By-Product Meal	37.83	21.49	24.26	17.53	8.85	9.03
Cellulose	15	14	12	14	10	10
Chicken Fat*	10	10	10	10	10	10
Fish Meal	5	5	5	5	5	5
Canola Oil	6.55	5.00	5.00	5.00	5.00	5.00
Celite	1	1	1	1	1	1
Vitamin/Mineral Premix	1	1	1	1	1	1
NaCl	0.3	0.3	0.3	0.3	0.3	0.3
Choline Chloride	0.1	0.1	0.1	0.1	0.1	0.1
Calcium Carbonate	0.05	0.05	0.05	0.05	0.05	0.05
Dicalcium Phosphate	0.05	0.05	0.05	0.05	0.05	0.38

*All values are expressed as % inclusion as fed. *Included addition of antioxidant Naturox (Kemin, Des Moines, IO USA).*

TABLE 2 | Proximate analyses of test diets after extrusion and fat coating.

	Rice diet	Lentil diet	Smooth pea diet	Fava bean diet	Wrinkled pea diet (4,140–4)	Wrinkled pea diet (Amigold)
DM (%)	89.78	90.43	89.90	90.62	89.33	89.69
Crude Protein (%)[a]	33.69	30.96	32.86	30.41	27.64	26.13
Crude Fiber (%)[b]	6.050	5.230	6.460	8.930	8.770	9.620
Fat (%)[c]	19.79	19.99	16.01	18.45	17.81	19.05
Ash (%)[d]	8.960	7.330	8.420	7.170	6.180	6.500
Metabolizable Energy (kcal/kg)[e]	3,913	4,121	3,806	3,855	3,920	3,856

Diets are listed in order of increasing fiber and amylose content from left to right. DM, dry matter; All % values are relative to dry matter except Metabolizable Energy (kcal/kg).
[a]Determined using a Nitrogen/Protein Analyzer (CN628, LECO Corporation, St. Joseph, MI, USA), with a conversion factor of 6.25.
[b]Determined by Central Testing Laboratory Ltd. (Winnipeg, Manitoba, Canada), following Crude Fiber Method by Ankom Technology (2017).
[c]Determined by Central Testing Laboratory Ltd. (Winnipeg, Manitoba, Canada), following AOCS Method Am 5-04.
[d]Determined by Central Testing Laboratory Ltd. (Winnipeg, Manitoba, Canada), following AOAC Method 942.05.
*[e]Determined using the ME equation for swine: (kcal/kg)=4151-(122*Ash)+(23*Crude Protein)+(38*Fat)-(64*Crude fiber)*[1.003-(0.0021* Crude Protein)].*

established protocols in dogs in our group (9). The amount of available carbohydrate in each diet was determined using a commercially obtained test kit (Available Carbohydrate Assay Kit, Megazyme, Bray, Ireland, see **Table 2**). Available carbohydrate was defined as total digestible starch (TDS) plus maltodextrins, sucrose, D-glucose, D-fructose and lactose. The available carbohydrate method measures glucose liberated *in vitro* after 4 h incubation (AOAC Method 2020.07). Blood glucose levels were monitored using a glucometer over a 5-h time period (time 0, 5, 10, 15, 20, 30, 45, 60, 90, 120, 150, 180, 210, 240, 270, 300) according to methods established in this group (10).

Sulfur Containing Amino Acid Plasma Analysis

Plasma sample analyses were conducted at a contract analytical laboratory (Amino Acid Laboratory, University of California Davis, Davis, CA, USA). Modified AOAC Official Method 994.12 alternative III was performed according to GLP (taurine and methionine recovery rates were 97–102%). Variances between duplicates were <5%. Cystine results were corrected by multiplying factor of 2 (recovery rate is about 50%).

Data Handling and Statistics

All data were tested for normality and outliers using the Kolomogorov-Smirnov test, Q-Q plots and box plots. Depending on the normality of the data either a repeated measure, 1-way ANOVA or a repeated measure, 1-way ANOVA on ranked data was then conducted followed by *post-hoc* Tukey's tests if significance was achieved. Differences were considered statistically significant at $p \leq 0.05$. Values obtained during the oral glucose tolerance test were not used for statistical analysis and were provided strictly for reference or in glycemic index calculations. Principal components analysis was also performed on the six dietary treatment groups (34 variables studied). Factors were reduced to two components and variables studied were reduced to 24 where ≥83% of the variance across the data was explained. Analyses were performed using SigmaPlot 12.0 and Systat 12.0 (Systat Software Inc. San Jose, CA, USA).

TABLE 3 | Cystine, methionine and taurine levels in test diets after extrusion and fat coating.

	Rice diet	Lentil diet	Smooth pea diet	Fava bean diet	Wrinkled pea diet (4,140–4)	Wrinkled pea diet (Amigold)
Cystine[a]	1.010	0.610	0.170	0.640	0.120	0.490
Methionine[a]	0.840	0.470	0.700	0.480	0.380	0.460
Cystine & Methionine[a]	1.850	1.080	0.870	1.120	0.500	0.950
Taurine[a]	0.140	0.090	0.120	0.090	0.070	0.070
LMWDF[b]	< 0.6	2.2	2.0	1.6	4.8	4.9
Soluble HMWDF[b]	0.9	1.3	1.2	1.7	1.5	1.9
Insoluble HMWDF[b]	15.2	18.1	16.7	20.6	21.3	21.8
TDF[b]	16.1	21.6	19.9	23.9	27.6	28.6
Amylose content[c]	5.1 ± 0.2	7.4 ± 0.2	7.6 ± 0.1	7.7 ± 0.1	13.9 ± 0.1	14.6 ± 0.1
Available carbohydrate (g/100g)[d]	23.9 ± 0.6	24.7 ± 0.3	25.2 ± 0.3	23.9 ± 0.3	22.4 ± 0.6	22.7 ± 0.4
Meal size fed to provide 1 g/kg available carbohydrate (g whole diet/kg dog)	4.2	4.0	4.0	4.2	4.5	4.4

Also shown are the contents of different fiber types, amylose, available carbohydrate and meal size needed to provide 1 g/kg available carbohydrate for each diet. Diets are listed in order of increasing fiber and amylose content from left to right. All values are expressed as % dry matter unless stated otherwise. LMWDF, low molecular weight dietary fiber; HMWDF, high molecular weight dietary fiber; TDF, total dietary fiber.

[a]Determined by Central Testing (Winnipeg, MB, Canada) using UPLC and ninhydrin detection.

[b]Determined by Eurofins (Toronto, ON, Canada) using AOAC 2011.25 dietary fiber method.

[c]Determined using an iodine colorimetric method of (26).

[d]Determined using Megazyme Available Carbohydrate Assay Kit following AOAC Method 2020.07.

TABLE 4 | Total tract apparent digestibility analyses of protein, fat, starch, cystine, methionine, cystine+methionine and taurine in the 6 different test diets formulated at 20% available carbohydrate with variable amounts of fiber, fed for 7 days.

	Rice	Lentil	Smooth pea	Fava bean	Wrinkled pea (4,140–4)	Wrinkled pea (Amigold)	*P*-Value
Protein**	84.35 ± 1.3[b]	81.49 ± 0.69[b]	80.85 ± 0.66[b]	79.21 ± 0.67[a,b]	78.90 ± 0.58[a,b]	72.95 ± 1.37[a]	< 0.001
Fat*	98.47 ± 0.18[c]	97.52 ± 0.30[b,c]	96.40 ± 0.65[a,b]	97.52 ± 0.27[b,c]	96.85 ± 0.42[a,b]	95.82 ± 0.48[a]	< 0.001
Starch**	97.71 ± 0.41[b,c]	97.72 ± 0.76[c]	97.77 ± 0.23[a,b,c]	98.28 ± 0.20[b,c]	89.68 ± 2.08[a,b]	87.74 ± 2.49[a]	< 0.001
Cystine*	89.76 ± 0.78[d]	84.91 ± 1.09[d]	41.80 ± 2.30[b]	86.10 ± 0.78[d]	6.52 ± 4.14[a]	73.31 ± 3.45[c]	< 0.001
Methionine**	79.22 ± 1.66[b]	76.27 ± 0.85[a,b]	83.29 ± 0.99[b]	75.80 ± 0.82[a,b]	64.72 ± 2.20[a]	53.56 ± 5.24[a]	< 0.001
Cystine + Methionine**	84.98 ± 1.01[c]	80.97 ± 0.94[b,c]	75.18 ± 1.08[a,b]	81.85 ± 0.73[b,c]	41.12 ± 6.47[a]	63.75 ± 4.07[a,b]	< 0.001
Taurine**	82.92 ± 1.83[a,b]	80.78 ± 11.58[b]	77.44 ± 3.09[a,b]	69.98 ± 2.19[a]	77.57 ± 5.96[a,b]	74.42 ± 5.11[a,b]	0.034

Diets are listed in order of increasing fiber and amylose content from left to right. All values are expressed as % of total tract apparent digestibility. Data is shown as Mean ± SEM; n = 8 dogs. Different letters indicate significant differences among diets using Tukey's post-hoc analysis (P < 0.05) after significant P-values reported in 1-Way Repeated Measures ANOVA and Friedman's One-Way ANOVA on Ranked Data**. Where same letters are indicated in a row, no significant differences among diets were detected.*

*Protein, and fat, determined by Central Testing (Winnipeg, MB Canada) as described in **Table 2**. Diet and fecal amino acids and starch were determined by Central Testing (Winnipeg, MB, Canada) using UPLC with ninhydrin detection and enzymatically using UV detection, respectively.*

RESULTS

Proximate and Amino Acid Analysis of Diets

As expected, crude fiber increased in the diets from the lowest rice control diet to the highest wrinkled pea diets (**Table 2**). Crude fat of the diets ranged from 16.01% to 19.99%, but was unrelated to dietary fiber (**Table 2**). Similarly, metabolizable energy ranged from 3,806 to 4,121 kcal/kg. In the test diets, crude protein (% dry matter) ranged from 26.13% to 33.69% which was well-above the AAFCO minimum of 18% dietary protein (25). However, dietary protein content slightly decreased with increasing fiber content, as higher levels of pulse ingredients were incorporated (**Table 2**). All test diets met or exceeded the AAFCO requirements of 0.33% methionine and 0.65% cystine+methionine (25), except the wrinkled pea CDC 4,140–4 diet which had 0.5% cystine+methionine (**Table 2**). Dietary methionine was highest in the rice diet at 0.84% on a dry matter basis, with all pulse-based diets containing lower methionine to a low of 0.38% for the high fiber CDC 4,140–4 wrinkled pea diet (**Table 2**). Dietary cystine ranged from a high of 1.01% for the rice diet to a low of 0.38% for the CDC 4,140–4 wrinkled pea diet, similar to the methionine results (**Table 2**). The grain-containing rice diet also had the highest level of taurine at 0.14% (**Table 2**). In contrast, the pulse containing diets had lower, varying amounts of taurine which ranged from 0.07% to 0.12% as shown in **Table 3**.

Fiber, Amylose and Carbohydrate Content of the Diets

Diets were formulated to contain 20% available carbohydrate and results of the available carbohydrate kit for diets showed good agreement with the target (**Table 3**). Available carbohydrate content (defined as the amount of glucose liberated by amyloglucosidase+pancreatin in 4 h *in vitro* at 37°C) in the formulated test diets ranged from 22.4% to 25.2%, as shown in **Table 3**. This available carbohydrate value was then used to calculate what meal size needed to be fed to provide 1 g available carbohydrate per kilogram body weight of the dogs during glycemic testing (**Table 3**). The same test diets prior to fat coating were tested for *in vitro* starch digestibility using Englyst methodology in separate study by this group (27). Uncoated wrinkled pea diets after extrusion (both Amigold and 4,140–4) had higher resistant starch content (15.1–19.0% on a dry starch basis) compared to the other pulse-based or rice-based diets (2.7–5.5% on a dry starch basis). Also, both wrinkled pea diets had lower gelatinized starch after extrusion (11.9–12.3% dry matter) compared with those of round pea, lentil, fava bean, and rice diets (17.1–19.6%; 9).

Dietary fiber analyses of the fat coated test diets used in this feeding study demonstrated that they had varying levels of dietary fiber (**Table 3**). Low molecular weight dietary fiber varied from < 0.6–4.9% for the rice and the wrinkled pea (Amigold) diets, respectively (**Table 3**). High molecular weight dietary fiber was subdivided into two categories: insoluble high molecular weight dietary fiber and soluble high molecular weight dietary fiber. The soluble high molecular weight dietary fiber varied from 0.9% to 1.9% (rice and Amigold wrinkled pea diets, respectively), while insoluble high molecular weight dietary fiber varied from 15.2% to 21.8% (rice and Amigold wrinkled pea diets, respectively; **Table 3**). Similarly, total dietary fiber ranged from 16.1% to 28.6% (rice and Amigold wrinkled pea diets, respectively; **Table 3**). Finally, the wrinkled pea (Amigold) pulse-containing diet had the highest amylose content (14.6%) while the rice-containing diet had the lowest level of amylose at 5.1% (**Table 3**).

Digestibility

Total tract apparent digestibility of crude protein varied across the diets (ANOVA, $p < 0.001$). Crude protein digestibility varied from 72.95% to 84.35%, the rice diet had the highest crude protein digestibility, and the wrinkled pea (Amigold) diet had the lowest (**Table 4**). For sulfur-containing amino acids, the total tract apparent digestibility of cystine, methionine, cystine+methionine and taurine varied among test diets (ANOVA, $p < 0.001$ for all but taurine where $p = 0.034$; **Table 4**). Variability was noted for cystine digestibility which ranged from 6.5% to 90% (**Table 4**). This variability was not due to diarrhea. Although fecal output and score (quality) were not quantitated in this study, qualitatively, no obvious changes were observed among diets. All other macronutrient (fat and starch) digestibility values were also different among diets (**Table 4**).

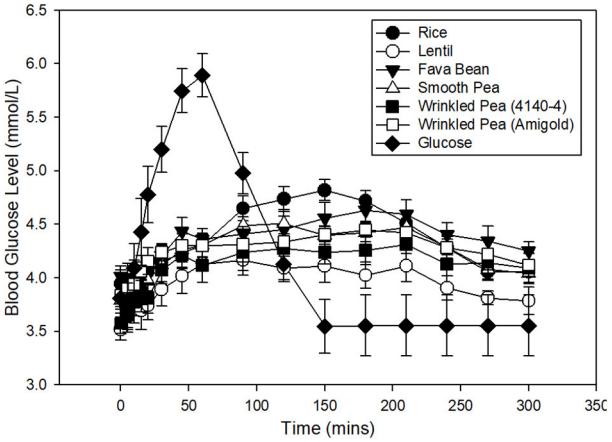

FIGURE 1 | Time course of blood glucose response in fasted dogs after oral glucose challenge (1 g glucose/kg body weight) after 7 days of feeding a commercial husbandry diet (filled circles). Time course of blood glucose response in fasted dogs after consuming a meal of each test diet (1 g available carbohydrate/kg body weight) is also shown. Dogs were fed each test diet formulated at 20% available carbohydrate with increasing levels of fiber and amylose (lowest to highest from top to bottom in figure legend) for 7 days prior to an overnight fast, followed by the test meal the next day. Data is shown as Mean ± SEM; $n = 8$ dogs.

Glycemic Response

Blood glucose increased from fasting after feeding glucose or a meal with 1 g available carbohydrate/kg body weight, then returned to baseline within 5 h in dogs (**Figure 1**). From these glycemic response figures, quantitative data and statistical analyses on peak, time to peak, area under the curve and glycemic index were calculated (**Table 5**). In dogs fed the commercial diet for 7 days, the fasting blood glucose level prior to the oral glucose tolerance test was 3.8 ± 0.2 mmol/L. After 7 days of feeding the test diets, fasting blood glucose had variation with diet in these same dogs (**Figure 1**; ANOVA, $p < 0.001$; **Table 5**). After ingestion of the glucose standard, glucose levels peaked at 52.5 ± 5.5 min with a blood glucose value of 6.3 ± 0.2 mmol/L (**Figure 1**, **Table 5**). Time to peak blood glucose was longer after a meal of a whole diet and the peak was lower when compared to the response to the glucose standard (**Figure 1**).

Dogs fed the lentil-based diet and wrinkled pea (4,140–4) diet for 7 days had the lowest fasting blood glucose levels (3.5 ± 0.1 mmol/L) and (3.6 ± 0.1 mmol/L), respectively (**Table 5**). The peak blood glucose was also different among diets (ANOVA, $p = 0.01$), while the time to peak was not different (ANOVA, $p = 0.20$). Dogs fed the rice diet had the highest peak blood glucose at 5.0 ± 0.09 mmol/L, while the lowest peak blood glucose was observed with the lentil diet at 4.4 ± 0.1 mmol/L (**Table 5**). The area under the blood glucose response curve (AUC) was different among diets (ANOVA, $p = 0.02$; **Table 5**), ranging from 810.9 ± 15.8 mmol/L x min for the rice diet to a low of 726.5 ± 21.7 mmol/L x min for the lentil diet (**Table 5**). Glycemic index values of the diets followed the AUC trend, with differences

TABLE 5 | Quantitative measures of glycemic response in fasted beagles after feeding a meal of different test diets.

	Glucose	Rice	Lentil	Smooth pea	Fava bean	Wrinkled pea (4,140-4)	Wrinkled pea (Amigold)	*P*-Value
Fasted Blood Glucose (mmol/L)*	3.8 ± 0.2	3.9 ± 0.1^{b}	3.5 ± 0.1^{a}	3.8 ± 0.06^{a}	4.0 ± 0.1^{b}	3.6 ± 0.1^{a}	3.8 ± 0.1^{b}	< 0.001
Peak (mmol/L)*	6.3 ± 0.2	5.0 ± 0.09^{b}	4.4 ± 0.1^{a}	4.7 ± 0.1^{a,b}	4.8 ± 0.1^{a,b}	4.5 ± 0.1^{a,b}	4.7 ± 0.1^{a,b}	0.01
Time to Peak (min)**	52.5 ± 5.5	135 ± 12.7^{a}	99.4 ± 18.6^{a}	91.9 ± 9.2^{a}	132.5 ± 20.9^{a}	111.6 ± 18.0^{a}	130 ± 22.8^{a}	0.2
AUC (mmol/L x min)*	849.3 ± 19.6	810.9 ± 15.8^{b}	726.5 ± 21.7^{a}	780.2 ± 14.0^{a,b}	792.6 ± 12.6^{a,b}	749.4 ± 18.8^{a,b}	776 ± 14.1^{a,b}	0.02
Glycemic Index*		95.7 ± 2.2^{c}	85.8 ± 2.8^{a}	92.2 ± 2.6^{b,c}	93.6 ± 2.2^{b,c}	88.3 ± 1.5^{a,b}	91.5 ± 1.2^{b,c}	< 0.001

Diets are listed in order of increasing fiber and amylose content from left to right. Data is taken from that shown in ***Figure 1****.*

Data is shown as Mean ± SEM; n = 8 dogs. Different letters indicate significant differences among diets using Tukey's post-hoc analysis (P < 0.05) after significant P-values reported in 1-Way Repeated Measures ANOVA and Friedman's One-Way ANOVA on Ranked Data**. Where same letters are indicated in a row, no significant differences among diets were detected. Glucose data was used to calculate glycemic index and is shown for reference, but was not used in the statistical analyses of the diets.*

among diets (ANOVA, $p < 0.001$; **Table 5**). The rice diet had the highest glycemic index of 95.7 ± 2.2, while the lowest glycemic index was observed in the lentil diet at 85.8 ± 2.8 (**Table 5**).

Sulfur Containing Amino Acids in Plasma

After 7 days of feeding each diet, fasting plasma taurine was different in the dogs after feeding different diets (ANOVA, $p = 0.021$; **Table 6**). Feeding rice and the lentil diets produced the highest plasma taurine levels in the dogs at 99 and 111 nmol/ml (equivalent to μmol/L), while feeding the fava bean diet produced the lowest plasma taurine at 73 nmol/ml (**Table 6**). Despite differences in dietary content of cystine and methionine (**Table 2**), after 7 days of feeding each diet, fasting levels of plasma methionine and half-cystine were not significantly different (**Table 6**). In contrast, plasma cysteine varied greatly among the dogs fed the different diets with the highest levels when the lentil and rice diets were fed.

Bile Acid Assay

Fecal bile acid content was significantly different among diets, with the rice diet having the highest value and the wrinkled pea 4,140–4 diet having the lowest (**Figure 2**).

Principal Components Analysis

In order to assess the relationships among the variables tested, a Principal Components Analysis (PCA) was run initially with all variables measured in all 6 test diets. The top 24 variables that were correlated to the first two components were retained in the final PCA analysis. These 24 variables explained 83.13% of variance in this final analysis (see **Table 7**). A factor loadings plot of these variables (**Figure 3A**) clustered variables with the greatest tendency to be positively related to each other. A plot of weighted factor scores for each of these 24 variables for each diet resulted in a graph where the rice diet was clearly separated from the two wrinkled pea diets, while fava bean, smooth pea and lentil diets were intermediate (**Figure 3B**). This confirms the order we predicted based on fiber and amylose content. In support of this, the greatest separation among the diets was along the x-axis showing Factor 1 scores (**Figure 3B**) where high positive scores indicated diets with high dietary content of chicken meal, crude protein, sulfur amino acids (cystine, methionine and taurine) as well as high fecal bile salt content, available carbohydrate and fat digestibility, but low values for dietary content of amylose and all fiber fractions (**Figure 3B**). The diets did not separate as much on the y-axis except for the lentil diet, but the 95% confidence intervals were very large for this diet and it did overlap with all diets on this axis (**Figure 3B**). High positive y-axis values for Factor 2 were associated with high fasting blood glucose, long time to peak blood glucose, high glycemic index and high plasma methionine values, but low plasma cystine, half-cysteine and taurine values.

DISCUSSION

The most important findings of this study were that the pulse-based, grain-free diets produced a lowered glycemic response, which could be utilized to promote increased satiety and

TABLE 6 | Plasma amino acid levels of taurine, half-cystine, cysteine and methionine observed in fasted dogs after 7 days of feeding each test diet.

	Rice	Lentil	Smooth pea	Fava bean	Wrinkled pea (4,140-4)	Wrinkled pea (Amigold)	*P*-Value
Half-cystine*	17.61 ± 0.85[a]	19.16 ± 1.62[a]	17.46 ± 1.14[a]	16.43 ± 1.42[a]	18.34 ± 1.59[a]	17.13 ± 1.12[a]	0.42
Cysteine**	132.5 ± 32.79[a]	2732.75 ± 139.75[b]	807.38 ± 279.88[a,b]	458.63 ± 213.27[a]	158.88 ± 213.27[a]	147.86 ± 28.58[a]	0.002
Methionine*	56.31 ± 3.13[a]	50.18 ± 2.09[a]	53.58 ± 2.08[a]	54.74 ± 2.51[a]	53.35 ± 3.13[a]	52.55 ± 3.54[a]	0.52
Taurine**	99.03 ± 11.90[a,b]	111.14 ± 18.04[b]	82.24 ± 8.67[a,b]	72.68 ± 12.74[a]	86.63 ± 10.95[a,b]	89.74 ± 15.36[a,b]	0.021

Diets are listed in order of increasing fiber and amylose content from left to right.
All values expressed nmol/ml (equivalent to μmol/L). Amino acids were measured at a contract analytical laboratory (Amino Acid Laboratory, University of California Davis, Davis, CA, USA) using modified AOAC Official Method 994.12 alternative III.
Data is shown as Mean ± SEM; n = 8 dogs. Different letters indicate significant differences among diets using Tukey's post-hoc analysis (P < 0.05) after significant P-values reported in 1-Way Repeated Measures ANOVA and Friedman's One-Way ANOVA on Ranked Data**. Where no letters are indicated in a row, no significant differences among diets were detected.*

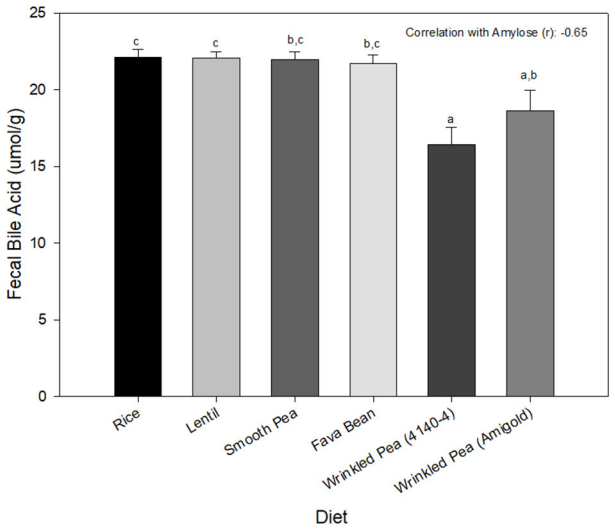

FIGURE 2 | Fecal bile acid content from dogs after 7 days of feeding each test diet. Diets are listed in order of increasing fiber and amylose content from left to right. Data is shown as Mean ± SEM; $n = 8$ dogs. Different letters indicate significant differences using Tukey's *post-hoc* analysis ($P < 0.05$) after 1-Way Repeated Measures ANOVA.

decreased risk of diabetes mellitus. This postprandial glycemic response was negatively associated with plasma taurine and cysteine levels, but positively associated with plasma methionine levels in dogs after 7-days of feeding each test diet. Surprisingly, fiber content (all fractions) and dietary amylose content were not strongly related to plasma sulfur amino acid levels. Instead, fiber and amylose were negatively correlated to digestibility of all macronutrients, including sulfur containing amino acids. Despite increasing levels of dietary fiber as amylose content increased among diets, excretion of fecal bile acids in this study unexpectedly decreased. In addition, on a short-term feeding basis (7 days), grain-free diets did not cause a detrimental impact on the plasma taurine status in dogs, despite decreased taurine digestibility. This study showed promising effects of grain-free, pulse-based diets that could be utilized for the improvement of health in dogs.

TABLE 7 | Principal Components Analysis of the top 24 variables examined in this study that explained 83% of the variation among diets.

	Component 1	Component 2
Total variance explained (%)	**56.03**	**27.095**
Variable	**Component loadings**	
Total Dietary Fiber	−0.992	−0.016
Crude Protein	0.990	0.014
Amylose	−0.968	0.007
Chicken By-Product Meal	0.962	0.115
Insoluble High Molecular Weight Dietary Fiber	−0.950	0.076
Low Molecular Weight Dietary Fiber	−0.949	−0.134
Dietary Taurine	0.938	0.231
Methionine Digestibility	0.909	−0.096
Protein Digestibility	0.901	−0.131
Starch Digestibility	0.889	−0.083
Soluble High Molecular Weight Dietary Fiber	−0.876	0.138
Fecal Total Bile Acids	0.847	−0.067
Dietary Methionine	0.839	0.329
Dietary Crude Fiber	−0.818	0.553
Available Carbohydrate	0.773	−0.331
Fat Digestibility	0.726	0.069
Fasting Blood Glucose Levels	0.244	0.904
Area Under the Curve (Glucose)	0.089	0.903
Plasma Cysteine Levels	0.258	−0.898
Glycemic Index	0.399	0.895
Plasma Methionine Levels	0.385	0.883
Plasma Half-Cystine	0.071	−0.880
Blood Glucose, Time to Peak	−0.118	0.740
Plasma Taurine Levels	0.263	−0.696

The strength of the relationship (r-value) for the two components, referred to as component loadings, are shown for each of these 24 variables.

Test Diet Properties and Relationship to Fiber Content

All test diets were formulated to be as similar as possible, while aiming to achieve ∼20% available carbohydrate. This produced desired variations among the diets regarding their dietary fiber and amylose content. Diets with increasing levels

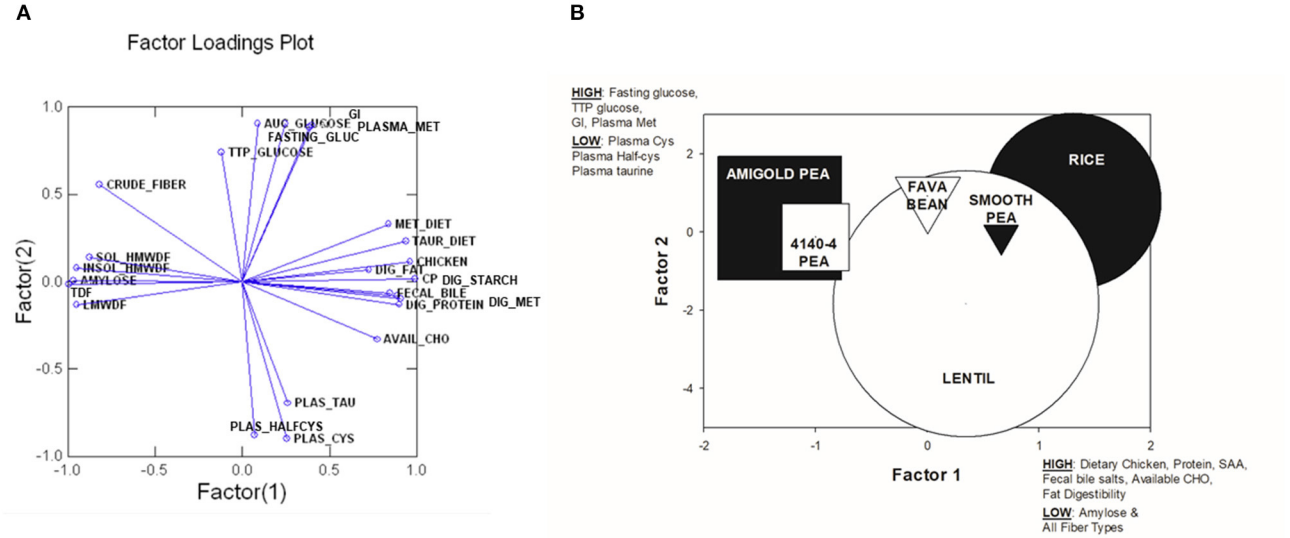

FIGURE 3 | Plots from Principal Components Analysis of the top 24 variables that explained the most variability among diets. **(A)** Factor loadings plot for factor 1 and factor 2. **(B)** A plot of weighted factor scores for components 1 vs. components 2 for each test diet in this study from the Principal Components Analysis. The size of the shape indicates the 95% confidence intervals and distance between shapes indicates greater difference among diets. Higher, positive scores for Factor 1 were associated with high dietary chicken content, dietary crude protein, dietary sulfur amino acid content (SAA; cysteine, methionine and taurine), fecal bile salts, dietary available carbohydrate and fat digestibility, but low amylose and all fiber types. In contrast, a high positive score for Factor 2 was associated with high fasting blood glucose, long time to peak blood glucose, high glycemic index and high plasma methionine, but low plasma cysteine, plasma half-cysteine and plasma taurine. SOL_HMWDF, soluble high molecular weight dietary fiber; INSOL_HMWDF, insoluble high molecular weight dietary fiber; TDF, total dietary fiber; LMWDF, low molecular weight dietary fiber; TTP_GLUCOSE, time to peak glucose; FASTING_GLUC, fasting blood glucose; AUC_GLUCOSE, area under the curve for blood glucose response; GI, glycemic index; PLASMA_MET, plasma methionine; MET_DIET, dietary level of methionine; TAUR_DIET, dietary level of taurine; DIG_FAT, total tract apparent digestibility of fat; DIG_STARCH, total tract apparent digestibility of starch; FECAL BILE, fecal bile salts; DIG_PROTEIN, total tract apparent digestibility of protein; DIG_MET, total tract apparent digestibility of methionine; AVAIL_CHO, available carbohydrate in diet; PLAS_TAU, plasma taurine; PLAS_HALFCYS, plasma half-cysteine; PLAS_CYS, plasma cystine.

of dietary fiber and amylose were among the diets that incorporated the largest amounts of pulse flour which provided plant protein and subsequently needed decreased amounts of animal protein (chicken meal) to be isonitrogenous. In addition, decreasing the amount of animal protein among diets produced decreasing levels of taurine in diets. Other studies confirmed that seafood and poultry products contained high concentrations of taurine, while plant products do not (28). Dietary fiber also did not appear to have any impacts on the other amino acids studied within the different diets such as cystine, methionine and cysteine + methionine. All pulse diets had lower levels of these amino acids in comparison to the rice diet due to pulses possessing limited content of these amino acids (6, 29). This could explain why there were variations of dietary cystine, methionine and cystine+methionine in the test diets.

In this study increasing amylose content among the different diets was associated with increasing levels of total dietary fiber. This was due to pulses with higher amylose levels containing larger amount of resistant starch, which is the fraction of starch that reaches the colon intact, as it is not digested in the small intestine (30). As reviewed by (30), resistant starch such as amylose is also viewed as dietary fiber, partially explaining the link between increased dietary fiber and amylose among diets. Increased amounts of amylose and dietary fiber influence nutrient digestibility and postprandial glucose responses (13). Thus, based on those diets we produced, our results support our hypothesis that the diets with higher fiber and lower animal-source protein exert negative effects on macronutrient and amino acid digestibility and produce lower glycemic responses. However, after 7-days of feeding diets, all dog plasma levels of cysteine, methionine and taurine were within normal range. Longer term studies are needed to confirm this.

Effect of Different Pulse-Based Diets on Glycemic Response

The diets for this study were chosen to demonstrate how increasing dietary fiber produced positive attributes on glycemic responses but might have negative impact on other nutrients in dogs. In humans, increased consumption of dietary fiber and amylose decreased postprandial blood glucose (31–34). High amylose pulse crops when used as an ingredient in dog diets minimized the risk of obesity and diabetes mellitus (13) and pulse-based diets have low glycemic properties in dogs [(9, 13, 35), Briens et al., unpublished]. However, a study systematically using a gradient of amylose content in dog diets had not yet been performed.

The current study demonstrates that while amylose and total dietary fiber content is clearly an important factor in decreasing glycemic response in dogs, the spurious low results with the lentil diet point likely contributed to the lack of correlation of all the glycemic endpoints to dietary fiber and amylose content.

In addition to dietary fiber content determining glycemic response, other factors include digestion rate, amount of diet ingested, processing factors and dietary composition (13). In this study, all diets had 22–25% total digestible starch. However, since wrinkled pea flour contains much lower starch levels (∼34%), the high amylose wrinkled pea diets contained up to 65% flour which contributed a large amount of fiber compared to the low amylose diets. In human studies, resistant starch is an indication of high levels of amylose and contributes to decreased starch digestibility and increased levels of total dietary fiber (36). In similar studies, changes in dietary fiber impacts glycemic response by slowing down the rate of passage of feed and the rate of hydrolysis on polysaccharides in starch (12, 37). Postprandial glucose responses are further impacted by dietary fiber as it is believed to prolong glucose absorption, thus reducing variations in glucose responses (13). Overall, glycemic response in this study was less impacted by the varying levels of amylose and dietary fiber within the different test diets and instead related to some other unidentified factor that was associated with high plasma taurine and cysteine. Further studies are needed to confirm this finding and explore how this happens.

Effect of Amylose vs. Fiber on Macronutrient and Amino Acid Digestibility

The results of the current study which found decreasing digestibility of all macronutrients (protein, fat and starch) with increasing dietary amylose and total dietary fiber agrees well-with a study conducted by (38). Digestibility of crude protein decreased as total dietary fiber consumption increased in dogs (38). This could be explained possibly by endogenous factors within the pulse flours having intrinsic interactions to form structures with starch, such as amylose, to limit the digestibility of protein (39). Similar to what is seen with protein digestion, lipids also interact with starches, creating a single-helical structure with amylose molecules and limits the enzymatic digestibility of starch (40). These amylose-lipid complexes that are resistant to starch digestion are formed during the exposure to elevated temperatures, which occur during the process of extrusion (41, 42). Due to the possibility of these processes, lipid digestibility in this study decreased as amylose levels and total dietary fiber increased. For example, a study in growing pigs demonstrated that increasing levels of fiber decreased the total tract apparent digestibility of both crude protein and fat (43).

In addition to amylose and dietary fiber impacting the digestibility of macronutrients, they also impact the digestibility of amino acids. In this study increasing levels of amylose and dietary fiber were negatively correlated to digestibility of cystine, methionine and taurine. Diets containing high amounts of dietary fiber not only lead to a greater possibility of sulfur amino acid excretion, but also greater microbial overgrowth and taurine assimilation by the microflora (18). This would be detected as increased total tract apparent digestibility, so is inconsistent with our observations. What this study could not determine is whether a particular fiber fraction or amylose content were driving changes in sulfur amino acid digestibility since all of fiber fractions and amylose were all equally negatively associated with digestibility.

Effect of Different Pulse-Based Diets on Plasma Levels of Sulfur-Containing Amino Acids

Unlike cats, dogs are able to synthesize taurine from the sulfur-containing amino acids cysteine and methionine (15). Thus, taurine is not considered an essential dietary nutrient for dogs, while methionine and methionine+cysteine have dietary minima in dogs (25). In this study, plasma taurine levels ranged from 73 to 111 nmol/mL in fasted dogs after 7 days of feeding test diets which falls within previously reported taurine reference ranges of 63–194 nmol/mL (16, 44–46). Other studies disagree on whether or not grain-free diets contribute to decreased plasma taurine levels in dogs. Some studies determined that dogs consuming grain-free diets have an increased prevalence of taurine deficiencies (16, 44), while others have noted no change or improvements in taurine status in dogs when consuming grain-free diets (47, 48). Despite lower dietary taurine content with increasing dietary fiber and amylose in the current study, plasma taurine remained within normal range. This could be due to the short-term nature of the current study (7 days per diet). Another important factor linked to a lack of consistent change in plasma taurine may be that taurine levels in target tissues such as the heart are more relevant and these tissue levels could be depleted before plasma levels of these free amino acids fall (17). Moreover, the beagle breed is not predisposed to either taurine deficiencies or dilated cardiomyopathy (19). Taken together, this study demonstrated that short-term consumption of both grain-containing and grain-free diets had no major effect on plasma taurine levels in dogs.

In this study, plasma levels of half-cystine ranged from 16 to 19 nmol/ml while plasma levels of methionine varied from 50 to 56 nmol/mL. Levels for both of these amino acids are lower than, but close to, the value reported in a dog study with 46 and 57 nmol/mL, respectively (46). Plasma cysteine values had much greater and unexpected variation from 132 to 2,732 nmol/mL among diets in the current study. Cysteine numbers are suspect for two reasons. First, cysteine is unstable after sample collection and rapidly forms disulfide bonds with itself to form the dimer called cystine or with other plasma proteins which are subsequently removed before analysis (personal communication from Amino Acid Laboratory, University of California Davis, Davis, CA, USA). Second, in this experiment, the cysteine data was more likely overestimated due

to interference during HPLC analysis from L-alpha-aminoadipic acid that co-eluted with cysteine (49). Coupled with the fact that dietary cystine levels did not vary that much, it seems likely that dietary fiber has no effect on plasma cysteine, methionine or taurine levels, at least after 7 days of feeding test diets.

Effect of Dietary Fiber and Amylose on Fecal Bile Acids

Contrary to the original hypothesis, fecal excretion of total bile acids decreased as dietary fiber and amylose increased in this study in beagles after 7 days of feeding test diets. The findings of this study disagree with reports that dietary fiber can bind bile acids within the intestinal lumen, leading to increased fecal excretion of bile acids (47, 50, 51). However, the results of this study agree with another report that grain-free diets did not lead to an increased excretion of bile acids in dogs (19). Soluble dietary fiber abundant in pulses, was proposed to lower taurine availability in companion animals (18). One of the major roles of taurine in dogs is conjugation with bile acids to form the predominant bile salt, taurocholate (17, 18). While soluble fibers bind bile acids to prevent their reabsorption through the entero-hepatic circulation, thereby lowering lipids (a beneficial health effect), this effect could also deplete taurine via taurocholate loss in the feces, leading to taurine wasting (17). Alternatively, legumes high in dietary fiber can also act as prebiotics for the gut microbiota, changing the microbial population composition and overgrowth, which could enhance taurine degradation in the small intestine before it can be absorbed (45). Future studies need to explore whether feeding periods >7 days cause increased bile salt loss, whether specific classes of bile acids are affected or whether the effects on intestinal microbiota lead to taurine depletion in dogs and if so, which dietary fiber components, if any, contribute to this loss.

STUDY STRENGTHS AND LIMITATIONS

A strength of this study was the detailed glycemic response data for each diet. Another strength of this study was the use of multiple pulses to study if dietary fiber and amylose are impacting variables in different pulses or if there is another component that should be studied. One limitation of the study design was the sample size of eight beagles. However, we combated this limitation by using a cross-over, repeated-measures design, allowing all dogs to rotate through all diets and be studied. Another limitation of this study could be insufficient duration of feeding each diet for plasma sulfur containing amino acids to change. Ongoing studies in this group are exploring effects of longer-term feeding periods but establishing what happens in shorter time frames is also important information. For digestibility measurements, a limitation was the use of total tract apparent digestibility method which was necessary since our studies require minimally invasive, non-lethal techniques to allow our dogs to be adopted into homes once retired. True digestibility can only be assessed through either lethal sampling to remove digesta along the length of the intestinal tract or through ileal sampling that requires surgical creation of a permanently disfiguring ileal cannula.

CONCLUSIONS

Pulses are beneficial at producing a low glycemic response in dogs and higher amylose pulses such as the wrinkled pea (4,140–4) have superior low glycemic properties in dogs. However, even pulses such as red lentil with relatively low amylose and dietary fiber could also be processed to produce a low glycemic diet. The trade-off for beneficial low glycemic properties of high dietary fiber and high amylose pulses is decreased macronutrient and amino acid digestibility. However, this study did not find high fiber or amylose to be associated with increased fecal bile acid secretion and instead observed a decrease. In addition, due to the limitations of diet formulation in this study, high-fiber pulse diets contained less animal-source protein and higher plant-based proteins compared with the rice diet, which could be another important factor leading to decreased digestibility of certain nutrients. However, despite decreased nutrient digestibility, plasma levels of sulfur-containing amino acids, including taurine remained within normal range after 7 days of feeding test diets, suggesting at least in the short term, that the benefits may outweigh any negative nutritional effects of high fiber canine diets.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by University of Saskatchewan Animal Research Ethics Board.

AUTHOR CONTRIBUTIONS

CQ: formulated and created test diets, designed and performed experiments, and analyzed data and co-wrote the paper. YR: material preparation, starch analyses, data curation, validation, and review & editing. TM: performed experiments and review & editing. YA: Conceptualization, funding acquisition, investigation, project administration, resources, and review & editing. LW: Supervised animal research, experimental design, funding acquisition, investigation, resources, project administration, review & editing, and co-wrote the paper. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2021.654223/full#supplementary-material

REFERENCES

1. Olatunde GA, Atungulu GG. Emerging pet food drying and storage strategies to maintain safety. *Food Feed Safety Systems Anal.* (2018), Chapter 3: 45–61. doi: 10.1016/B978-0-12-811835-1.00003-8
2. Dzanis DA. The association of american feed control officials dog and cat food nutrient profiles: substantiation of nutritional adequacy of complete and balanced pet foods in the United States. *J Nutr.* (1994) 124(suppl. 12):2535S. doi: 10.1093/jn/124.suppl_12.2535S
3. Suarez L, Peña C, Carretón E, Juste MC, Bautista-Castaño I, Montoya-Alonso JA. Preferences of owners of overweight dogs when buying commercial pet food. *J Animal Physiol Animal Nutr.* (2012) 96:655–9. doi: 10.1111/j.1439-0396.2011.01193.x
4. Pirsich W, von Hardenberg LM, Theuvsen L. The pet food industry: an innovative distribution channel for marketing feed products from welfare friendly production to consumers? *Int J Food System Dynamics.* (2017) 8:250–61. doi: 10.18461/ijfsd.v8i3.836
5. Prantil LR, Heinze CR, Freeman LM. Comparison of carbohydrate content between grain-containing and grain-free dry cat diets and between reported and calculated carbohydrate values. *J Feline Med Surg.* (2018) 20:349–55. doi: 10.1177/1098612X17710842
6. Singh N. Pulses: an overview. *J Food Sci Technol.* (2017) 54:853–7. doi: 10.1007/s13197-017-2537-4
7. Mudryj AN, Yu N, Aukema HM. Nutritional and health benefits of pulses. *Appl Physiol Nutr Metabol.* (2014) 39:1197–204. doi: 10.1139/apnm-2013-0557
8. Ren Y, Setia R, Warkentin TD, Ai Y. Functionality and starch digestibility of wrinkled and round pea flours of two different particle sizes. *Food Chem.* (2021) 336:127711. doi: 10.1016/j.foodchem.2020.127711
9. Adolphe JL, Drew MD, Huang Q, Silver TI, Weber LP. Postprandial impairment of flow-mediated dilation and elevated methylglyoxal after simple but not complex carbohydrate consumption in dogs. *Nutr Res.* (2012) 32:278–84. doi: 10.1016/j.nutres.2012.03.002
10. Adolphe JL, Silver TI, Childs H, Drew MD, Weber LP. Short-term obesity results in detrimental metabolic and cardiovascular changes that may not be reversed with weight loss in an obese dog model. *Bri J Nutr.* (2014) 112:647–56. doi: 10.1017/S0007114514001214
11. Thomas DE, Brotherhood JR, Brand JC. Carbohydrate feeding before exercise: effect of glycemic index. *Int J Sports Med.* (1991) 12:180–6. doi: 10.1055/s-2007-1024664
12. Nguyen P, Dumon H, Biourge V, Pouteau E. Glycemic and insulinemic responses after ingestion of commercial foods in healthy dogs: influence of food composition. *J Nutr.* (1998) 128:2654S. doi: 10.1093/jn/128.12.2654S
13. Carciofi AC, Takakura FS, De-Oliveira LD, Teshima E, Jeremias JT, Brunetto MA, et al. Effects of six carbohydrate sources on dog diet digestibility and post-prandial glucose and insulin response. *J Animal Physiol Animal Nutr.* (2008) 92:326–36. doi: 10.1111/j.1439-0396.2007.00794.x
14. Englyst KN, Englyst HN, Hudson GJ, Cole TJ, Cummings JH. Rapidly available glucose in foods: an *in vitro* measurement that reflects the glycemic response. *Am J Clin Nutr.* (1999) 69:448–54. doi: 10.1093/ajcn/69.3.448
15. Backus RC, Ko KS, Fascetti AJ, Kittleson MD, MacDonald KA, Maggs DJ, et al. Low plasma taurine concentration in Newfoundland dogs is associated with low plasma methionine and cyst (e) ine concentrations and low taurine synthesis. *J Nutr.* (2006) 136:2525–33. doi: 10.1093/jn/136.10.2525
16. Kaplan JL, Stern JA, Fascetti AJ, Larsen JA, Skolnik H, Peddle GD, et al. Taurine deficiency and dilated cardiomyopathy in golden retrievers fed commercial diets. *PLoS ONE.* (2018) 13:e0209112. doi: 10.1371/journal.pone.0209112
17. Sanderson SL, Gross KL, Ogburn PN, Calvert C, Jacobs G, Lowry SR, et al. Effects of dietary fat and L-carnitine on plasma and whole blood taurine concentrations and cardiac function in healthy dogs fed protein-restricted diets. *Am J Vet Res.* (2001) 62:1616–23. doi: 10.2460/ajvr.2001.62.1616
18. Mansilla WD, Marinangeli CP, Ekenstedt KJ, Larsen JA, Aldrich G, Columbus DA, et al. Special topic: The association between pulse ingredients and canine dilated cardiomyopathy: addressing the knowledge gaps before establishing causation. *J Animal Sci.* (2019) 97:983–97. doi: 10.1093/jas/sky488
19. Pezzali JG, Acuff HL, Henry W, Alexander C, Swanson KS, Aldrich CG. Effects of different carbohydrate sources on taurine status in healthy beagle dogs. *J Animal Sci.* (2020) 98:skaa010. doi: 10.1093/jas/skaa010
20. Ko KS, Fascetti AJ. Dietary beet pulp decreases taurine status in dogs fed low protein diet. *J Animal Sci Technol.* (2016) 58:29. doi: 10.1186/s40781-016-0112-6
21. Fascetti AJ, Reed JR, Rogers QR, Backus RC. Taurine deficiency in dogs with dilated cardiomyopathy: 12 cases (1997–2001). *J Am Vet Med Asso.* (2003) 223:1137–41. doi: 10.2460/javma.2003.223.1137
22. Sales J, Janssens GPJ. Acid-insoluble ash as a marker in digestibility studies: a review. *J Animal Feed Sci.* (2003) 12:383–401. doi: 10.22358/jafs/67718/2003
23. Association of Official Analytical, Chemists. *Association of Official Analytical Chemists Official Methods of Analysis*. 21st ed. Washington, DC: AOAC (2019).
24. Baldwin K, Bartges J, Buffington T, Freeman LM, Grabow M, Legred J, et al. AAHA nutritional assessment guidelines for dogs and cats. *J Am Animal Hosp Asso.* (2010) 46:285–96. doi: 10.5326/0460285
25. Association of American Feed Controls Organization AAFCO. AAFCO Methods for Substantiating Nutritional Adequacy of Dog and Cat Foods. AAFCO Model Pet Food and Specialty Pet Food Regulations PF2, 4, 7, 8, 9 and/or 10 (2013). Association of American Feed Control Officials. Official Publication. Champaign, IL.
26. Chrastil J. Improved colorimetric determination of amylose in starches or flours. *Carboh Res.* (1987) 159:154–8. doi: 10.1016/S0008-6215(00)90013-2
27. Ren Y, Quilliam C, Weber LP, Warkentin TD, Tulbek MC, Ai Yl. *Development of Low-Glycemic Pet Foods From Normal and High-Amylose Pulse Varieties. Cereals & Grains 20 Online Meeting by Cereals & Cereal Grains Association.* (2020). Available online at: https://aaccnet.confex.com/aaccnet/2020/meetingapp.cgi/Paper/5606. (accessed March 16, 2021).

28. Spitze AR, Wong DL, Rogers QR, Fascetti AJ. Taurine concentrations in animal feed ingredients; cooking influences taurine content. *J Animal Physiol Animal Nutr.* 87:251–62. doi: 10.1046/j.1439-0396.2003.00434.x
29. Tiwari BK, Singh N. *Pulse Chemistry and Technology*. Royal Society of Chemistry (2012). Cambridge.
30. Newberry M, Berbezy P, Belobrajdic D, Chapron S, Tabouillot P, Regina A, et al. High-amylose wheat foods: a new opportunity to meet dietary fiber targets for health. *Cereal Foods World.* (2018) 63:188–93. doi: 10.1094/CFW-63-5-0188
31. Behall KM, Hallfrisch J. Plasma glucose and insulin reduction after consumption of breads varying in amylose content. *Eur J Clin Nutr.* (2002) 56:913–20. doi: 10.1038/sj.ejcn.1601411
32. Behall KM, Howe JC. Effect of long-term consumption of amylose vs amylopectin starch on metabolic variables in human subjects. *Am J Clin Nutr.* (1995) 61:334–40. doi: 10.1093/ajcn/61.2.334
33. Behall KM, Scholfield DJ, Yuhaniak I, Canary J. Diets containing high amylose vs amylopectin starch: effects on metabolic variables in human subjects. *Am J Clin Nutr.* (1989) 49:337–44. doi: 10.1093/ajcn/49.2.337
34. Granfeldt Y, Drews A, Björck I. Arepas made from high amylose corn flour produce favorably low glucose and insulin responses in healthy humans. *J Nutr.* (1995) 125:459–65.
35. Adolphe JL, Drew MD, Silver TI, Fouhse J, Childs H, Weber LP. Effect of an extruded pea or rice diet on postprandial insulin and cardiovascular responses in dogs. *J Animal Physiol Animal Nutr.* (2015) 99:767–76. doi: 10.1111/jpn.12275
36. Li H, Gidley MJ, Dhital S. High-amylose starches to bridge the "fiber gap": development, structure, and nutritional functionality. *Compr Rev Food Sci Food Safety.* (2019) 18:362–79. doi: 10.1111/1541-4337.12416
37. Wolever TM. Relationship between dietary fiber content and composition in foods and the glycemic index. *Am J Clin Nutr.* (1990) 51:72–5. doi: 10.1093/ajcn/51.1.72
38. Beloshapka AN, Cross TWL, Swanson KS. Graded dietary resistant starch concentrations on apparent total tract macronutrient digestibility and fecal fermentative end-products and microbial populations of healthy adult dogs. *J Animal Sci.* (2020). 99(1):1–9. doi: 10.1093/jas/skaa409
39. Rooney LW, Pflugfelder RL. Factors affecting starch digestibility with special emphasis on sorghum and corn. *J Animal Sci.* (1986) 63:1607–23. doi: 10.2527/jas1986.6351607x
40. Debet MR, Gidley MJ. Three classes of starch granule swelling: Influence of surface proteins and lipids. *Carbo Polymer.* (2006) 64:452–65. doi: 10.1016/j.carbpol.2005.12.011
41. Seneviratne HD, Biliaderis CG. Action of α-amylases on amylose-lipid complex superstructures. *J Cereal Sci.* (1991) 13:129–43. doi: 10.1016/S0733-5210(09)80030-1
42. Lin S, Hsieh F, Huff HE. Effects of lipids and processing conditions on degree of starch gelatinization of extruded dry pet food. *LWT-Food Sci Technol.* (1997) 30:754–61. doi: 10.1006/fstl.1997.0271
43. Zhang W, Li D, Liu L, Zang J, Duan Q, Yang W, et al. The effects of dietary fiber level on nutrient digestibility in growing pigs. *J Animal Sci Biotechnol.* (2013) 4:17. doi: 10.1186/2049-1891-4-17
44. Ontiveros ES, Whelchel BD, Yu J, Kaplan JL, Sharpe AN, Fousse SL, et al. Development of plasma and whole blood taurine reference ranges and identification of dietary features associated with taurine deficiency and dilated cardiomyopathy in golden retrievers: A prospective, observational study. *PLos ONE.* (2020) 15:e0233206. doi: 10.1371/journal.pone.0233206
45. Tôrres CL, Backus RC, Fascetti AJ, Rogers QR. Taurine status in normal dogs fed a commercial diet associated with taurine deficiency and dilated cardiomyopathy. *J Animal Physiol Animal Nutr.* (2003) 87:359–72. doi: 10.1046/j.1439-0396.2003.00446.x
46. Delaney SJ, Kass PH, Rogers QR, Fascetti AJ. Plasma and whole blood taurine in normal dogs of varying size fed commercially prepared food. *J Animal Physiol Animal Nutr.* (2003) 87:236–44. doi: 10.1046/j.1439-0396.2003.00433.x
47. Donadelli RA, Pezzali JG, Oba PM, Swanson KS, Coon C, Varney J, et al. A commercial grain-free diet does not decrease plasma amino acids and taurine status but increases bile acid excretion when fed to labrador retrievers. *Trans Animal Sci.* (2020) 4:txaa141. doi: 10.1093/tas/txaa141
48. Adin D, DeFrancesco TC, Keene B, Tou S, Meurs K, Atkins C, et al. Echocardiographic phenotype of canine dilated cardiomyopathy differs based on diet type. *J Vet Cardiol.* (2019) 21:1–9. doi: 10.1016/j.jvc.2018.11.002
49. Tôrres CL, Miller JW, Rogers QR. Determination of free and total cyst (e) ine in plasma of dogs and cats. *Vet Clin Pathol.* (2004) 33:228–33. doi: 10.1111/j.1939-165X.2004.tb00378.x
50. Garcia-Diez F, Garcia-Mediavilla V, Bayon JE, Gonzalez-Gallego J. Pectin feeding influences fecal bile acid excretion, hepatic bile acid and cholesterol synthesis and serum cholesterol in rats. *J Nutr.* (1996) 126:1766–71. doi: 10.1093/jn/126.7.1766
51. Stratton-Phelps M, Backus RC, Rogers QR, Fascetti AJ. Dietary rice bran decreases plasma and whole-blood taurine in cats. *J Nutr.* (2002) 132:1745S. doi: 10.1093/jn/132.6.1745S

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The Effect of Fermentation of High- or Low-Tannin Fava Bean on Glucose Tolerance, Body Weight, Cardiovascular Function, and Blood Parameters in Dogs After 7 Days of Feeding: Comparison With Commercial Diets With Normal vs. High Protein

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Fava bean, which is available in high- and low-tannin varieties, is not an approved pet food ingredient and was not included in the "assumed to be safe" category based on its ability to cause favism and hemolytic anemia in susceptible humans. The effects of 7-day feeding of test canine diets containing moderate protein (∼27%) were compared with two control commercial diets with normal (NP, grain-containing, ∼25% protein) or high protein (HP, grain-free, ∼41% protein). Fava bean diets were formulated either with or without *Candida utilis* fermentation processing to reduce antinutritional factors. Glucose tolerance, body weight, cardiovascular function, and blood parameters were investigated in beagles fed the NP or HP diets or a randomized, crossover, 2 × 2 Latin square design of the fava bean diets: unfermented high-tannin (UF-HT), fermented high-tannin (FM-HT), unfermented low-tannin (UF-LT), and fermented low-tannin (FM-LT). After 7 days, HP decreased red blood cells (RBC) ($P < 0.05$) compared with NP, while FM increased RBC compared with UF. HP increased blood bicarbonate, calcium, phosphorus, urea, cholesterol, and albumin:globulin ratio while decreasing bilirubin, liver enzymes, and total protein. Sodium:potassium ratio was increased in UF-HT, decreased in FM-HT, and intermediate in LT regardless of fermentation. Blood phosphorus was increased in HT. Blood amylase was increased in FM-HT and decreased in FM-LT, being intermediate in UF regardless of fava bean variety. Blood direct bilirubin was decreased in HT regardless of fermentation. Of note, left ventricular end-systolic volume and cardiac output were increased in NP compared with HP-fed dogs, but were normal and had no significant differences among the fava bean diets. As expected, plasma taurine, cystine, and cysteine levels were increased in HP- compared with NP-fed dogs. Plasma cysteine

levels were increased in HT- compared with LT-fed dogs and in FM- compared with UF-fed dogs. Taken together, these results show that fava bean appears to be safe as a dog food ingredient at least in the short term, and its nutritional value appears improved by fermentation. Moreover, blood chemistry parameters and cardiovascular function were impacted by protein content which merits further investigation with longer term feeding trials.

Keywords: fava bean (*Vicia faba*), *Candida utilis*, fermentation, cardiovascular function, blood chemistry, glucose tolerance, domestic dog (*Canis familiaris*)

INTRODUCTION

Fava bean (*Vicia faba* L.) has been regarded as a healthy, sustainable alternative for partially replacing animal protein sources in human diets (1). The varieties of fava bean are divided by their tannin levels such as low or normal/high tannin content which affects taste (2). Because pet owners are increasingly matching their own nutritional choices with that of their pets, incorporation of fava beans in pet diets as a source of carbohydrates and protein has been considered. Fava bean safety in dogs has been scarcely explored and is not approved as a feed ingredient for pet food by the American Association of Feed Control Organization (AAFCO) yet due to concerns about potential toxicity from antinutritional factors (3). Further complicating the approval of a novel pulse ingredient such as fava beans for use in pet food, the US Food and Drug Administration (FDA) reported in July 2018, cases in which dilated cardiomyopathy (DCM) was observed in dogs fed grain-free diets, i.e., food formulated with potatoes and pulse ingredients instead of grains (4). DCM is recognized as the second most common type of genetically linked cardiac disease in the dog and is most prevalent in large or giant breeds (5). Dobermans, Boxers, Great Danes, Newfoundlands, Irish Wolfhounds, English Cocker Spaniels, and Portuguese Water Dogs are the breeds with the highest prevalence of DCM (6–9). Moreover, Golden Retrievers and American Cocker Spaniels have recently emerged as being predisposed to taurine deficiency (10, 11). DCM is described as a primary myocardial disorder causing systolic dysfunction with secondary ventricular dilation, regular or decreased wall thickness, and increased cardiac mass due to myocyte enlargement (12). However, while pulse ingredients in grain-free diets have been suggested as causally linked to DCM, the actual link between them has not been definitively demonstrated to date (13–15). Despite an acknowledgment by the FDA in November 2020 that the link between DCM and diet in dogs is multi-factorial and not due solely to pulses, this issue remains unresolved, leaving veterinarians, and pet owners wary of pulse-containing dog foods.

The best evidence so far for a link between DCM and nutrition in dogs is through taurine insufficiency (16, 17). Taurine is important to cardiac health because it participates in the reabsorption of calcium by the sarcoplasmic reticulum and enhances the sensitivity of the myofilaments to calcium (18). Therefore, as reviewed by Mansilla et al. (13), because calcium is a key component of cardiac contraction, in cases of taurine absence due to reduced synthesis or low intake of taurine and/or its precursors, the cardiac muscle tissue is unable to properly contract and presumably develops DCM. Dog diets do not require taurine and taurine is not considered essential since dogs can synthesize taurine from sulfur-containing amino acids such as cysteine and methionine (19). Grain-free and pulse-containing diets are higher in fermentable fiber which has been demonstrated to decrease protein digestibility though either an increased fermentation of the sulfur-containing amino acids leading to decreased bioavailability or to higher fecal excretion (20, 21). Previous studies have reported that high fermentable dietary fiber in dogs increases the need for dietary taurine or taurine precursors (e.g., methionine and cysteine) due to increased fecal excretion of taurocholate (the predominant bile acid in dogs), leading to fecal loss of taurine (22, 23).

Fava beans have numerous antinutritional factors such as condensed tannins, trypsin inhibitor activity, lectins, and pyrimidine glucosides (24). Tannins are known to cause reduction of protein and energy digestibility (3). The pyrimidine glucosides (vicine and convicine) lead to favism which is a blood disorder caused when fava beans are eaten by humans with genetic mutations of glucose-6-phosphate dehydrogenase (G6PD), leading to decreased G6PD activity and reduced ability of red blood cells (RBCs) to produce ATP and regenerate glutathione (25). This then makes the red blood cell susceptible to oxidative damage, leading to rapid RBC death and acute anemia when uncooked fava beans are consumed. There are no reports of favism in dogs to the best of our knowledge. However, because of this concern, fava beans cannot be placed in the "generally assumed to be safe" category by AAFCO and thus is not currently used in pet food until proven otherwise.

Abbreviations: ALP, alkaline phosphatase; ALT, alanine aminotransferase; AUC, area under the curve; CK, creatine kinase; CO, cardiac output; CP, crude protein; DCM, dilated cardiomyopathy; DB, direct bilirubin; DBP, diastolic blood pressure; DWT, left ventricular diastolic wall thickness; EDV, left ventricular end-diastolic volume; EF, ejection fraction; ESV, left ventricular end-systolic volume; FM, fermented; FMD, flow-mediated dilation; G6PD, glucose-6-phosphate dehydrogenase; GGP, gamma-glutamyltransferase; GLDH, glutamate dehydrogenase; HP, high protein; HR, heart rate; HT, high-tannin fava bean variety; IB, indirect bilirubin; LT, low-tannin fava bean variety; $LVID_d$, left ventricular end-diastolic diameter; $LVID_s$, left ventricular end-systolic diameter; MV, maximum velocity; NP, normal protein; RBC, red blood cell; SBP, systolic blood pressure; SV, stroke volume; SWT, left ventricular systolic wall thickness; TB, total bilirubin; UF, unfermented; VTI, velocity time integral; WBC, white blood cell.

Fermentation is a processing technique well-known for improving health, functional, and nutraceutical effects of foods (26). Fermentation also has been positively associated with enhanced nutritional quality of pulses by reducing their levels of anti-nutritional factors (27–29). For example, fermentation of fava bean with *Lactobacillus plantarum* reduced the content of the antinutritional factors vicine and convicine by more than 90% while increasing the amount of free amino acids and enhancing protein digestibility (30). Likewise, Rizzello et al. (31) demonstrated a complete degradation of the pyrimidine glycosides in *L. plantarum*–fermented fava bean flour after 48 h, which indicates the usefulness of bioprocessing techniques for industrial fava bean detoxification. In comparison, some yeast organisms in the *Candida* clade have the potential to synthesize and increase taurine content while other yeasts and bacteria do not (32). This current study explored the use of fermentation with the yeast *Candida utilis* to both reduce antinutritional factors and increase taurine content.

The objective of this study was to determine if short-term (7-day) feeding of beagles with a moderate protein diet that has 30% inclusion of fava bean flour would show altered glucose tolerance, body weight, cardiovascular function, and blood parameters when contrasted to commercial diets with normal vs. high protein. Two commercial diets with normal or high protein content and four different fava bean-containing diets with low- (LT) or high-tannin (HT) content were fed, both varieties with and without fermentation. We hypothesized that pulse-based diets would impair cardiovascular health due to the low taurine, cysteine, or methionine levels and high fiber content. Moreover, fermentation of fava bean flour with *C. utilis* would enhance diet quality and, consequently, health of dogs.

MATERIALS AND METHODS

Fava Bean Ingredients and Fermentation Protocol

Low-tannin (Snowdrop) and HT (Florent) fava bean varieties, genotypes grown in Saskatchewan (2), were dehulled and ground into flour using a 400-μm screen. Fermentation of each variety was adapted from methodology used previously in pea flour in our laboratory (33). Briefly, *C. utilis* (ATCC 9950) was maintained in sterile 80% (v/v) glycerol solution at −80°C, then reactivated on YGC agar plates when needed (Yeast Extract Glucose Chloramphenicol Agar; catalogue number 95765; Sigma Aldrich, St Louis, MO). Seeded plates were incubated at 30°C for 72 h. Two loops of colonies were transferred using a flame-sterilized platinum needle to a 250-ml sterile conical flask containing 100 ml of YPD liquid medium (Yeast Peptone Dextrose—A1374501; ThermoFisher, Waltham, MA). The flask was incubated in a horizontal shaker incubator (30°C) at 120 rpm for 12 to 15 h. After this period, 10 ml of the cultured yeast mass was transferred into a 500-ml sterile conical flask containing 250 ml of YPD liquid medium. The medium containing the yeast was then incubated on a horizontal shaker (30°C) at 120 rpm for an additional 12 to 15 h. Twenty-kilogram batches of each fava bean variety were mixed with the yeast broth, ammonia, and sterile water to form a soft dough, then fermented in an adapted cement mixer with temperature maintained at 30°C. The fermentation slurry was mixed for 3 min every hour for 72 h. Samples were collected every 24 h, and serial dilutions of mixture plated on sterile agar plates, then incubated at 30°C for 72 h to verify yeast viability and count throughout the process. The fermented fava bean flour was subsequently dried in an oven (60°C) for 48 h at the WCVM before transport to the University of Saskatchewan Canadian Feed Research Centre (North Battleford, Canada) for grinding of the fermented flours, followed by mixing of all test diets (both fermented and unfermented fava bean flours), extruding, and vacuum coating with fat to produce the final dry kibble format of the diets to be used in the feeding trials.

Animals and Diets

All animal use and procedures were approved by the University of Saskatchewan Animal Care Committee (Animal Utilization Protocol #20190055) and adhered to the Canadian Council on Animal Care. Eight neutered beagles, four males and four females, at ideal body weight (8.8 ± 1.9 kg) and a mean age of 2.6 ± 1.0 years, were obtained from King Fisher International (Toronto, ON, Canada) or Marshall Bioresources (North York, NY, USA) and housed at the Western College of Veterinary Medicine (Saskatoon, SK, Canada). The animals were housed individually in 1.1 × 2.7-m kennels with outdoor kennel access at night and during feeding but kept in a group kennel area during the day. Dogs were walked or socialized with volunteers for at least 1 h every day. When not on trial, dogs were fed a commercial adult maintenance dry diet (Purina Dog Chow; Ralston Purina Co, St Louis, MI) in amounts that maintained each individual dog at ideal condition (score of 4–6 on a 9-point scale) in conjunction with energy requirements stated in the National Research Council guidelines (34). All dogs were acclimated to procedures with rewards before the start of experiments to minimize stress, thus no anesthetics or sedatives were used in this study.

In total, six diets were tested. Low- or high-tannin fava bean flours were used at 30% inclusion, in either fermented or unfermented formats with diet formulations indicated in **Supplementary Table 1**. The four fava bean test diets were unfermented low-tannin [UF-LT; 27% crude protein (CP)], fermented low-tannin (FM-LT; 28% CP), unfermented high-tannin (UF-HT; 27% CP), and fermented high-tannin (FM-HT; 28% CP). Insoluble ash (Celite) was included at 1% as a non-digestible marker. Diets were formulated in accordance with the nutrient guidelines for adult dog maintenance set by AAFCO to be nutritionally balanced before being extruded under identical process conditions (35). The two additional diets tested were commercial diets containing normal-protein (NP; Purina Dog Chow, St. Louis, MI; 24% CP) or high-protein content (HP; GO! Solutions Carnivore, Richmond, BC; 41% CP). Ingredient lists for the commercial diets are shown in **Supplementary Table 2**. All six diets were randomly sub-sampled and sent for proximate analysis (Central Testing, Winnipeg, MB, Canada) (**Supplementary Table 3**). The analyzed content of crude fiber, non-fiber carbohydrates, metabolizable

energy, vicine, and convicine of UF-LT, UF-HT, FM-LT, and FM-HT diets are 1.1, 0.5, 0.5, and 0.5%; 60.7, 62.1, 61.6, and 61.8%; 3.7, 3.8, 3.8, and 3.8 kcal/g; 1.8, 1.8, 0.4, and 0.6 mg/g; and 0.5, 0.6, 0.1, and 0.2 mg/g, respectively. The analyzed content of crude fiber, non-fiber carbohydrates, and metabolizable energy of NP and HP diets are 0.8 and 1.1%, 53.0 and 29.8%, and 4.0 and 4.1 kcal/g, respectively. Dogs were fed each diet for 7 days, with the NP or HP diets fed during the first and sixth weeks, respectively. From weeks 2 to 5, using a randomized, crossover, 2 × 2 Latin square design, the UF-LT, FM-LT, UF-HT, and FM-HT diets were fed. Commercial diets were not included in the crossover design because the fava bean–based diets were formulated to contain the same nutrient profile to enable a reasonable comparison. In contrast, NP and HP diets have different ingredients at unknown inclusion levels (specific formulation not available on label), making direct comparisons difficult. Dogs were weighed at the beginning of the experiment and after each feeding week. The amount of diet allotted per dog per day was calculated to be isocaloric with the daily requirement for that dog using standard equations that determine the energy requirements for individual dog maintenance [maintenance energy (ME in kcal)] = [(70 × $BW^{0.7}$) × 1.6], daily portions divided, and dogs fed twice daily (at 08:30 and 16:30 h). Bowls were removed before the next meal and any uneaten food was weighed and recorded. All dogs generally consumed all food portioned in each meal within 5–10 min, with no palatability issues noted (Morris, Reis & Weber, unpublished).

Digestibility Protocol

After a 5-day feeding period on each fava bean–based diet, feces were collected during the subsequent 2 days of each period (total of 7 days on each diet). Collected feces were labeled and frozen at −20°C until analysis. Feces were thawed, homogenized, and pooled by dog (i.e., two samples from each dog pooled per dietary treatment). Before laboratory testing, feces were dried in a forced air oven at 55°C for 72 h and ground in a cutting mill with a 1-mm sieve. Diets and feces were analyzed (Central Testing, Winnipeg, MB) according to AOAC standards (36) for dry matter by oven-drying the sample, non-fiber carbohydrates, crude protein applying the Kjeldahl method, and acid-hydrolyzed fat. Gross energy (GE) content of diets was determined using a bomb calorimeter. The equation below was used to calculate the apparent digestibility coefficients of dry matter, crude protein, non-fiber carbohydrates, fat, gross energy, methionine, cysteine, and taurine for each fava bean–based diet using Celite as a non-digestible marker (37):

$$\text{Digestibility}(\%) = 100 - [100 \times (\text{Mfeed} \times \text{Cfeces} / \text{Mfeces} \times \text{Cfeed})]$$

Where M feed and M feces represent concentrations of index compound in feed and feces, respectively; C feed and C feces represent concentrations of components of interest in feed and feces, respectively.

Oral Glucose Tolerance Test and Blood Analysis

After 7 days of feeding each diet, dogs were fasted for 8 h then a syringe with 10 ml/kg body weight of a 10% glucose solution (1 g/kg BW glucose) fed to each dog by placing in the back of the mouth for an oral glucose tolerance test conducted at the same time each day. Before glucose feeding, the fasted dogs were aseptically catheterized using a peripheral intravenous catheter equipped with an extension tube inserted into the cephalic vein. Blood samples (∼0.2 ml) were taken before feeding glucose (time 0) and at 15, 30, 60, and 90 min after feeding. Blood glucose was measured using a glucometer (OneTouch Ultra 2; LifeScan, Johnson & Johnson, New Brunswick, NJ) with a minimum of duplicate readings for each time or until two consistent readings were obtained. The extension tube was filled with blood and initial blood discarded before glucometer reading to ensure no contamination of blood with anticoagulant solution. Subsequently, after each blood sample was obtained, the catheter was flushed with a sterile citrate solution to prevent clotting. The trapezoidal method was used to determine the incremental area under the curve (AUC) for the glucose response (38). Peak concentration and time to peak concentration for glucose were also calculated.

Additional fasting blood samples (8 ml) were taken using collection tubes with and without EDTA. Complete blood cell count [red (RBC) and white (WBC) blood cell counts] and chemistry panel [cholesterol; total (TB), direct (DB), and indirect bilirubin (IB); alkaline phosphatase (ALP); alanine aminotransferase (ALT); creatine kinase (CK); gamma-glutamyltransferase (GGP); glutamate dehydrogenase (GLDH); total protein, albumin (A), globulin (G), and A:G] were analyzed at Prairie Diagnostic Services (Saskatoon, SK). Moreover, 3-ml subsamples of EDTA-tube blood from fasted animals were centrifuged at 2,000 rpm for 10 min for plasma collection. Plasma samples were kept at −80°C until further analyses of methionine, cystine, cysteine, and taurine content (UC Davis Amino Acid Lab, Davis, CA). Plasma amino acid concentrations were analyzed in an automated amino acid analyzer via cation-exchange high-pressure liquid chromatography separation and ninhydrin-reactive colorimetric detection (39–42).

Cardiac Function, Blood Pressure, and Vascular Health

After 7 days of feeding each diet, dogs were tested for cardiovascular health. All ultrasound measurements were performed and analyzed by one individual. Before ultrasound, blood pressure was taken using a high-definition canine/feline oscillometer (VET HDO High Definition Oscillometer, Babenhausen, Germany). An average of two readings with good agreement was used to determine diastolic and systolic pressures. Endpoints of flow-mediated dilation were used as an indicator of vascular health and included brachial artery diameter during baseline, during inflation of a blood pressure cuff placed distal to the brachial artery, and at the time of peak dilation (30 s) after cuff release, as previously determined by our research group in dogs (43, 44). Echocardiography endpoints were used to assess cardiac function included heart rate (HR), stroke volume (SV), and cardiac output (CO) (44, 45). Moreover, left ventricular end-diastolic volume (EDV), left ventricular end-systolic volume (ESV), ejection fraction (EF), left ventricular diastolic free

TABLE 1 | Body weight, food portion, and body condition score (BCS) of dogs fed diets formulated with either low or high tannin fava beans without (UF) or with (FM) fermentation, or normal (NP) vs. high (HP) protein commercial diets for 7 days each*.

Item	Commercial			Low tannin		High tannin			*P*-value[a]		
	NP	HP	SEM	UF	FM	UF	FM	SEM	*P*	FB	FM
Body weight (kg)	8.45	8.32	0.88	8.31	8.32	8.33	8.28	0.88	0.16	0.88	0.66
Food portion (g/day)	188	163	22.12	188	188	188	188	23.59	<0.01	1.00	1.00
BCS	4.62	4.50	0.18	4.56	4.62	4.55	4.50	0.18	0.35	0.34	0.95

*Eight mixed-gender, neutered beagles were fed the NP or HP diets during the first and sixth weeks, respectively. From weeks 2 to 5, using a randomized, crossover, 2 × 2 Latin square design, 4 diets differing in fava bean variety and fermentation were compared as follows: unfermented (UF) high tannin, fermented (FM) high tannin, UF low tannin, and FM low tannin. Values expressed as means (n = 8). SEM = pooled standard error of the mean.

[a]P, P-value between commercial diets with different protein content (NP vs. HP); FB, fava bean (low tannin vs. high tannin); FM, fermentation (UF vs. FM). The interaction between FB and FM was not significant for any of the parameters measured (P > 0.10).

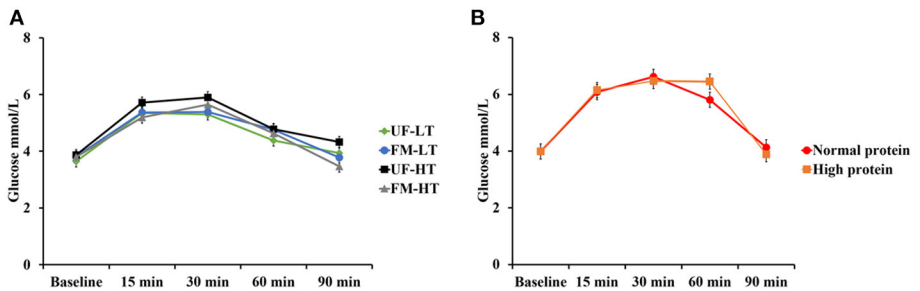

FIGURE 1 | Blood glucose responses to the oral glucose tolerance test in dogs fed either low (LT) or high (HT) tannin fava bean–based diets without (UF) or with (FM) fermentation **(A)**, or normal vs. high protein commercial diets **(B)**. Eight mixed-gender, neutered beagles were fed the NP, or HP diets during the first and sixth weeks, respectively. From weeks 2 to 5, using a randomized, crossover, 2 × 2 Latin square design, 4 diets differing in fava bean variety and fermentation were compared as follows: UF-HT, FM-HT, UF-LT, and FM-LT. Fasting and peak glucose levels, time to peak, and area under the curve (AUC) were not influenced by dietary treatments ($P > 0.10$).

wall thickness (DWT), left ventricle systolic free wall thickness (SWT), systolic blood pressure (SBP), diastolic blood pressure (DBP), velocity time integral for bloodflow through the mitral valve (VTI), and maximum velocity of bloodflow through the mitral valve (MV) were also obtained. Flow-mediated dilation and echocardiography were measured using a SonoSite Edge II ultrasound (Fujifilm SonoSite, Bothell, WA) with detection using a P10x transducer (8–4 Hz) to detect cardiac endpoints and the L38xi (10–5 Hz) transducer for vascular imaging. Flow-mediated dilation was calculated using the following equation:

$$\%\text{FMD} = 100\% \times [(\text{maximum diameter postcuff release}) - (\text{baseline diameter})]/(\text{baseline diameter}).$$

Two-dimensional ultrasonography was used to measure left ventricular volume using the left parasternal apical two- and four-chamber views in diastole and systole (46). Two-dimensional guided M-mode echocardiography was used to obtain a right parasternal short-axis view of the heart at the level of the papillary muscles (46). Measurements of left ventricular end-diastolic inner diameter ($LVID_d$) and left ventricular end-systolic inner diameter ($LVID_s$) were also compiled from all dogs and normalized to body weight according to methodology described by Cornell et al. (47).

Statistical Analysis

Analyses were performed using SAS (version 9.4; SAS Institute, Cary, NC). Before performing all analyses, the data were explored for normality and outliers using the PROC UNIVARIATE model in SAS and the Shapiro–Wilk test. One-way ANOVA was used to compare differences among diets among normal vs. high protein commercial diets and two-way ANOVA was used to compare parameters for fava bean–based diets (fixed effects being fava bean variety and fermentation). In the present study, we used mixed-sex dogs to control the variation possibly associated with this factor. Previous studies from our group have not detected sex-related differences among (spayed/neutered) dogs (44) and because sex-related differences are not related to the purposes of the present study, we did not include this factor in the model. All *post-hoc* analyses were performed using the Fisher least significant difference (LSD) method. Differences were considered significant at $P < 0.05$.

RESULTS

Body Weight, Meal Portion, and Body Condition Score

Body weight (BW), BCS, and meal portion data are presented in **Table 1**. No significant effect ($P > 0.05$) of dietary protein content (NP vs. HP) was observed on BCS or BW after 7 days of feeding in

TABLE 2 | Fasting and peak blood glucose levels, time to peak, and area under the curve of dogs fed diets formulated with either low or high tannin fava beans without (UF) or with (FM) fermentation, or normal (NP) vs. high (HP) protein commercial diets for 7 days each*.

Item	Commercial			Low tannin		High tannin			*P*-value[a]		
	NP	HP	SEM	UF	FM	UF	FM	SEM	*P*	FB	FM
Fasting blood glucose level (mmol/L)	3.98	3.99	0.19	3.62	3.87	3.77	3.74	0.16	0.97	0.96	0.37
Peak blood glucose level (mmol/L)	7.02	6.96	0.35	5.69	5.81	6.33	5.80	0.23	0.92	0.15	0.36
Time to peak (min)	37.50	41.25	7.15	31.53	30.09	26.26	32.31	5.56	0.76	0.78	0.68
Area under the curve (mmol/L × min)	321.98	324.38	15.50	273.59	275.07	282.47	265.02	9.26	0.91	0.95	0.41

Eight mixed-gender, neutered beagles were fed the NP or HP diets during the first and sixth weeks, respectively. From weeks 2 to 5, using a randomized, crossover, 2 × 2 Latin square design, 4 diets differing in fava bean variety and fermentation were compared as follows: unfermented (UF) high tannin, fermented (FM) high tannin, UF low tannin, and FM low tannin. Values expressed as means (n = 8). SEM = pooled standard error of the mean.

[a]*P, P-value between commercial diets with different protein content (NP vs. HP); FB, fava bean (low tannin vs. high tannin); FM, fermentation (UF vs. FM). The interaction between FB and FM was not significant for any of the parameters measured (P > 0.10).*

beagles. Within commercial diets, meal portion (163–187 g/day) was significantly smaller ($P < 0.05$) in HP compared with NP-fed dogs to restrict meal size to an isocaloric amount among diets. Within fava bean–based diets, there was no effect of either FM, FB, or the interaction between FM and FB on body weight, BCS, or meal portion ($P > 0.10$).

Glucose Tolerance

Time course of blood glucose responses to the oral glucose tolerance test are shown in **Figure 1** with fasting and peak glucose levels (mmol/L), time to peak (min), and area under the curve (mmol/L × min) data shown in **Table 2**. There was no effect ($P > 0.10$) of dietary protein content (NP vs. HP) and no effect of FM, FB, or the interaction between FM and FB on fasting and peak glucose levels, time to peak, or AUC.

Red and White Blood Cell Count

White blood cell and RBC of beagles after feeding each diet for 7 days are shown in **Figure 2**. Dogs fed HP diets showed decreased RBC compared with NP-fed dogs ($P < 0.05$). Conversely, WBC tended to increase in dogs fed HP diets compared with NP-fed dogs ($P < 0.10$). Within fava bean–based diets, FM increased RBC regardless of FB ($P < 0.05$) and no effect ($P > 0.10$) of either FM, FB, or the interaction between FM and FB was observed on WBC.

Blood Parameters of Hepatic Function

Blood parameters indicative of hepatic function in beagles after feeding test diets for 7 days are shown in **Table 3**. Within commercial diets, serum cholesterol was increased, while bilirubin parameters (TB, DB, and IB) were decreased in dogs fed HP diets compared with NP-fed dogs ($P < 0.05$). Furthermore, serum ALP and ALT were decreased in dogs fed HP diets compared with NP-fed dogs ($P < 0.05$). Moreover, total serum protein was decreased in dogs fed HP diets compared with NP-fed dogs, primarily due to a decrease in serum globulin, which elevated the albumin:globulin ratio ($P < 0.05$). There was no effect of dietary protein content on GGT, GLDH, CK, and albumin ($P > 0.10$). Among fava bean–based diets, DB was higher in dogs fed LT compared to HT-fed dogs regardless of FM ($P < 0.05$). There was no significant effect ($P > 0.10$) of either FM, FB, or the interaction between FM and FB on any other blood parameter of hepatic function measured.

Blood Electrolytes

Blood electrolytes from beagles after feeding each test diet for 7 days are shown in **Table 4**. Within commercial diets, serum bicarbonate, Ca, and P were increased in dogs fed HP compared with NP-fed dogs ($P < 0.05$). There was no effect ($P > 0.10$) of serum protein content on Na, K, Na:K, Cl, anion gap, or Mg. Among fava bean–based diets, serum P was increased in dogs fed HT compared with LT-fed dogs, regardless of FM ($P < 0.05$). Furthermore, FM tended to increase serum P compared with UF, regardless of FB ($P < 0.05$). There was an interaction between FB and FM for serum K and Na:K ($P < 0.05$). Dogs fed FM-HT diets showed the highest serum K, UF-HT the lowest, with FM-LT and UF-LT being intermediate. Consequently, dogs fed UF-HT diets showed the highest serum Na:K, and FM-HT the lowest, with FM-LT and UF-LT being intermediate. There was no significant effect ($P > 0.10$) of either FM, FB, or the interaction between FM and FB on serum Na, Cl, bicarbonate, anion gap, Ca, and Mg.

Blood Urea and Creatinine

Blood urea and creatinine of beagles after feeding each test diet for 7 days are shown in **Figure 3**. Serum urea was increased ($P < 0.05$) while creatinine tended to increase ($P < 0.10$) in dogs fed HP compared with NP-fed dogs. There was no significant effect ($P > 0.10$) of either FM, FB, or the interaction between FM and FB on serum urea and creatinine.

Digestive Enzymes in Blood

After 7 days of feeding each test diet to dogs, there was an interaction between FB and FM, where serum amylase was highest in FM-HT, lowest in FM-LT, but intermediate in UF-LT and UF-HT ($P < 0.05$; **Figure 4**). However, there was no significant effect of either FM or FB on serum amylase ($P > 0.10$). Moreover, dietary protein content did not significantly impact serum amylase ($P > 0.10$).

Cardiovascular Function

Cardiovascular function parameters of beagles after 7 days of feeding each test diet are shown in **Table 5**. Dogs fed

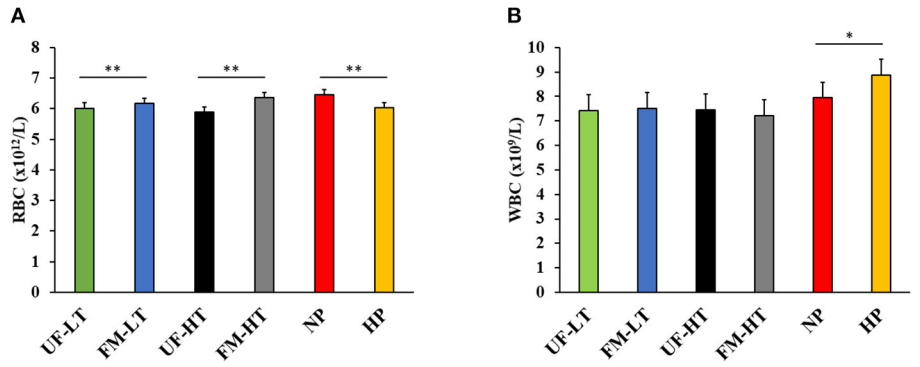

FIGURE 2 | Red [RBC; **(A)**] and white [WBC; **(B)**] blood cell counts in dogs fed either low (LT) or high (HT) tannin fava bean–based diets without (UF) or with (FM) fermentation, or normal vs. high protein commercial diets. Eight mixed-gender, neutered beagles were fed the NP, or HP diets during the first and sixth weeks, respectively. From weeks 2 to 5, using a randomized, crossover, 2 × 2 Latin square design, 4 diets differing in fava bean variety and fermentation were compared as follows: UF-HT, FM-HT, UF-LT, and FM-LT *$P < 0.10$; **$P < 0.05$.

TABLE 3 | Blood parameters of hepatic function in dogs fed diets formulated with either low or high tannin fava beans without (UF) or with (FM) fermentation, or normal (NP) vs. high (HP) protein commercial diets for 7 days each*.

Item	Commercial			Low tannin		High tannin			*P*-value[a]		
	NP	HP	SEM	UF	FM	UF	FM	SEM	*P*	FB	FM
Cholesterol (mmol/L)	4.17	4.41	0.36	3.00	3.14	3.05	3.01	0.26	0.03	0.61	0.50
TB (μmol/L)	0.76	0.27	0.10	0.67	0.62	0.57	0.53	0.16	<0.01	0.55	0.78
DB (μmol/L)	0.43	0.18	0.07	0.37	0.38	0.28	0.21	0.08	0.01	0.05	0.65
IB (μmol/L)	0.33	0.09	0.07	0.30	0.23	0.20	0.32	0.12	0.02	0.96	0.80
ALP (U/L)	62.87	51.12	10.53	70.62	70.87	74.87	68.25	11.69	0.02	0.80	0.34
GGT (U/L)	0.37	0.68	0.30	2.25	1.62	1.75	2.37	0.74	0.31	0.84	1.00
ALT (U/L)	24.87	20.12	1.23	28.50	29.75	28.37	26.75	3.13	<0.01	0.27	0.89
GLDH (U/L)	4.75	4.50	0.45	5.25	6.12	5.12	4.50	1.22	0.51	0.28	0.87
CK (U/L)	148.25	197.37	19.65	116.27	143.32	165.23	163.52	17.73	0.06	0.10	0.51
Total protein (g/L)	52.75	51.62	1.65	50.75	51.00	51.12	51.37	1.49	0.03	0.80	0.86
Albumin (A; g/L)	31.75	32.75	1.36	32.12	32.25	32.25	32.25	1.32	0.06	0.89	0.89
Globulin (G; g/L)	21.00	18.87	0.76	18.62	18.75	18.75	19.12	0.62	<0.01	0.41	0.62
A:G	1.52	1.75	0.08	1.73	1.74	1.71	1.69	0.08	<0.01	0.52	0.85

**Eight mixed-gender, neutered beagles were fed the NP or HP diets during the first and sixth weeks, respectively. From weeks 2 to 5, using a randomized, crossover, 2 × 2 Latin square design, 4 diets differing in fava bean variety and fermentation were compared as follows: unfermented (UF) high tannin, fermented (FM) high tannin, UF low tannin, and FM low tannin. Values expressed as means (n = 8). SEM, pooled standard error of the mean; TB, total bilirubin; DB, direct bilirubin; IB, indirect bilirubin; ALP, alkaline phosphatase; GGT, gamma-glutamyl transferase; ALT, alanine aminotransferase; GLDH, glutamate dehydrogenase; CK, creatine kinase.*

[a]P, P-value between commercial diets with different protein content (NP vs. HP); FB, fava bean (low tannin vs. high tannin); FM, fermentation (UF vs. FM).

HP had decreased left ventricular end-systolic volume (ESV) and cardiac output (CO) compared with NP-fed dogs ($P < 0.05$). Dogs fed FM diets tended to have decreased maximum velocity (MV) of passive ventricular filling (the early or E wave) compared with UF-fed dogs regardless of FB ($P < 0.10$). There was no significant effect of protein content or either FM, FB, or the interaction between FM and FB on left ventricular end-diastolic volume (EDV), stroke volume (SV), heart rate (HR), ejection fraction (EF), left ventricular diastolic wall thickness (DWT), left ventricular systolic wall thickness (SWT), systolic blood pressure (SBP), diastolic blood pressure (DBP), flow-mediated dilation (FMD), velocity time integral for ventricular filling (VTI) (E and A wave combined), and MV (A wave) ($P > 0.10$). Moreover, there was no significant effect of dietary treatments on $LVID_d$ and $LVID_s$ ($P > 0.10$).

Digestibility

Apparent total tract digestibility in beagles fed fava bean–based diets are shown in **Table 6**. Dogs fed FM diets had decreased fat digestibility and increased non-fiber carbohydrates digestibility compared with UF diets regardless of FB ($P < 0.05$). There was no significant effect of FM, FB, or the interaction between FM and

TABLE 4 | Blood electrolytes (mmol/L) of dogs fed diets formulated with either low or high tannin fava beans without (UF) or with (FM) fermentation, or normal (NP) vs. high (HP) protein commercial diets for 7 days each*.

Item	Commercial			Low tannin		High tannin			*P*-value[a]			
	NP	HP	SEM	UF	FM	UF	FM	SEM	*P*	FB	FM	FB × FM
Na	146.50	147.25	0.54	146.34	145.67	146.62	146.26	0.55	0.24	0.36	0.28	0.71
K	4.80	4.87	0.07	4.65^{b,c}	4.62^{b,c}	4.56^{c}	4.74^{b}	0.06	0.52	0.70	0.13	0.01
Na:K	30.50	30.00	0.56	31.26^{b,c}	31.49^{b,c}	32.19^{b}	31.08^{c}	0.51	0.54	0.47	0.23	0.04
Cl	113.63	113.88	0.60	112.13	111.54	111.65	112.21	0.65	0.62	0.81	0.97	0.12
HCO_3-	19.12	20.50	0.38	20.28	19.22	19.92	20.03	0.63	0.04	0.73	0.48	0.38
Anion gap	18.62	17.87	0.47	18.52	19.64	19.53	19.02	0.92	0.26	0.83	0.73	0.35
Ca	2.44	2.49	0.03	2.45	2.46	2.47	2.45	0.03	0.01	0.70	0.37	0.22
P	1.49	1.67	0.03	1.34	1.47	1.48	1.53	0.04	<0.01	0.05	0.08	0.42
Mg	0.88	0.81	0.08	0.85	0.82	0.83	0.84	0.01	0.56	0.87	0.55	0.11

*Values expressed as means (n = 8).
[a]P, P-value between commercial diets with different protein content (NP vs. HP); FB, fava bean (low tannin vs. high tannin); FM, fermentation (UF vs. FM).
[b,c]Means within a same row with no common superscript differ significantly ($P < 0.05$).

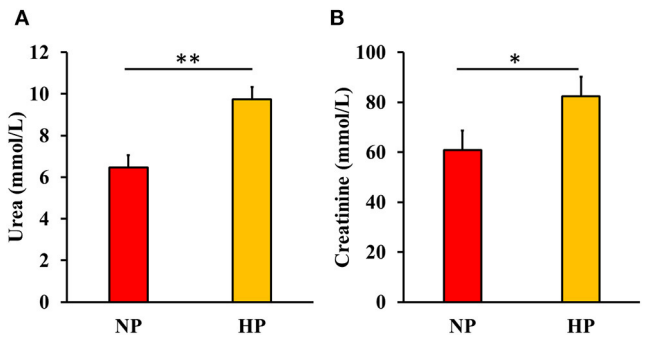

FIGURE 3 | Blood urea **(A)** and creatinine **(B)** content in dogs fed either normal (NP) or high protein (HP) commercial diets. Eight mixed-gender, neutered beagles were fed the NP, or HP diets during the first and sixth weeks, respectively *$P < 0.10$; **$P < 0.05$. There was no effect of either fava bean (FB), fermentation (FM), or the interaction between FB and FM on urea and creatinine content ($P > 0.05$).

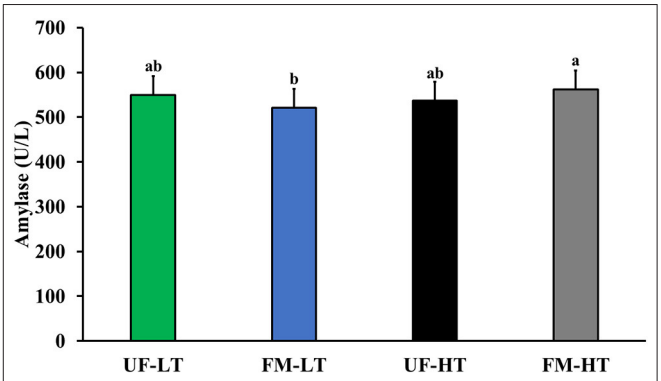

FIGURE 4 | Blood amylase content in dogs fed either low (LT) or high (HT) tannin fava bean–based diets without (UN) or with (FM) fermentation. From weeks 2 to 5, using a randomized, crossover, 2 × 2 Latin square design, 8 mixed-gender, neutered beagles were fed 4 diets differing in fava bean variety and fermentation as follows: UF-HT, FM-HT, UF-LT, and FM-LT. Bars with no common letter (a, b) differ significantly ($P < 0.05$). There was no effect of dietary protein content on serum amylase content ($P > 0.05$).

FB on digestibility of crude protein, gross energy, methionine, cysteine, or taurine ($P > 0.10$).

Plasma Amino Acid Levels

Plasma amino acid concentrations in beagles after 7 days of feeding each test diet are shown in **Table 7**. Dogs fed HP diets had increased taurine, cystine, and cysteine concentrations compared with NP-fed dogs ($P < 0.05$). There was no significant effect of dietary protein content on plasma methionine concentration ($P > 0.10$). Plasma cysteine was increased in HT-fed dogs compared with LT-fed dogs regardless of fermentation ($P < 0.05$). There was no significant effect of fava bean variety on plasma taurine, cystine, and methionine levels ($P > 0.05$). However, fermentation increased plasma cysteine ($P < 0.05$) and tended to decrease plasma cystine ($P < 0.10$) regardless of fava bean variety. There were no significant interactions between fava bean variety and fermentation on plasma amino acid levels ($P > 0.10$).

DISCUSSION

The objective of this study was to determine if neutered, mixed-gender, adult beagles fed diets with 30% inclusion of fava bean flour would show altered nutrient digestibility, glucose tolerance, overall health, cardiovascular function, and plasma amino acid levels when contrasted to commercial diets with normal vs. high protein. Fava bean diets had moderate protein levels, but were formulated to be near the AAFCO dietary minimums for methionine (0.33% inclusion) or cystine + methionine (0.65% inclusion) (35). This was done intentionally to cause more rapid changes in sulfur-containing amino acid levels in the dogs and potentially cause early, reversible impairments in cardiac function despite using only a 7-day feeding period for each test diet. All fava bean diets in this study met the cystine + methionine AAFCO minimum, but all were slightly below the

TABLE 5 | Cardiovascular function parameters of dogs fed diets formulated with either low or high tannin fava beans without (UF) or with (FM) fermentation, or normal (NP) vs. high (HP) protein commercial diets for 7 days each*.

Item	Commercial			Low tannin		High tannin			*P*-value[a]		
	NP	HP	SEM	UF	FM	UF	FM	SEM	*P*	FB	FM
EDV (ml)	24.25	21.33	2.50	19.36	16.84	16.86	17.33	1.89	0.42	0.60	0.59
ESV (ml)	8.67	4.37	1.32	2.97	2.36	2.59	2.42	0.60	0.05	0.79	0.52
$LVID_d$ ($cm/BW^{1/3}$)	0.96	0.88	0.07	0.92	0.85	0.83	0.90	0.08	0.43	0.85	0.99
$LVID_s$ ($cm/BW^{1/3}$)	0.47	0.43	0.05	0.37	0.37	0.36	0.36	0.04	0.58	0.79	1.00
SV (ml)	18.62	16.96	1.37	16.39	14.59	14.29	14.91	1.41	0.41	0.53	0.68
CO (L/min)	1.84	1.41	0.14	1.41	1.34	1.41	1.21	0.18	0.04	0.72	0.44
EF (%)	79.38	83.13	3.49	85.88	86.44	85.56	87.31	3.20	0.46	0.89	0.60
HR (bpm)	97.75	93.13	5.05	102.83	91.17	101.29	91.50	5.55	0.53	0.91	0.07
DWT (cm)	0.83	0.79	0.07	0.85	0.89	0.90	0.88	0.05	0.72	0.57	0.90
SWT (cm)	1.52	1.35	0.13	1.50	1.37	1.48	1.39	0.08	0.38	0.99	0.17
SBP (mmHg)	136.12	128.93	5.52	131.31	118.06	127.75	123.25	7.95	0.37	0.91	0.27
DBP (mmHg)	76.88	72.57	6.58	67.42	61.87	63.80	62.50	5.57	0.65	0.79	0.54
FMD (%)	13.25	6.78	4.03	6.88	10.35	6.54	9.66	4.35	0.27	0.90	0.45
VTI (E wave) (cm)	9.87	9.23	2.14	7.49	8.43	9.08	8.97	1.23	0.83	0.40	0.74
VTI (A wave) (cm)	1.10	0.82	0.22	1.02	1.13	1.40	1.21	0.21	0.39	0.30	0.84
MV (E wave) (cm/s)	87.80	82.46	13.30	72.08	65.86	83.00	80.40	6.58	0.76	0.51	0.06
MV (A wave) (cm/s)	44.31	37.03	4.76	40.67	35.75	49.14	41.32	5.21	0.30	0.19	0.23

**Eight mixed-gender, neutered beagles were fed the NP or HP diets during the first and sixth weeks, respectively. From weeks 2 to 5, using a randomized, crossover, 2 × 2 Latin square design, 4 diets differing in fava bean variety and fermentation were compared as follows: unfermented (UF) high tannin, fermented (FM) high tannin, UF low tannin, and FM low tannin. Values expressed as means (n = 8). SEM, pooled standard error of the mean; EDV, left ventricular end-diastolic volume; ESV, left ventricular end-systolic volume; $LVID_d$, left ventricular end diastolic diameter; $LVID_s$, left ventricular end systolic diameter; SV, stroke volume; HR, heart rate; CO, cardiac output; EF, ejection fraction; DWT, left ventricle diastolic wall thickness; SWT, left ventricle systolic wall thickness; SBP, systolic blood pressure; DBP, diastolic blood pressure; FMD, flow-mediated dilation; VTI (E wave), velocity time integral (E wave); VTI (A wave), velocity time integral (A wave); MV (E wave), maximum velocity (E wave); MV (A wave), maximum velocity (A wave).*

[a]P, P-value between commercial diets with different protein content (NP vs. HP); FB, fava bean (low tannin vs. high tannin); FM, fermentation (UF vs. FM). The interaction between FB and FM was not significant for any of the parameters measured.

TABLE 6 | Apparent total tract digestibility of dogs fed diets formulated with either low or high tannin fava beans without (UF) or with (FM) fermentation for 7 days each*.

Item	Low tannin		High tannin			*P*-value[a]	
	UF	FM	UF	FM	SEM	FB	FM
Crude protein	85.74	86.80	85.91	84.74	1.044	0.37	0.95
Fat	91.93	89.15	92.44	89.15	1.262	0.66	0.01
Non-fiber carbohydrates	95.94	96.59	96.09	96.64	0.214	0.76	0.05
Gross energy	92.02	92.19	91.85	91.61	0.347	0.29	0.91
Methionine	85.87	84.56	83.28	83.32	1.299	0.15	0.62
Cysteine	84.38	87.34	88.26	87.34	1.491	0.21	0.50
Taurine	86.68	84.08	84.88	84.87	1.545	0.74	0.41

**From weeks 2 to 5, using a randomized, crossover, 2 × 2 Latin square design, 8 mixed-gender, neutered beagles were fed 4 diets differing in fava bean variety and fermentation as follows: unfermented (UF) high tannin, fermented (FM) high tannin, UF low tannin, and FM low tannin. Values expressed as means (n = 8). SEM, pooled standard error of the mean.*
[a]FB, fava bean (low tannin vs. high tannin); FM, fermentation (UF vs. FM). The interaction between FB and FM was not significant for any of the parameters measured.

minimum for methionine alone due to variations in methionine content of the fava beans from reported literature values that were used for diet formulation (26).

Lack of Evidence for Toxicity From Fava Beans, Digestibility, Glucose Tolerance, and Antinutritional Factors

Fava beans are pulses, a subset of legumes. Other legumes such as peas have been increasingly included in dog diets as a protein and fiber source (48, 49). Pulse ingredients have been controversially associated with grain-free diets and the occurrence of DCM in dogs (13). Specific to fava beans, however, is the additional association from vicine and convicine antinutritional factors with hemolytic anemia in susceptible humans (50). Worries about potential dog toxicity have prevented its AAFCO approval as a dog food ingredient thus far. Fermentation has been used as a valuable approach to reduce anti-nutritional factors in pulses, including trypsin inhibitors, hemagglutinins, and saponins. Moreover, *Candida* species have the potential to

TABLE 7 | Plasma amino acid levels (nmol/ml) of dogs fed diets formulated with either low or high tannin fava beans without (UF) or with (FM) fermentation, or normal (NP) vs. high (HP) protein commercial diets for 7 days each*.

Item	Commercial			Low tannin		High tannin			*P*-value[a]		
	NP	HP	SEM	UF	FM	UF	FM	SEM	*P*	FB	FM
Taurine	60.83	116.38	13.576	79.81	74.91	90.88	87.39	9.953	0.01	0.22	0.65
Cystine	10.83	13.33	0.774	16.75	14.75	16.38	13.75	1.467	0.04	0.59	0.08
Cysteine	54.00	472.50	20.401	85.75	137.25	112.62	153.87	35.863	<0.01	0.05	<0.01
Methionine	55.20	51.50	2.203	44.57	45.86	44.67	41.00	2.551	0.26	0.36	0.64

*Eight mixed-gender, neutered beagles were fed the NP or HP diets during the first and sixth weeks, respectively. From weeks 2 to 5, using a randomized, crossover, 2 × 2 Latin square design, 4 diets differing in fava bean variety and fermentation were compared as follows: unfermented (UF) high tannin, fermented (FM) high tannin, UF low tannin, and FM low tannin. Values expressed as means (n = 8). SEM, pooled standard error of the mean.

[a]P, P-value between commercial diets with different protein content (NP vs. HP); FB, fava bean (low tannin vs. high tannin); FM, fermentation (UF vs. FM). The interaction between FB and FM was not significant for any of the parameters measured.

synthesize and increase taurine content (32) as well as improve protein digestibility through its breakdown into amino acids by fermentative microorganisms (51). This current study is the first to show that fermentation with *C. utilis* can successfully reduce the vicine/convicine content in fava bean flour, but had no effect on taurine content, protein digestibility, or amino acid digestibility. Of interest, however, is the observation that fermented fava bean diets both caused increases in plasma cysteine after 7 days of feeding, an effect that does not seem to relate to dietary levels and has no current explanation that should be explored in future studies.

The low-tannin variety used in the present study, Snowdrop, is known to have tannin levels as low as 1% (52), while Florent, which was the high tannin variety used in the present study, has been classified as a normal (higher) tannin genotype in Canada (2). Generally, other antinutritional factors tend to be high in varieties with high tannins, but both fava bean varieties used in the present study had high concentration of the antinutritional factors vicine and convicine. The main issue associated with high tannin content in the diet is related to reduced bioavailability of nutrients in the gastrointestinal tract (53, 54). Despite not evaluating bioavailability in the present study, there was no effect of fava bean variety on digestibility values of any nutrient measured. This implies that despite the potential negative effects of tannins on, specially, protein digestibility (55), we do not have evidence to show a clear detrimental effect of tannins. However, within-animal variation in digestive response to tannins and low sample size may have prevented detection of an impact on digestibility (56). Interestingly, fermentation was able to dramatically reduce both the vicine and convicine content in fava bean–based diets, regardless of variety. In a recent study, fermentation with *L. plantarum* degraded the pyrimidine glycosides in fava bean flour within 48 h, which reduced the toxicity of the fermented fava bean as assessed through *ex vivo* assays on human blood (31). In the present study, there was no impairment in glucose tolerance in dogs fed fermented fava bean (FM) diets as revealed by the lack of effect on glucose baseline and peak levels, time to peak, and AUC. This leads to the conclusion either that fava beans do not have any effect on glucose utilization in dogs or that 7 days is not sufficient to alter glucose utilization. However, it should be noted that the four fava bean test diets in this study were fed sequentially in a crossover design. By the end of these four feeding periods, beagles had been fed fava bean–based diets for a month, with no change in glucose utilization from the previous NP period. Moreover, RBC content was unchanged in dogs fed fava beans compared with the NP diet. Taken together, because anemia is a primary sign of favism and none of the fava bean–based diets caused anemia in the dogs in the current study, it can be concluded that fava beans are not toxic in dogs. While AAFCO requires a 6-month feeding study for fava beans to be approved as a pet food ingredient, this study provides initial indications that they are a safe dog food ingredient.

The present study also provided other indication of fermentation of fava bean flour with *C. utilis* enhancing diet quality and consequently health in dogs. Fermentation significantly decreased the digestibility of fat and increased the digestibility of non-fiber carbohydrates. It has been shown in fish that the concentration of carbohydrates in the gut are inversely related to fat digestibility (57) and this is exacerbated when large amounts of starch are present (58). Surprisingly, amylase content in blood was increased in FM-HT compared with FM-LT with both unfermented diets being intermediate. This indicates that the fermentability of fava bean varieties may be different, which might be associated with the carbohydrate composition, releasing increased or decreased amounts of starch when fermented (59). Finally, dogs fed fermented diets showed increased RBC levels compared with those fed unfermented fava bean diets. Low RBC in young dogs is a common occurrence as RBC lifespan is shorter and young RBC contain less hemoglobin when compared with aging RBC (60). An increased RBC content in fermented diet–fed dogs could be associated with an accelerated production of blood cells and increased health that should be further explored in future studies.

Effect of High Dietary Protein Diet on Dog Health

The results of the current study using commercial diets also provide indications that high dietary protein can negatively affect overall health of dogs. Previous studies have reported that high protein dog diets may negatively impact gastrointestinal health, pre-disposing dogs to diarrhea (61). While diarrhea was not observed with the HP commercial diet tested in the current study,

excessive protein intake has been reported to increase proteinuria and overload kidneys, potentially decreasing the overall health of dogs (62), and this is consistent with the higher serum cholesterol, urea, and creatinine observed with the HP diet in the current study. High serum cholesterol in dogs is consistent with a positive relationship reported between protein intake and cholesterol in humans (63). Moreover, bilirubin measurements (TB, DB, and IB) were decreased in HP-fed dogs compared with NP, which agrees with a recent study showing higher bilirubin concentrations in young pigs fed a protein restricted diet compared with a control diet (64). On the other hand, potential benefits of the HP diet in the current study come from the observed decrease in ALP and ALT in dogs fed HP compared with NP. In dogs, higher ALT content is directly associated with hepatocyte membrane damage and necrosis, whereas ALP is positively associated with biliary stasis (65, 66). Increased ALT and ALP in NP dogs may be interpreted as poor hepatic function (67). It should be noted that while trends for changes in blood parameters can be interpreted as positive or negative, all values for all end-points measured in this study fell within clinical norms and thus all dogs were maintained in a healthy state. Future studies using longer feeding periods are required to determine if trends continue and become clinically significant compared to that observed after 7 days in the current study.

Cardiac Function, Cysteine, Methionine, and Taurine

One of the main hypotheses of the present study was that pulse-based diets with their higher fiber would cause decreases in plasma taurine, cysteine, or methionine levels, subsequently leading to impaired cardiac contractility or enlargement of the heart consistent with DCM. However, after 7 days of feeding each fava bean–based diet, no significant adverse changes were detected in cardiac or vascular function in the current study. Longer feeding trials are needed to confirm that there are no effects. However, what did change in the short term was ESV that was increased in NP-fed dogs compared with HP-fed dogs, but without changes in ventricular chamber size (LVID). Changes in cardiac chamber size would be surprising after only 7 days, thus a functional change such as impaired contractility and reduce cardiac output (CO) is not expected to explain the higher ESV. In fact, the NP-fed dogs instead had higher CO that may be due to a non-significant trend for both HR and SV to increase. This is consistent with a similar non-significant trend for both systolic and diastolic blood pressure to increase in NP-fed dogs compared with HP-fed dogs, suggesting a generalized increase in sympathetic outflow in the NP-fed dogs. Higher sympathetic tone and blood pressure increases afterload and impairs emptying of the left ventricle at the end of systole (i.e., higher ESV), a reliable indicator of impaired systolic function (68).

The proximate analysis of diets showed no major differences in crude fiber content between laboratory-formulated fava bean–based diets and commercial diets. The main differences among diets were observed for cysteine content, which was low in the fava bean diets. Also, methionine and taurine content were highest in HP diets compared with all fava bean–based diets and the NP diet. If reduced dietary taurine levels were driving cardiac changes, then it would have been expected that the NP diet should have led to adverse cardiac changes and this may agree with what we observed. Future studies should explore longer feeding periods and address whether more susceptible dog breeds than beagles produce a stronger relationship between taurine and cardiac impairment.

The fava bean–based diets and the NP commercial diet tested in the current study all had methionine content that was just below the AAFCO minimum. Dogs can synthesize taurine from cysteine or methionine (69), but all three of these amino acids tend to be low or limiting when plant-based protein sources such as pulses are used. Compounding this problem, pulses are high in resistant starch and fiber. Previous studies in dogs have reported that high fiber diets decrease protein digestibility and increase fecal bile acid excretion in feces (70). Because taurocholate is the major bile salt excreted by dogs, the net effect of high fiber has been reported to deplete taurine and impair digestion of protein that contains cysteine and methionine needed to replace it. However, none of the diets tested caused any significant drop in plasma levels of taurine, cysteine, or methionine and levels remained above the reference range throughout the study (27). In fact, fermented fava beans (both varieties) led to significant increases in plasma cysteine, suggesting a potentially beneficial health effect of fermentation processing. In contrast, the high protein (HP) commercial diet led to higher plasma levels of cysteine and taurine, but no change in plasma methionine compared with NP diet, suggesting that 7 days was sufficient time to cause some alterations of blood levels of these amino acids. Cysteine results, however, should be cautiously interpreted due to its unstable nature and an interfering substance during HPLC analysis of this amino acid that could have led to overestimation of cysteine levels (71). Further analysis using a different method would be necessary to confirm the cysteine results.

STRENGTH AND LIMITATIONS

A strength of this study was that the fava bean–based diets that were made in our laboratory were tested against two popular commercial brands, giving a more realistic context to the results. Another strength was the use of a Latin square, crossover design where the same dogs were tested on each diet, reducing variability and increasing power with the small sample size of this experiment. However, an important limitation of this study was the duration, which is insufficient to cause major structural cardiac changes, but was long enough to change plasma levels of sulfur-containing amino acid levels, at least in response to high dietary protein. Moreover, the results of this study using young, healthy adult beagles may not apply to older, large breed dogs with genetic susceptibility to taurine deficiency. However, we would predict that changes in taurine and sulfur-containing amino acids would be even more pronounced in these breeds or in older dogs. A last limitation of the present study was the experimental design which does not allow the statistical comparison between commercial and fava bean-based diets.

CONCLUSION AND IMPLICATIONS

Most importantly, fava bean–based diets did not cause hemolytic anemia and did not alter glucose handling in dogs after 7 days of feeding, thus fava beans appear safe as a dog food ingredient. In contrast, the high-protein grain-free commercial diet adversely altered blood chemistry compared with the normal protein, grain-containing commercial diet we tested. Moreover, the normal protein, grain-based diet appeared to cause excess sympathetic tone, a trend that if it were to continue with long-term feeding, might lead to adverse changes in cardiac health that are distinct from DCM. On the other hand, fermentation with *C. utilis* looks promising to reduce antinutritional factors and potentially improve health through improvements in nutrient digestibility and increased RBC levels in dogs. Studies using longer feeding periods are needed to determine whether these short-term changes are sustained to produce clinically significant changes in dogs.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by University of Saskatchewan Animal Research Ethics Board.

AUTHOR CONTRIBUTIONS

LGR, ML, and LW designed the study. LGR, TM, and CQ conducted the study. LGR and LAR performed data analysis. LGR and LW wrote the article. LW was responsible for final content of the article. All authors contributed to the interpretation of the results throughout the study and have read and approved the article.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2021.653771/full#supplementary-material

REFERENCES

1. de Boer J, Aiking H. On the merits of plant-based proteins for global food security: marrying macro and micro perspectives. *Ecol Econ.* (2011) 70:1259–65. doi: 10.1016/j.ecolecon.2011.03.001
2. Fleury D, Barker B. *Faba Bean Variety Report 2015/16.* (2015). Available online at: https://saskpulse.com/files/general/151026_Faba_bean_variety_report2.pdf (accessed February 21, 2021).
3. Rahate KA, Madhumita M, Prabhakar PK. Nutritional composition, anti-nutritional factors, pretreatments-cum-processing impact and food formulation potential of faba bean (*Vicia faba* L.): a comprehensive review. *LWT.* (2021) 138:110796. doi: 10.1016/j.lwt.2020.110796
4. U.S. Food and Drug Administration. *FDA Investigation Into Potential Link Between Certain Diets and Canine Dilated Cardiomyopathy.* (2019). Available online at: https://www.fda.gov/animal-veterinary/outbreaks-and-advisories/fda-investigation-potential-link-between-certain-diets-and-canine-dilated-cardiomyopathy (accessed February 21, 2021).
5. Dutton E, López-Alvarez J. An update on canine cardiomyopathies – is it all in the genes? *J Small Anim Pract.* (2018) 59:455–64. doi: 10.1111/jsap.12841
6. Monnet E, Orton EC, Salman M, Boon J. Idiopathic dilated cardiomyopathy in dogs: survival and prognostic indicators. *J Vet Intern Med.* (1995) 9:12–7. doi: 10.1111/j.1939-1676.1995.tb03266.x
7. Borgarelli M, Santilli RA, Chiavegato D, D'Agnolo G, Zanatta R, Mannelli A, et al. Prognostic indicators for dogs with dilated cardiomyopathy. *J Vet Intern Med.* (2006) 20:104–10. doi: 10.1111/j.1939-1676.2006.tb02829.x
8. Werner P, Raducha MG, Prociuk U, Sleeper MM, Van Winkle TJ, Henthorn PS. A novel locus for dilated cardiomyopathy maps to canine chromosome 8. *Genomics.* (2008) 91:517–21. doi: 10.1016/j.ygeno.2008.03.007
9. Martin MWS, Stafford Johnson MJ, Celona B. Canine dilated cardiomyopathy: a retrospective study of signalment, presentation and clinical findings in 369 cases. *J Small Anim Pract.* (2009) 50:23–9. doi: 10.1111/j.1748-5827.2008.00659.x
10. Kramer GA, Kittleson MD, Fox PR, Lewis J, Pion PD. Plasma taurine concentrations in normal dogs and in dogs with heart disease. *J Vet Intern Med.* (1995) 9:253–8. doi: 10.1111/j.1939-1676.1995.tb01076.x
11. Bélanger MC, Ouellet M, Queney G, Moreau M. Taurine-Deficient dilated cardiomyopathy in a family of golden retrievers. *J Am Anim Hosp Assoc.* (2005) 41:284–91. doi: 10.5326/0410284
12. Maron BJ, Towbin JA, Thiene G, Antzelevitch C, Corrado D, Arnett D, et al. Contemporary definitions and classification of the cardiomyopathies: an American heart association scientific statement from the council on clinical cardiology, heart failure and transplantation committee; quality of care and outcomes research and functional genomics and translational biology interdisciplinary working groups; and council on epidemiology and prevention. *Circulation.* (2006) 113:1807–16. doi: 10.1161/CIRCULATIONAHA.106.174287
13. Mansilla WD, Marinangeli CPF, Ekenstedt KJ, Larsen JA, Aldrich G, Columbus DA, et al. Special topic: the association between pulse ingredients and canine dilated cardiomyopathy: addressing the knowledge gaps before establishing causation. *J Anim Sci.* (2019) 97:983–97. doi: 10.1093/jas/sky488
14. McCauley SR, Clark SD, Quest BW, Streeter RM, Oxford EM. Review of canine dilated cardiomyopathy in the wake of diet-associated concerns. *J Anim Sci.* (2020) 98:skaa155. doi: 10.1093/jas/skaa155
15. Kaplan JL, Stern JA, Fascetti AJ, Larsen JA, Skolnik H, Peddle GD, et al. Taurine deficiency and dilated cardiomyopathy in golden retrievers fed commercial diets. *PLoS ONE.* (2018) 13:e0209112. doi: 10.1371/journal.pone.0209112
16. Fascetti AJ, Reed JR, Rogers QR, Backus RC. Taurine deficiency in dogs with dilated cardiomyopathy: 12 cases (1997–2001). *J Am Vet Med Assoc.* (2003) 223:1137–41. doi: 10.2460/javma.2003.223.1137

17. Backus RC, Ko KS, Fascetti AJ, Kittleson MD, MacDonald KA, Maggs DJ, et al. Low plasma taurine concentration in newfoundland dogs is associated with low plasma methionine and cyst(e)ine concentrations and low taurine synthesis. *J Nutr.* (2006) 136:2525–33. doi: 10.1093/jn/136.10.2525
18. Bakker AJ, Berg HM. Effect of taurine on sarcoplasmic reticulum function and force in skinned fast-twitch skeletal muscle fibres of the rat. *J Physiol.* (2002) 538:185–94. doi: 10.1113/jphysiol.2001.012872
19. Sanderson SL. Taurine and carnitine in canine cardiomyopathy. *Vet Clin Small Anim Pract.* (2006) 36:1325–43. doi: 10.1016/j.cvsm.2006.08.010
20. Kim SW, Rogers QR, Morris JG. Dietary antibiotics decrease taurine loss in cats fed a canned heat-processed diet. *J Nutr.* (1996) 126:509–15. doi: 10.1093/jn/126.2.509
21. Kim SW, Rogers QR, Morris JG. Maillard reaction products in purified diets induce taurine depletion in cats which is reversed by antibiotics. *J Nutr.* (1996) 126:195–201. doi: 10.1093/jn/126.1.195
22. O'Máille ER, Richards TG, Short AH. Acute taurine depletion and maximal rates of hepatic conjugation and secretion of cholic acid in the dog. *J Physiol.* (1965) 180:67–79.
23. Story JA, Kritchevsky D. Bile acid metabolism and fiber. *Am J Clin Nutr.* (1978) 31:S199–202. doi: 10.1093/ajcn/31.10.S199
24. Liener IE. Antinutritional factors in legume seeds: state of the art. *Recent Adv Res Antinutritional Factors Legume Seeds.* 1st ed. (1989) 6–13.
25. Luzzatto L, Arese P. Favism and glucose-6-phosphate dehydrogenase deficiency. *N Engl J Med.* (2018) 378:60–71. doi: 10.1056/NEJMra1 708111
26. Frias J, Peñas E, Martinez-Villaluenga C. Chapter 16 - ". In: Frias J, Martinez-Villaluenga C, Peñas E, editors. *Fermented Foods in Health and Disease Prevention.* Cambridge, MA: Academic Press (2017). p. 385–416. doi: 10.1016/B978-0-12-802309-9.00016-9
27. Coda R, Rizzello CG, Gobbetti M. Use of sourdough fermentation and pseudo-cereals and leguminous flours for the making of a functional bread enriched of γ-aminobutyric acid (GABA). *Int J Food Microbiol.* (2010) 137:236–45. doi: 10.1016/j.ijfoodmicro.2009.12.010
28. Curiel JA, Coda R, Centomani I, Summo C, Gobbetti M, Rizzello CG. Exploitation of the nutritional and functional characteristics of traditional Italian legumes: the potential of sourdough fermentation. *Int J Food Microbiol.* (2015) 196:51–61. doi: 10.1016/j.ijfoodmicro.2014.11.032
29. Santana FC, Empis J. Bacterial removal of quinolizidine alkaloids from *Lupinus albus* flours. *Eur Food Res Technol.* (2001) 212:217–24. doi: 10.1007/s002170000221
30. Coda R, Melama L, Rizzello CG, Curiel JA, Sibakov J, Holopainen U, et al. Effect of air classification and fermentation by *Lactobacillus plantarum* VTT E-133328 on faba bean (*Vicia faba* L.) flour nutritional properties. *Int J Food Microbiol.* (2015) 193:34–42. doi: 10.1016/j.ijfoodmicro.2014.10.012
31. Rizzello CG, Losito I, Facchini L, Katina K, Palmisano F, Gobbetti M, et al. Degradation of vicine, convicine and their aglycones during fermentation of faba bean flour. *Sci Rep.* (2016) 6:32452. doi: 10.1038/srep32452
32. Hébert A, Forquin-Gomez M-P, Roux A, Aubert J, Junot C, Heilier J-F, et al. New insights into sulfur metabolism in yeasts as revealed by studies of *Yarrowia lipolytica*. *Appl Environ Microbiol.* (2013) 79:1200–11. doi: 10.1128/AEM.03259-12
33. Curso Almeida P. *Effects of Pea Starch Yeast Fermentation on Glycemic Index, Palatability, Metabolic Status and Intestinal Health of Dogs and Cats Fed a Pea-Based Diet.* [dissertation/master's thesis], Saskatoon (SK): University of Saskatchewan (2020).
34. National Research Council. *Nutrient Requirements of Dogs and Cats*. Washington, DC: The National Academies Press (2006).
35. Association of American Feed Control Officials. *Official Publication*. Champaign, IL: AAFCO (2017).
36. AOAC. *Association of Official Analytical Chemists Official Methods of Analysis*. 21st ed. Washington, DC: AOAC (2019).
37. Zhang F, Adeola O. Techniques for evaluating digestibility of energy, amino acids, phosphorus, and calcium in feed ingredients for pigs. *Anim Nutr.* (2017) 3:344–52. doi: 10.1016/j.aninu.2017.06.008
38. Wolever TM, Jenkins DJ, Jenkins AL, Josse RG. The glycemic index: methodology and clinical implications. *Am J Clin Nutr.* (1991) 54:846–54. doi: 10.1093/ajcn/54.5.846
39. Delaney SJ, Kass PH, Rogers QR, Fascetti AJ. Plasma and whole blood taurine in normal dogs of varying size fed commercially prepared food. *J Anim Physiol Anim Nutr.* (2003) 87:236–44. doi: 10.1046/j.1439-0396.2003.00433.x
40. Tôrres CL, Backus RC, Fascetti AJ, Rogers QR. Taurine status in normal dogs fed a commercial diet associated with taurine deficiency and dilated cardiomyopathy. *J Anim Physiol Anim Nutr.* (2003) 87:359–72. doi: 10.1046/j.1439-0396.2003.00446.x
41. Spitze AR, Wong DL, Rogers QR, Fascetti AJ. Taurine concentrations in animal feed ingredients; cooking influences taurine content. *J Anim Physiol Anim Nutr.* (2003) 87:251–62. doi: 10.1046/j.1439-0396.2003.00434.x
42. Heinze CR, Larsen JA, Kass PH, Fascetti AJ. Plasma amino acid and whole blood taurine concentrations in cats eating commercially prepared diets. *Am J Vet Res.* (2009) 70:1374–82. doi: 10.2460/ajvr.70.11.1374
43. Raitakari OT, Celermajer DS. Research methods in human cardiovascular pharmacology. *Br J Clin Pharmacol.* (2000) 50:397–404. doi: 10.1046/j.1365-2125.2000.00277.x
44. Adolphe JL, Drew MD, Huang Q, Silver TI, Weber LP. Postprandial impairment of flow-mediated dilation and elevated methylglyoxal after simple but not complex carbohydrate consumption in dogs. *Nutr Res.* (2012) 32:278–84. doi: 10.1016/j.nutres.2012.03.002
45. Otto CM, Schwaegler RG, Freeman RV, Linefsky J. *Echocardiography Review Guide E-Book: Companion to the Textbook of Clinical Echocardiography*. Philadelphia, PA: Elsevier Health Sciences. (2019) 456p.
46. Lang RM, Bierig M, Devereux RB, Flachskampf FA, Foster E, Pellikka PA, et al. Recommendations for chamber quantification: a report from the American society of echocardiography's guidelines and standards committee and the chamber quantification writing group, developed in conjunction with the European association of echocardiography, a branch of the European society of cardiology. *J Am Soc Echocardiogr.* (2005) 18:1440–63. doi: 10.1016/j.echo.2005.10.005
47. Cornell CC, Kittleson MD, Torre PD, Häggström J, Lombard CW, Pedersen HD, et al. Allometric scaling of m-mode cardiac measurements in normal adult dogs. *J Vet Intern Med.* (2004) 18:311–21. doi: 10.1111/j.1939-1676.2004.tb02551.x
48. Butterwick RF, Markwell PJ, Thorne CJ. Effect of level and source of dietary fiber on food intake in the dog. *J Nutr.* (1994) 124:2695S–700S. doi: 10.1093/jn/124.suppl_12.2695S
49. Rice JE, Ihle SL. Effects of diet on fecal occult blood testing in healthy dogs. *Can J Vet Res.* (1994) 58:134–7.
50. Champ MM-J. Non-nutrient bioactive substances of pulses. *Br J Nutr.* (2002) 88:307–19. doi: 10.1079/BJN2002721
51. Malcolmson L, Han J. pulse processing and utilization of pulse ingredients in foods. In: Dahl WJ, editor. *Health Benefits of Pulses*. Cham: Springer International Publishing (2019). p. 129–49. doi: 10.1007/978-3-030-12763-3_9
52. Wei X. *Effects of Short-Term Germination and Autoclaving on Selected Compounds in Faba Bean and Faba Bean Applications in Low-Fat Pork Bologna.* [Dissertation/master's thesis], Saskatoon (SK): University of Saskatchewan (2019).
53. Addisu S. Effect of dietary tannin source feeds on ruminal fermentation and production of cattle; a review. *Online J Anim Feed Res.* (2016) 6:42–56.
54. Bunglavan SJ, Dutta N. Use of tannins as organic protectants of proteins in digestion of ruminants. *J Livest Sci.* (2013) 4:67–77.
55. Berard NC, Wang Y, Wittenberg KM, Krause DO, Coulman BE, McAllister TA, et al. Condensed tannin concentrations found in vegetative and mature forage legumes grown in western Canada. *Can J Plant Sci.* (2011) 91:669–75. doi: 10.4141/cjps10153
56. Hagerman AE, Robbins CT, Weerasuriya Y, Wilson TC, McArthur C. Tannin chemistry in relation to digestion. *J Range Manag.* (1992) 45:57–62. doi: 10.2307/4002526
57. Storebakken T, Shearer KD, Refstie S, Lagocki S, McCool J. Interactions between salinity, dietary carbohydrate source and carbohydrate concentration on the digestibility of macronutrients and energy in rainbow trout (*Oncorhynchus mykiss*). *Aquaculture.* (1998) 163:347–59. doi: 10.1016/S0044-8486(98)00259-2
58. Skrede G, Storebakken T, Skrede A, Sahlstrøm S, Sørensen M, Shearer KD, et al. Lactic acid fermentation of wheat and barley whole meal flours improves

digestibility of nutrients and energy in Atlantic salmon (*Salmo salar* L.) diets. *Aquaculture.* (2002) 210:305–21. doi: 10.1016/S0044-8486(01)00851-1
59. Çalişkantürk Karataş S, Günay D, Sayar S. *In vitro* evaluation of whole faba bean and its seed coat as a potential source of functional food components. *Food Chem.* (2017) 230:182–8. doi: 10.1016/j.foodchem.2017.03.037
60. Bush BM. *Interpretation of Laboratory Results for Small Animal Clinicians.* Oxford: Blackwell Scientific Publications Ltd (1991) 515p.
61. Hang I, Heilmann RM, Grützner N, Suchodolski JS, Steiner JM, Atroshi F, et al. Impact of diets with a high content of greaves-meal protein or carbohydrates on faecal characteristics, volatile fatty acids and faecal calprotectin concentrations in healthy dogs. *BMC Vet Res.* (2013) 9:201. doi: 10.1186/1746-6148-9-201
62. Burkholder WJ, Lees GE, LeBlanc AK, Slater MR, Bauer JE, Kashtan CE, et al. Diet modulates proteinuria in heterozygous female dogs with x-linked hereditary nephropathy. *J Vet Intern Med.* (2004) 18:165–75. doi: 10.1111/j.1939-1676.2004.tb00157.x
63. Pasiakos SM, Lieberman HR, Fulgoni VL. Higher-Protein diets are associated with higher HDL Cholesterol and Lower BMI and waist circumference in US adults. *J Nutr.* (2015) 145:605–14. doi: 10.3945/jn.114.205203
64. Fisher KD, Scheffler TL, Kasten SC, Reinholt BM, Eyk GR van, Escobar J, et al. Energy dense, protein restricted diet increases adiposity and perturbs metabolism in young, genetically lean pigs. *PLoS ONE.* (2013) 8:e72320. doi: 10.1371/journal.pone.0072320
65. Willard MD, Twedt DC. Gastrointestinal, pancreatic, and hepatic disorders. *Small Anim Clin Diagn Lab Methods.* 5th ed. (2012) 191–225. doi: 10.1016/B978-1-4377-0657-4.00009-0
66. Lawrence YA, Dangott LJ, Rodrigues-Hoffmann A, Steiner JM, Suchodolski JS, Lidbury JA. Proteomic analysis of liver tissue from dogs with chronic hepatitis. *PLoS ONE.* (2018) 13:e0208394. doi: 10.1371/journal.pone.0208394
67. Lucena R, Novales M, Blanco B, Hernández E, Ginel PJ. Effect of probiotic *Enterococcus faecium* SF68 on liver function in healthy dogs. *J Vet Intern Med.* (2019) 33:2628–34. doi: 10.1111/jvim.15625
68. Kittleson MD, Kienle RD. *Small Animal Cardiovascular Medicine.* Milton: Elsevier Canada (1998) 603p.
69. Harrison M, Thomas G, Gilham M, Gray K, Colyer A, Allaway D. Short-term determination and long-term evaluation of the dietary methionine requirement in adult dogs. *Br J Nutr.* (2020) 123:1333–44. doi: 10.1017/S0007114520000690
70. Pezzali JG, Acuff HL, Henry W, Alexander C, Swanson KS, Aldrich CG. Effects of different carbohydrate sources on taurine status in healthy beagle dogs. *J Anim Sci.* (2020) 98:1–9. doi: 10.1093/jas/skaa010
71. Tôrres CL, Miller JW, Rogers QR. Determination of free and total cyst(e)ine in plasma of dogs and cats. *Vet Clin Path.* (2008) 33:228–33. doi: 10.1111/j.1939-165X.2004.tb00378.x

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Partial Substitution of Maize for Sorghum With or Without Supplemental Hydrolysable Tannins on Digestibility and Postprandial Glycemia in Adult Dogs

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The effect of partial substitution of maize for sorghum, containing condensed tannins (CT), with or without the addition of a purified hydrolysable tannin extract (HT), on dog apparent digestibility and glycemic response were evaluated. The trial was conducted with eight adult dogs distributed in four treatments: (M) 50% maize; (MS) 25% maize + 25% sorghum; (MHT) 50% maize + 0.10% HT; (MSHT) 25% maize + 25% sorghum + 0.10% HT; in a balanced incomplete Latin square design in three periods, with two dogs per diet, per period. Data were analyzed by ANOVA procedure and glycemic response by repeated measures ANOVA over time ($P < 0.05$). The phenolic compounds analyzed were not detected after extrusion process, with a reduction mainly in diets containing sorghum. There were no differences in the digestibility coefficients of nutrients and energy between the dietary treatments ($P > 0.05$). Fecal and urinary characteristics were not changed by the addition of sorghum and HT ($P > 0.05$). The fecal score remained within the ideal classification as hard, dry, firm stools. A moderate increase in fecal pH was observed on dogs fed diets containing sorghum ($P = 0.0948$). Additionally, the partial replacement of maize for sorghum associated or not with HT do not alter the glycemic aspects evaluated among dietary treatments ($P > 0.05$). Availability of nutrients from maize and sorghum were similar. Tannins did not interfere in the nutritional capacity of the ingredients.

Keywords: carbohydrate, tannins, phenolic compounds, digestibility, postprandial response

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INTRODUCTION

Cereal grains are widely used in pet food as sources of energy due to its starch content. In addition, starch is fundamental for appropriate extrusion process and kibble characteristics, such as expansion, cellular structure formation, and crispness (1). The most common carbohydrate sources used for companion animal products are rice, maize, and sorghum due to its wide distribution, easy acquisition, and low cost. Besides, all these ingredients are well-accepted, digested, and metabolized for dogs.

However, the evaluation of functional ingredients with appropriate nutritional characteristics that combines some beneficial effect, such as modulation of postprandial glycemic response, has been the major goal in pet food in the past years. The increasing prevalence of diseases such as obesity and diabetes are the main reasons to find other ingredients with functional properties for companion animals (2, 3). This highlights the importance of selecting ingredients based on their nutritional potential and their effects on digestion and metabolism.

In this way, sorghum becomes an interesting alternative to rice and maize because the grain contains functional properties such as groups of phenolic compounds. The most part of them are condensed tannins, secondary compounds of plant metabolism that may be active to improve health. There are some studies relating tannins as an antimicrobial, antiparasitic, antioxidant, anti-inflammatory, and antiviral agent (4). Tannins are classified as condensate (CT) or hydrolysable (HT). CTs, also known as proanthocyanidins, are polymers of flavan-3-ols and flavan-3,4-diols and, after oxidation, yields anthocyanidins (5), while HT are composed of simple phenols, gallotannins, and elagitannins, which, after hydrolysis, yields gallic and ellagic acid (6). Sorghum contains only CT (7). HT can be found in leaves, flowers, twigs, bark of some plants, and as a purified commercial extract. Additionally, sorghum varieties are divided according to their genetics and chemical composition. Sorghum type I have low level of phenols and tannins (0.28 g/kg of tannins), while types II and III present 4.48 and 11.95 g/kg of tannins, respectively (8).

In opposite to its beneficial effects, tannins can form complexes with substances present in saliva promoting astringency and food refusal (9). In addition, tannins can inhibit enzymes and form complexes with carbohydrates, proteins, and metal ions, thus impairing digestibility (10). Indeed, some studies had showed controversial results; some did not find differences between dogs fed maize- or sorghum-based diets (11, 12), while others found some differences on digestibility of dry matter (DM), organic matter, and crude protein (CP) (13), or even an increase on digestibility of CP, gross energy, and DM compared to maize diet (14). Regarding fecal characteristics, studies demonstrated that there are no differences between diets based on maize and sorghum. Finally, Carciofi et al. (11) demonstrated that sorghum-based diet promoted later postprandial meal response in dogs, thus reducing glycemic peak and favoring glycemic control. Concerning HT, Teixeira et al. (15) observed an increase on the fecal dry matter of dogs fed a diet based on rice and HT and a reduction in metabolizable energy on dogs fed a rice, sorghum, and HT diet.

We went through the evaluation of sorghum and rice blends and its effects on digestibility and glycemic index in dogs. The replacement of rice with sorghum reduced the digestibility of protein and also reduced the metabolizable energy content of the diets (15). As a partial replacement of rice, no variation in the glycemic response was observed.

Based on the fact that maize does not contain significant amounts of CT than sorghum does, we hypothesized that by mixing both, we could improve the content of tannins in the final diet, and then, it can have an impact on the glycemic index of the final diet. In addition, the impact of HT on maize must be tested to evaluate if it changes either digestibility or glycemic index. Therefore, this study evaluated the effects of the partial substitution of maize with sorghum containing CT plus the addition of a HT extract on the digestibility of nutrients and energy, fecal, and urinary characteristics, and lastly the glycemic response in adult dogs.

MATERIALS AND METHODS

All animal care and handling procedures were approved by The Institutional Animal Care and Use Committee at the Universidade Federal do Rio Grande do Sul, protocol number 26275.

Animals

Eight healthy adult Beagle (four males and four females), coming from the Animal Science Department, Universidade Federal do Rio Grande do Sul, Porto Alegre, Brazil, were used in this study. They were all intact, between 2 and 3 years old, weighing 12.4 ± 0.97 kg, with a body condition score (BCS) ranging from 4.5 to 5.5 out of 9 points (16), made by a single trained person, and free of endo- and ectoparasites. All dogs were regularly immunized and submitted to clinical and laboratory tests to measure complete blood count (CBC) and to perform biochemical and coproparasitological analyses before the start of the study. The dogs were housed in individual stainless steel metabolic cages (1.0 × 1.0 × 1.5 m) equipped with a feces and urine collector, feeders, and drinkers, in a controlled room at 24°C, with a light/dark cycle of 14:10 h. The adaptation and positive reinforcement were used to avoid stress during the assay. 6 months before starting the trial, dogs were adapted to the metabolic cages and blood collections. During this period, they were fed twice daily inside the metabolic cages and stayed there all through the night. During the day, dogs remained in a patio playing all together for socialization. In the morning and afternoon, before being fed, dogs were set over a table for 3 min. Dogs were safely held, and a blood collection was simulated with no needle introduction; then, dogs were pet and received food. Between each trial, dogs were rested for 15 days, maintained in the patio, and playing together. At the end of the trials, dogs were castrated and given for adoption.

Diets

Maize was partially substituted with sorghum as a way of introducing CT into the diets. Additionally, purified HT obtained from a commercial extract of the chestnut bark (*Castanea sativa*) was included into the diets. The extract was a water-soluble fine brown powder containing HT, hydrolysable polyphenols, cellulose, hemicellulose, simple sugars, lignin, minerals, and 8% moisture; its fiber content was <3%, it had a relative density of 0.5–0.6 g/mL and pH <4.0. Four experimental diets were formulated and extruded to be isonutritives: (M) 50% maize; (MS) 25% maize + 25% sorghum; (MHT) 50% maize + 0.10% HT; (MSHT) 25% maize + 25% sorghum + 0.10% HT (**Tables 1**, **2**). The dogs were fed twice a day (at 0830 and 1,700 h) to meet the energetic and nutritional requirements of adult dogs, as recommended by the NRC (17). Food intake was adjusted

TABLE 1 | Chemical composition of sorghum (*Sorghum bicolor* L. Moench).

Item, % DM basis	Sorghum
Dry matter	86.9
Starch	63.6
Crude protein	7.59
Total dietary fiber	15.3
Fat	2.56
Ash	1.42
Crude fiber	0.72
Gross energy, kcal/kg	4.446
Polyphenol tannins, %	4.8
Polyphenol non-tannins, %	2.6

DM, dry matter.

according to the body weight, weekly, in order to maintain the BCS in 5 points out of 9. The leftovers were collected, weighed, and discounted to calculate consumption. Water was provided *ad libitum*.

Experiment 1: Digestibility Assay

Experimental Design

The assay was conducted as a balanced incomplete Latin square design as a model proposed by Ai et al. (18). Eight dogs were assigned in four treatments and three 10-day periods, with two dogs per treatment in each period, for a total of six replications per treatment, according to the recommendations of the American Association of Feed Control Officials protocol (19). The model for the balanced incomplete Latin square design (8 × 3) was:

$$\text{yij(k)} = \mu + \text{Timej} + \text{Dogj} + \tau\text{k} + \varepsilon\text{ij(k)}$$

in which yij(k) is observation ijk, μ is the overall mean, Time is the effect of row, Dog is the effect of column j, τk is the fixed effect of treatment k, and εij(k) is the random error with mean 0 and variance σ2. Gender (female and male) was used as a criterion for blocking, and body weight was used to randomize them in the treatments. Each period lasted 10 days, with 5 days for adaptation to the cage and experimental diet, followed by 5 days of total feces and urine collection and measurement of fecal and urinary pH. Between each period, 15 days of rest were provided to the dogs so they could exercise. In the rest period, dogs were fed M diet.

Sample Procedure

To establish the beginning and the end of each period of feces and urine collection, a gelatin capsule containing 1 g of iron oxide (III) Fe_2O_3 was orally given to the dogs. Feces were collected for 5 days, every 3 h except night time (12 h), and scored as follows: 1 = very hard and dry stool; 2 = hard, dry, firm stool; 3 = soft, moist stool, well formed; 4 = soft and shapeless stool; and 5 = liquid stool, diarrhea. The fecal score analysis was conducted by a single trained person using the WALTHAM Feces Scoring System (20). After daily collection, feces were weighed and stored in a freezer at −20°C until the end of the trial to perform analysis. Total urine collection was performed daily in the morning and then stored in plastic bottles containing 1 g of thimol (Synth, Diadema, Brazil), and the pH was measured. The urine total volume was measured and kept in a freezer at −20°C until analysis. The fecal pH was measured immediately after collection using 2 g of fresh feces diluted in 20 mL of distilled water using a portable pH meter (Digimed DM-22, Campo Grande, Brazil).

TABLE 2 | Ingredients and chemical composition of experimental diets.

Ingredient, % as is	Treatments			
	M	MS	MHT	MSHT
Maize	56.6	28.4	56.6	28.7
Sorghum	–	26.7	–	26.2
Hydrolysable tannins	–	–	0.10	0.10
Wheat bran	10.0	14.0	10.0	14.0
Poultry byproducts meal	10.5	13.5	10.3	12.8
Bovine meat and bone meal	8.60	7.60	8.70	7.50
Poultry fat	5.20	4.60	5.20	4.70
Corn gluten 60% CP	5.00	1.10	5.00	1.90
Flaxseed	1.00	0.90	1.00	0.90
Digest[a]	1.50	1.50	1.50	1.50
Cellulose	0.90	1.00	0.90	1.00
Premix mineral/vitamin[b]	0.40	0.40	0.40	0.40
Salt	0.40	0.40	0.40	0.40
Potassium chloride	0.03	–	0.03	–
Analyzed chemical composition, %DM basis				
Dry matter	92.3	91.6	91.0	92.3
Starch	39.9	41.5	41.0	39.4
Crude protein	17.0	16.5	17.6	18.5
Acid hydrolyzed fat	9.25	8.54	9.33	8.09
Ash[c]	5.78	5.97	5.79	5.87
Crude fiber[c]	3.40	3.40	3.40	3.40
Total dietary fiber	21.4	21.3	20.7	23.1
Gross energy, kcal/kg	4,804	4,812	4,776	4,842
Gelatinization index of starch, %	87.9	93.1	97.2	84.9

M, maize; MS, maize + sorghum; MHT, maize + hydrolysable tannins; MSHT, maize + sorghum + hydrolysable tannins.

[a] *DTECH 8L, S.P.F. Argentina S.A., Argentina.*

[b] *Premix (supplied per kilogram of diet): vitamin A (10,800 UI), vitamin D3 (980 UI), vitamin E (60 mg), vitamin K3 (4.8 mg), vitamin B1 (8.1 mg), vitamin B2 (6.0 mg), vitamin B6 (6.0 mg), 12 vitamin (30 mcg), pantothenic acid (12 mg), niacin (60 mg), folic acid (0.8 mg), biotin (0.084 mg), manganese (7.5 mg), zinc (100 mg), iron (35 mg), copper (7.0 mg), cobalt (10 mg), iodine (1.5 mg), selenium (0.36 mg), choline (2.400 mg), taurine (100 mg), and antioxidant BHT (150 mg).*

[c] *Calculated values.*

Chemical Analysis

Stool from each dog was thawed, homogenized, and dried in forced-air oven at 55°C for 72 h, according to the recommendations of the Association of Official Analytical Chemists (21). Feces, sorghum, and diets were ground through a 1-mm screen in a Wiley hammer mill (DeLeo Equipamentos Laboratoriais, Porto Alegre, Brazil) and analyzed for dry matter (DM—AOAC 934.01), acid hydrolyzed fat (AHF—AOAC 954.02; model 170/3, Fanem, Saõ Paulo, Brazil), crude protein

TABLE 3 | Phenolic compounds present in ingredients and experimental diets.

Item	Total tannins[a]	Condensed tannins[b]	Total phenols[a]
Ingredients (raw material)			
Maize	2.98	0.01	5.73
Sorghum	18.4	30.7	27.5
Wheat bran	2.96	ND	5.69
Diets (after extrusion)			
M	2.38 (1.87)	0.10 (0.01)	4.43 (3.60)
MS	2.50 (5.85)	0.40 (7.76)	4.34 (9.27)
MHT	2.50 (1.90)	0.09 (0.01)	4.57 (3.66)
MSHT	2.94 (5.73)	0.78 (7.56)	5.12 (9.08)

ND, not detected; M, maize; MS, maize + sorghum; MHT, maize + hydrolysable tannins; MSHT, maize + sorghum + hydrolysable tannins.
[a]Values are expressed as g of tannic acid^{-1} kg DM.
[b]Values are expressed as g of leucocyanidin kg^{-1} DM.
Calculated values in parentheses based on the content of each ingredient, in % DM basis.

(CP—AOAC 954.01; model TE 036/2, Tecnal, Piracicaba, Brazil), crude fiber (CF—AOAC 962.10; model MA 450/8, Marconi, Piracicaba, Brazil), and ash (21). Diets and sorghum were analyzed for total dietary fiber, according to Prosky et al. (22), and starch, according to Karkalas (23). The model for the gelatinization index of starch was:

$$Gelatinization\ \ index\ (\%) = \frac{(total\ starch - resistant\ starch)}{(total\ starch)} \times 100$$

Urine samples were thawed and homogenized, and 150-mL aliquots were lyophilized (Micromodulyi-Fis; Thermo Fisher Scientifics Inc., Maryland, USA) for analysis of DM and gross energy (GE). Another 50-mL aliquot was collected for analysis of CP. Dietary, fecal, and urinary GE were determined using isoperibolic bomb calorimetry (calorimeter model C2000 basic, Ika-werke, Staufen, Germany). All analyses were performed in duplicate, assuming a coefficient of variation <1% for energy and <5% for the other analyzes. Ingredients, diets (**Table 3**), and feces were analyzed for phenolic compounds by gravimetric tests; total phenols and tannins were measured according to Makkar et al. (24) and CT based on Porter et al. (25). Based on the content of total tannins, condensed tannins, and total phenols in maize, sorghum, and wheat bran, an estimated value was calculated for each of the experimental diets (**Table 3**) in order to compare with the results obtained with the gravimetric tests.

Statistical Analyses

Data were analyzed using the ANOVA procedure of SAS 9.4 (SAS Inst. Inc., Cary, NC). Means were compared using Tukey's test at 5% probability ($P < 0.05$). The $P < 0.10$ was considered a tendency.

Experiment 2: Postprandial Glycemia Assay

The dogs and the dietary treatments were the same as previously described for the digestibility assay.

Experimental Design

The dogs were adapted to the experimental diets for 11 days, then were fasted for 12 h inside the metabolic cages before starting the first blood collection. Immediately before starting the experiment, the cephalic vein was cannulated with a catheter BD ANGIOCATH 22″ (Becton, Dickinson and Company do Brasil, Curitiba, Brazil). Then, 1 mL of blood was collected in a tube containing 0.05 mL of sodium fluoride (LABTEST, Lagoa Santa, Brazil); this sample was used to determine the baseline glycemia at time 0. Dogs were fed, and dietary consumption was performed within 5 min. Sequential collections were started over 8 h after total consumption, at 5, 10, 15, 30, 45, 60, 90, 120, 180, 240, 300, 360, 420, and 480 min after food consumption. After each collection, the catheter was washed out with heparinized solution, and before each new collection, about 0.3 mL of blood was discarded.

Chemical Analyses

The tubes were centrifuged at 3,000 *g* for 10 min, and plasma was transferred to 1.5-mL Eppendorf tubes, cooled between 2 and 4°C, and analyzed in sequence. Blood glucose was analyzed by the enzymatic colorimetric method according to the manufacturer's instructions (Wiener Lab Group, Rosário, Argentina). All samples were analyzed in duplicate.

Statistical Analyses

The results were analyzed using repeated measures of variance on SAS 9.4 (SAS Inst. Inc., Cary, NC). The area under the curve (AUC), basal, maximum, average, and minimum glycemia, and maximum glycemic increase was calculated, and the means of each treatment were compared by Tukey's test ($P < 0.05$).

RESULTS

All experimental diets were well-consumed by the dogs without refusals.

There was a reduction in the phenolic compounds content, mainly in the diets with sorghum, MS and MSHT, after the extrusion (**Table 3**).

Dogs fed the diet containing both types of tannins together, CT and HT, consumed more phenol and tannins ($P < 0.05$) (**Table 4**). Phenolic compounds have not been found in feces of dogs fed the diets with HT, MHT, and MSHT, thus leading to the 100% apparent digestibility coefficients shown in **Table 4**.

Dogs fed the experimental diets containing sorghum consumed less fat daily ($P = 0.0009$) (**Table 5**). The addition of sorghum containing CT or HT to the dietary treatments did not promote any differences in the coefficients of digestibility of nutrients and energy ($P > 0.05$).

No differences were observed in the fecal and urinary characteristics analyzed ($P > 0.05$), except a tendency of diets with sorghum to increase fecal pH ($P = 0.0948$) (**Table 6**). Feces remained with adequate characteristics such as hard, dry, and firm stool. Dogs fed diets containing sorghum and HT had darkened feces and urine.

The glycemic items evaluated, e.g., area under the curve, basal, maximum, average, and minimum glycemia, and maximum

TABLE 4 | Phenolic compounds intake and digestibility of dogs fed the experimental diets.

Item	Treatments				*P*-value	SEM
	M	MS	MHT	MSHT		
Daily phenolic intake, g/day						
Total phenols[1]	9.69[b]	8.97[b]	9.79[ab]	10.9[a]	0.0124	0.74
Total tannins[1]	5.21[b]	5.17[b]	5.35[b]	6.28[a]	0.0029	0.41
Condensed tannins[2]	0.22[c]	0.83[b]	0.19[c]	1.67[a]	<0.0001	0.08
Apparent total tract digestibility, %						
Total phenols[1]	95.2[b]	96.4[b]	100[a]	100[a]	<0.0001	0.30
Total tannins[1]	94.9[b]	95.3[b]	100[a]	100[a]	<0.0001	0.29
Condensed tannins[2]	97.3[c]	98.2[b]	100[a]	100[a]	<0.0001	0.28

M, maize; MS, maize + sorghum; MHT, maize + hydrolysable tannins; MSHT, maize + sorghum + hydrolysable tannins; SEM, standard error of the mean.
[1]*Values are expressed as g of tannic acid^{-1} kg DM.*
[2]*Values are expressed as g of leucocyanidin kg^{-1} DM.*
[a,b,c]*Means in the same row with different lowercase letters are significantly different ($P < 0.05$).*

TABLE 5 | Nutrient intake and digestibility of nutrients and energy of dogs fed the experimental diets.

Item	Treatments				*P* value	SEM
	M	MS	MHT	MSHT		
Daily nutrient intake, g/day						
DM	219	207	214	213	0.6606	15.5
OM	206	194	202	201	0.6344	14.6
AHF	20.2[a]	17.7[b]	20.0[a]	17.3[b]	0.0009	1.35
CP	37.2	34.1	37.7	39.4	0.0853	2.75
CF	7.44	7.03	7.28	7.26	0.6578	0.53
NFE	149	143	144	144	0.7376	10.5
Ash	12.6	12.3	12.4	12.5	0.9387	0.90
GE, kcal/day	1,051	995	1,023	1,034	0.7761	74.7
ME, kcal/day	164	158	164	160	0.5495	12.0
Apparent total tract digestibility, %						
DM	78.7	80.6	81.4	78.2	0.1363	2.81
OM	82.0	83.4	83.4	81.7	0.3642	2.02
AHF	85.8	86.2	88.3	83.2	0.0766	3.00
CP	78.1	78.4	82.0	79.5	0.1612	3.15
NFE	82.5	84.2	84.7	82.1	0.1457	2.41
GE	81.6	83.0	83.9	81.3	0.4170	2.05
Nutritional value, kcal/kg						
ME	3,755	3,831	3,834	3,755	0.3366	110

M, maize; MS, maize + sorghum; MHT, maize + hydrolysable tannins; MSHT, maize + sorghum + hydrolysable tannins; SEM, standard error of the mean; DM, dry matter; OM, organic matter; AHF, acid hydrolyzed fat; CP, crude protein; CF, crude fiber; NFE, nitrogen-free extractive; GE, gross energy; ME, metabolizable energy.
[a,b]*Means in the same row with different lowercase letters are significantly different ($P < 0.05$).*

glycemic increase, did not differ between the dietary treatments ($P > 0.05$) (**Table 7**). The average postprandial glycemic curves for the dietary treatments are illustrated in **Figure 1**.

DISCUSSION

Functional ingredients are highly pursued and evaluated in both human and animal nutrition. The main reason is that, besides nutritional value, these ingredients contain compound with metabolic effects, either promoting satiety or controlling the peak of glycemia after a meal, for example. The search for ingredients with these characteristics is valuable for pet food production, since obesity rates have increased over the years accompanied by associated diseases, such as diabetes mellitus. Based on previous evidence that sorghum containing tannins may interfere with digestive enzyme activity (26), we aimed to investigate the effects of condensed tannins, naturally present

TABLE 6 | Fecal and urinary characteristics of dogs fed the experimental diets.

Item	Treatments				*P*-value	SEM
	M	MS	MHT	MSHT		
Fecal characteristics						
DM, %	33.5	34.3	35.8	35.6	0.3263	2.71
Output, g/day	141	118	110	131	0.2043	21.8
Output, g/day (DM)	46.7	40.2	39.5	46.7	0.2794	7.46
Fecal score[1]	2.23	2.12	2.02	2.01	0.3064	0.24
pH	6.34[b]	6.44[a]	6.34[b]	6.54[a]	0.0948	0.17
Urinary characteristics						
Volume, mL/day[2]	261	293	254	293	0.7620	99.9
pH	7.24	7.36	7.18	7.21	0.4611	0.29

M, maize; MS, maize + sorghum; MHT, maize + hydrolysable tannins; MSHT, maize + sorghum + hydrolysable tannins; SEM, standard error of the mean; DM, dry matter.
[1]*Scored as: 1 = very hard and dry stool, 2 = hard, dry, firm stool, 3 = soft, moist stool, well formed, 4 = soft and shapeless stool, 5 = liquid stool, diarrhea.*
[2]*Mean volume produced in 5 days.*
[a,b]*Means in the same row with different lowercase letters are significantly different ($P < 0.10$).*

TABLE 7 | Area under the curve without basal glycemic area (AUC), plasma basal glucose concentration, plasma glucose concentration, and values of the glycemic peak of dogs fed the experimental diets.

Item	Treatments				*P*-value	SEM
	M	MS	MHT	MSHT		
AUC total (0–480) mg/dl min	39,628	38,633	40,585	38,884	0.7211	3,168
Basal glycemia, mg/dl	82.0	80.3	83.3	81.3	0.9621	10.0
Maximum glycemia, mg/dl	95.5	97.0	101	96.0	0.8122	11.5
Average glycemia, mg/dl	82.7	80.2	83.8	82.0	0.8606	7.51
Minimum glycemia, mg/dl	70.0	66.7	69.7	69.5	0.5915	4.68
Maximum glycemic increase, mg/dl	13.5	16.7	18.0	14.7	0.8621	9.44

M, maize; MS, maize + sorghum; MHT, maize + hydrolysable tannins; MSHT, maize + sorghum + hydrolysable tannins; SEM, standard error of the mean; AUC, area under the curve.

in sorghum, added to hydrolysable tannins on digestibility and whether these phenolic compounds could modulate postprandial glycemia, as it can affect absorption.

The inclusion of tannins may affect the palatability. The intake of tannins is related to a sensation known as astringency, caused by the interaction between salivary proline-rich protein, mucosal epithelium of the oral cavity, and tannins and characterized by the formation of complexes and precipitates, decreasing the saliva's lubricity and resulting in dryness of the mouth (9). However, Mole et al. (27) noted that dogs and cats produced small amounts of salivary proline-rich proteins, and they did not have tannin affinity *in vitro*. This may explain the good acceptance and voluntary intake of the experimental diets formulated to contain condensed and hydrolysable tannins (MS, MHT, and MSHT).

Phenolic compounds intake and metabolism in this study indicate that these molecules disappeared after extrusion, since after the thermal process, only a small content of total tannins and phenols and condensed tannins was detected by the method used. This fact was highlighted after a comparison between the estimated values for the diets and the values obtained by the analytical method, in which the negative impact of extrusion on tannins and phenols detection was found. The amount of phenolic compounds in all diets was similar after extrusion despite the presence of sorghum and HT. In fact, phenolic compounds can be thermally sensitive and are highly disposed to degradation during the heating process (28). Phenolic compounds exist both in free, extractable by solvent solutions, and bound, covalently bound with cell wall components and non-soluble in organic solvents, form mainly in carbohydrates requiring acid or alkaline hydrolysis prior to the extraction. Since approximately 80% of the phenolic compound present in sorghum is the bound form, this cereal requires treatment to increase accessibility and perform its bioactive functions (29). According to Masisi et al. (30), the biological potential of phenolic compounds depends on their bioaccessibility, absorption in the gastrointestinal tract, and their bioavailability *in vivo*. Therefore, it is indispensable to select the most appropriate heat treatment that minimizes potential losses in order to guarantee the bioactive responses.

The phenolic compounds content can be affected negatively by heat treatment according to the genotype of sorghum, mainly due to differences in flavonoid composition, and in general, extrusion is more deleterious than other types of cooking (31). On the other hand, extrusion decreased total

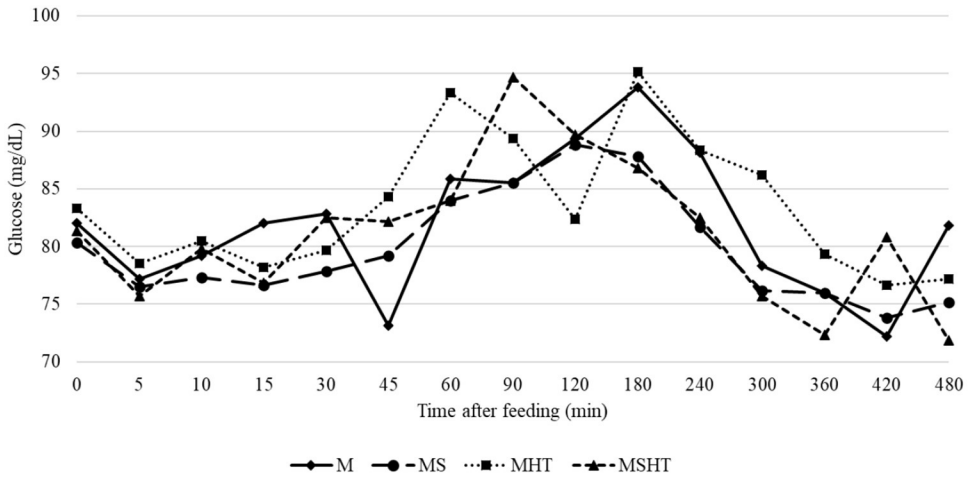

FIGURE 1 | Average postprandial glycemic curves of dogs fed the experimental diets (mg/dl). M, maize; MS, maize + sorghum; MHT, maize + hydrolysable tannins; MSHT, maize + sorghum + hydrolysable tannins.

proanthocyanidins and their oligomers and polymers, possible due to the complexation of proanthocyanidins with other molecules, and increased the amount of proanthocyanidins monomers and dimers, suggesting that this heat treatment helps the cleavage of high molecular weight compounds into lower molecular weight (31, 32). The increasing in monomer and dimer proanthocyanidins is important since they have greater bioavailability and effectiveness on oxidative stress (33). However, sorghum functional effects as antioxidant are attributed to the oligomers (34).

In this study, we selected sorghum containing CT and replaced maize with sorghum in order to improve the amount of CT in the diets. In addition, we used an HT obtained in the form of purified powder to easily incorporate in the formulation. However, due to its structure as esters, HTs are rapidly hydrolyzed in gallic acid or hexahydroxydiphenic acid and the parent polyol (35). In opposite, although CT can be degraded to anthocyanidins, under mild or anaerobic conditions, it remains stable (25). Based on our results, it is possible that extrusion modified total proanthocyanidins and their oligomers and polymers into other conjugated forms that were not possible to detect. Additionally, the lack of standardized and commercial methods makes the identification and quantification of HT and its metabolites difficult. Specific analytical methods are needed in order to allow the correct quantification to evaluate its effects *in vivo* (9). However, processing must be an important part of the disappearance of CT. The consumption of CT in the MS diet was ∼50% of the consumption in diet MSHT, which was not expected. Adding these results to those of starch digestibility, it is possible to observe that MSHT presents a starch gelatinization index of 10% lower than those found in the MS diet. Considering that the diets were milled in the same place, the cooking processing must be the probable source of this variation. Therefore, heat and pressure can have some impact on the CT.

In the current study, no negative effects on digestibility were detected by the addition of sorghum containing CT or with the supplementation of HT. Similar results were obtained by Carciofi et al. (11), who did not find differences in nutrient intake and digestibility between dogs fed diets containing maize or sorghum. In addition, Kore et al. (36) did not observe differences in intake and digestibility of nutrients in dogs fed diets containing maize or sorghum when evaluating alternative cereals to rice. However, Twomey et al. (14) demonstrated that the inclusion of sorghum on dog diet reduced the digestibility coefficients of protein, dry matter, and gross energy compared to dogs fed maize-based diet. In addition, Murray et al. (13) did not found differences in ileal digestibility of nutrients between diets based on maize or sorghum, but the total tract digestibility of dry matter, organic matter, and crude protein were decreased in dogs fed the sorghum diet. This occurs probably because tannins, especially proanthocyanidins, have a property of forming complexes and inhibiting activity of enzymes, such as pectinase, amylase, lipase, protease, and β-galactosidase, impairing absorption of proteins and carbohydrates (37).

The comparison of alternative cereals, such as sorghum, with cereals containing a large amount of available starch leads to differences in the digestibility coefficients of nutrients and energy. This was evidenced in several studies comparing rice vs. sorghum and maize and may be attributed to structural differences in the starch–protein matrix of these cereals that, according to Hoseney (38), forms very strong bonds between the protein and starch components of maize and sorghum, which finally results in starch resistant to digestion (39). Teixeira et al. (15) observed impairing of apparent digestibility of dry matter, organic matter, crude protein, and fat, as well as on digestible and metabolizable energy of dogs fed diets with partial substitution of rice by sorghum.

In fact, the appropriate processing of cereals includes grinding to a particle size, known as mean geometric diameter (MGD), which is directly associated to starch gelatinization during extrusion. As reported by Bazolli et al. (12), smaller particle sizes allowed higher starch gelatinization and digestibility for diets based on maize and sorghum compared to coarsely ground

maize and sorghum. Thus, correct grinding and extrusion at high temperatures allows to reduce the resistant starch present on maize and sorghum, improving its availability. All four experimental diets had starch gelatinization degree above 80%, which indicates adequate cooking during the extrusion.

Neither fecal or urinary characteristics were altered by the consumption of diets containing sorghum and HT. Due to its capacity to form complexes with enzymes, thus reducing the digestibility of nutrients, it was expected that the addition of sorghum promoted an increase in the fecal volume and a decrease in the DM of feces. In line with our findings, several studies have also found no differences between dogs fed diets containing maize and sorghum regarding fecal characteristics, such as fecal score, fecal pH, and fecal DM (11, 13, 14), with fecal score remaining as hard, dry, and formed stool as how we classified the stools in the present study. Down et al. (40) demonstrated that wild rodents consuming diets containing CT produced a more alkaline urine. This evidence probably could be found if we completely replaced maize with sorghum. There was a tendency to observe higher pH in dogs fed diets containing sorghum. The only aspect that differed in dogs fed diets containing CT or HT from the dogs fed the M diet was the darker coloration of feces and urine produced by dogs fed diets MS, MHT, and MSHT. It indicates that these phenolic compounds are absorbed by dogs, and their components are excreted in both feces and urine.

Finally, the glycemic parameters evaluated in this study did not change as we expected. Based on the results obtained by Carciofi et al. (11), in which dogs fed diets containing sorghum presented higher later meal response, characterized by a long and flat glycemic area under the curve, we hypothesized that dogs consuming diets with sorghum containing CT and HT would present a different response over the postprandial glycemia. The variety of sorghum selected to formulate our diets was considered as a high tannin grain (4.8% of polyphenol tannins). The contrasting results may be explained by the differences in the diets' chemical composition, including type of starch and granule structure and the fiber content. On the study conducted by Carciofi et al. (11), they replaced completely the source of starch by sorghum, and the levels of fiber, especially total dietary fiber (TDF), were not maintained similar between the diets, in which the maize-based diet had 9.4% of TDF while the sorghum diet had 14.1% of TDF. In the current study, the experimental diets were formulated to be isonutritive, so the levels of TDF were kept closer (M = 21.4%, MS = 21.3%, MHT = 20.7%, and MSHT = 23.1% of TDF). The total fiber could play a greater role in the digestion and glycemia compared to the tannins.

According to Clifford and Scalbert (41), compared to CT, the HT ones interact with proteins and form less stable bonds that can be metabolized by the intestinal microbiota, making them more soluble. Additionally, HT are absorbed mainly in the small intestine and less fermented in the colon (42). Thus, supplementation with HT in the current study may have been completely metabolized and, thus, not enough to promote some modulation in the glycemic response.

Based on the results obtained in this study and previously, many factors are associated with the modulation of the postprandial glycemic response in dogs. The use of sorghum, containing CT, and the purified HT extract did not negatively impact the digestibility of nutrients and energy and the fecal and urinary characteristics evaluated as previously reported in some studies. In fact, extrusion appears to have some impact on phenolic compounds that are not recovered by the same analytical method. Thus, the phenolic compounds could not be related to any interference in the evaluated responses since they were not detected in the same amount supplied in the diets before extrusion. The partial substitution of maize for sorghum and the addition of HT do not interfere in the postprandial glycemia response in adult dogs. Maize and sorghum are capable of providing similar amounts of nutrients to dogs. The substitution of 50% of maize for sorghum can be a practice applicable in diets without losing the nutritional value of the final diet.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by The Institutional Animal Care and Use Committee at the Universidade Federal do Rio Grande do Sul, protocol number 26275.

AUTHOR CONTRIBUTIONS

LTe, LTr, and AK designed the study. LTr acquired the grant and provided logistical support and graduate student mentorship. LTe, LTr, and GM conducted the animal trial and sample collection. CP conducted statistical analysis. CP, LTe, and LTr wrote the manuscript. All authors have read and approved the manuscript.

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REFERENCES

1. Crane SW, Griffin RW, Messent PR. Introduction to commercial pet foods. In: Hand MS, Thatcher CD, Remillard RL, Roudebush P, Lewis LD, editors. *Small Animal Clinical Nutrition*. 4th ed. Topeca, KS: Mark Morris Institute (2000). p. 111–26.
2. Brunetto MA, Sá FC, Nogueira SP, Gomes MDOS, Pinarel AG, Jeremias JT, et al. The intravenous glucose tolerance and postprandial glucose tests may present different responses in the evaluation of obese dogs. *Br J Nutr*. (2011) 106:S194–S7. doi: 10.1017/S0007114511000870
3. Laflamme DP. Companion animals symposium: obesity in dogs and cats: what is wrong with being fat?. *J Anim Sci*. (2012) 90:1653–62. doi: 10.2527/jas.2011-4571
4. Huang QQ, Liu XL, Zhao GQ, Hu TM, Wang YX. Potential and challenges of tannins as an alternative to in-feed antibiotics for farm animal production. *Anim Nutr*. (2018) 4:137–50. doi: 10.1016/j.aninu.2017.09.004
5. Costa CTC, Bevilaqua CML, Morais SM, Vieira LS. Taninos e sua utilização em pequenos ruminantes. *Rev Bras Pl Med*. (2008) 10:108–16.
6. Etuk EB, Okeudo NJ, Esonu BO, Udedibie ABI. Antinutritional factors in sorghum: chemistry, mode of action and effects on livestock and poultry. *Online J Anim Feed Res*. (2012) 2:113–9.
7. Dicko MH, Gruppen H, Traoré AS, Voragen AG, Van Berkel WJ. Sorghum grain as human food in Africa: relevance of content of starch and amylase activities. *Afr J Biotechnol*. (2006) 6:384–95.
8. Dykes L, Rooney LW. Sorghum and millet phenols and antioxidants. *J Cereal Sci*. (2006) 44:236–51. doi: 10.1016/j.jcs.2006.06.007
9. Arapitsas P. Hydrolyzable tannin analysis in food. *Food Chem*. (2012) 135:1708–17. doi: 10.1016/j.foodchem.2012.05.096
10. Kumari M, Jain S. Tannin: an antinutrient with positive effect to manage diabetes. *Res J Recent Sci*. (2012) 1:4.
11. Carciofi AC, Takakura FS, De-Oliveira LD, Teshima E, Jeremias JT, Brunetto MA, et al. Effects of six carbohydrate sources on dog diet digestibility and post-prandial glucose and insulin response. *J Anim Physiol Anim Nutr*. (2008) 92:326–36. doi: 10.1111/j.1439-0396.2007.00794.x
12. Bazolli RS, Vasconcellos RS, De-Oliveira LD, Sá FC, Pereira GT, Carciofi AC. Effect of the particle size of maize, rice, and sorghum in extruded diets for dogs on starch gelatinization, digestibility, and the fecal concentration of fermentation products. *J Anim Sci*. (2015) 93:2956–66. doi: 10.2527/jas.2014-8409
13. Murray SM, Fahey GC, Merchen NR, Sunvold GD, Reinhart GA. Evaluation of selected high-starch flours as ingredients in canine diets. *J Anim Sci*. (1999) 77:2180–6. doi: 10.2527/1999.7782180x
14. Twomey LN, Pluske JR, Rowe JB, Choct M, Brown W, Pethich DW. The replacement value of sorghum and maize with or without supplemental enzymes for rice in extruded dog foods. *Anim Feed Sci Techol*. (2003) 108:1–4. doi: 10.1016/S0377-8401(03)00168-8
15. Teixeira L, Pinto CFD, Kessler AdeM, Trevizan L. Effect of partial substitution of rice with sorghum and inclusion of hydrolyzable tannins on digestibility and postprandial glycemia in adult dogs. *PLoS ONE*. (2019) 14:e0208869. doi: 10.1371/journal.pone.0208869
16. Laflamme D. Development and validation of a body condition score system for dogs: a clinical tool. *Canine Pract*. (1997) 22:10–5.
17. National R.esearch Council. *Nutrient Requirements of Dogs and Cats*. Washington, DC: The National Academy Press (2006).
18. Ai M, Li K, Liu S, Lin DKJ. Balanced incomplete Latin square designs. *J Stat Plan Inference*. (2013) 143:1575–82. doi: 10.1016/j.jspi.2013.05.001
19. Association of American Feed Control Officials. *Association of American Feed Control Officials Official Publication*. Oxford, IN: Association of American Feed Control Officials (2019).
20. Moxham G. Waltham feces scoring system–A tool for veterinarians and pet owners: How does your pet rate? *WALTHAM Focus*. (2001) 11:24–5.
21. Association of Official Analytical Chemists (AOAC). *Official Methods of Analysis*. 16th ed. Arlington, USA: Association of Official Analytical Chemists (1995).
22. Prosky L, Asp NG, Schweizer TF, DeVries JW, Furda I. Determination of insoluble and soluble dietary fiber in foods and foods products: collaborative study. *J Assoc Off Anal Chem*. (1992) 75:360–7. doi: 10.1093/jaoac/75.2.360
23. Karkalas J. An improved enzymic method for the determination of native and modified starch. *J Sci Food Agri*. (1985) 36:1019–27. doi: 10.1002/jsfa.2740361018
24. Makkar HPS, Blummel M, Borowy NK, Becker K. Gravimetric determination of tannins and their correlation with chemical and protein precipitation methods. *J Sci Food Agric*. (1993) 61:161–5. doi: 10.1002/jsfa.2740610205
25. Porter LJ, Hrstich LN, Chan BC. The conversion of cyanidins and prodelphinidins to cyanidin and delphinidin. *Phytochemistry*. (1986) 25:223–30. doi: 10.1016/S0031-9422(00)94533-3
26. Nyamambi B, Ndlovu LR, Read JS, Reed JD. The effects of sorghum proanthocyanidins on digestive enzyme activity in vitro and in the digestive tract of chicken. *J Sci Food Agric*. (2000) 80:2223–31. doi: 10.1002/1097-0010(200012)80:15<2223::AID-JSFA768>3.0.CO;2-I
27. Mole S, Butler LG, Iason G. Defense against dietary tannin in herbivores: a survey for proline rich salivary proteins in mammals. *Biochem Syst Ecol*. (1990) 18:287–93. doi: 10.1016/0305-1978(90)90073-O
28. Zhang R, Khan SA, Chi J, Wei Z, Zhang Y, Deng Y, et al. Different effects of extrusion on the phenolic profiles and antioxidant activity in milled fractions of brown rice. *LWT*. (2018) 88:64–70. doi: 10.1016/j.lwt.2017.09.042
29. Acosta-Estrada BA, Gutiérrez-Uribe JA, Serna-Saldívar SO. Bound phenolics in foods, a review. *Food Chem*. (2014) 152:46–55. doi: 10.1016/j.foodchem.2013.11.093
30. Masisi K, Beta T, Moghadasian MH. Antioxidant properties of diverse cereal grains: a review on in vitro and in vivo studies. *Food Chem*. (2016) 196:90–9. doi: 10.1016/j.foodchem.2015.09.021
31. Cardoso LdeM, Pinheiro SS, de Carvalho CWP, Queiroz VAV, de Menezes CB, Moreira AVB, et al. Phenolic compounds profile in sorghum processed by extrusion cooking and dry heat in a conventional oven. *J Cereal Sci*. (2015) 65:220–6. doi: 10.1016/j.jcs.2015.06.015
32. Awika JM, Dykes L, Gu L, Rooney LW, Prior RL. Processing of sorghum (Sorghum bicolor) and sorghum products alters procyanidin oligomer and polymer distribution and content. *J Agric Food Chem*. (2003) 51:5516–21. doi: 10.1021/jf0343128
33. Cardoso LdeM, Pinheiro SS, Martino HSD, Pinheiro-Sant'Ana HM. Sorghum (Sorghum bicolor L.): nutrients, bioactive compounds, and potential impact on human health. *Crit Rev Food Sci Nutr*. (2017) 57:372–90. doi: 10.1080/10408398.2014.887057
34. Beecher GR. Proanthocyanidins: biological activities associated with human health. *Pharm Biol*. (2004) 42:2–20. doi: 10.3109/13880200490893474
35. Haslam E. Vegetable tannins. In: Swain T, Harborne JB, VanSumere CF, editors. *Biochemistry of Plants Phenolics (Recent Advances in Phytochemistry)*. NY: Plenum Press (1979). p. 475–523. doi: 10.1007/978-1-4684-3372-2_15
36. Kore KB, Pattanaik AK, Sharma AdK. Evaluation of alternative cereal sources in dog diets: effect on nutrient utilization and hindgut fermentation characteristics. *J Sci Food Agric*. (2009) 89:2174–80. doi: 10.1002/jsfa.3698
37. Smeriglio A, Barreca D, Bellocco E, Trombetta D. Proanthocyanidins and hydrolysable tannins: occurrence, dietary intake and pharmacological effects. *Br J Pharmacol*. (2017) 174:1244–62. doi: 10.1111/bph.13630
38. Hoseney RC. Minor constituents of cereals. In: *Principles of Cereal Science and Technology*, 2nd ed. St. Paul, MN: American Association of Cereal Chemists (1994). p. 81–101.
39. Rowe JB, Choct M, Pethick DW. Processing cereal grains for animal feeding. *Aust J Agric Res*. (1999) 50:721–36. doi: 10.1071/AR98163
40. Downs CT, McDonald PM, Brown K, Ward D. Effects of Acacia condensed tannins on urinary parameters, body mass, and diet choice of an Acacia specialist rodent, Thallomys nigricauda. *J Chem Ecol*. (2003) 29:845–58. doi: 10.1023/a:1022975531372
41. Clifford M, Scalbert A. Ellagitannins—nature, occurrence and dietary burden. *J Sci Food Agric*. (2000) 80:1118–25. doi: 10.1002/(SICI)1097-0010(20000515)80:7<1118::AID-JSFA570>3.0.CO;2-9

42. Serrano J, Puupponen- Pimiä R, Dauer A, Aura AM, Saura-Calixto F. Tannins: current knowledge of food sources, intake, bioavailability and biological effects. *Mol Nutr Food Res*. (2009) 53:S310–29. doi: 10.1002/mnfr.200900039

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Use of Legumes and Yeast as Novel Dietary Protein Sources in Extruded Canine Diets

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The popularity of plant-based protein sources has increased as consumer demand for grain-free and novel protein sources increase. Minimal research has been conducted as regards to use of legumes and yeast and their effects on acceptability and digestibility in canine diets. The objective of this study was to evaluate macronutrient apparent total tract digestibility (ATTD), gastrointestinal tolerance, and fermentative end-products in extruded, canine diets. Five diets were formulated to be isocaloric and isonitrogenous with either garbanzo beans (GBD), green lentils (GLD), peanut flour (PFD), dried yeast (DYD), or poultry by-product meal (CON) as the primary protein sources. Ten adult, intact, female beagles (mean age: 4.2 ± 1.1 yr, mean weight: 11.9 ± 1.3 kg) were used in a replicated, 5 × 5 Latin square design with 14 d periods. Each experimental period consisted of 10 d of diet adaptation, followed by 4 d of total fecal and urine collection. A fasted, 5 ml blood sample was collected at the end of each period and analyzed for serum metabolites and complete blood count. Serum metabolites were within normal ranges and all dogs remained healthy throughout the study. Fecal quality, evaluated on a 5-point scale, was considered ideal. Macronutrient ATTD was similar among dietary treatments, with diets highly digestible (>80%). Total fecal branched-chain fatty acid concentrations were highest ($P < 0.05$) for DYD (23.4 μmol/g) than GLD (16.1 μmol/g) and PFD (16.0 μmol/g) but not different ($P > 0.05$) than other treatments. The plant-based protein treatments had greater ($P < 0.05$) total fecal short chain fatty acid (SCFA) concentrations (average 627.6 μmol/g) compared with CON (381.1 μmol/g). Fecal butyrate concentration was highest ($P < 0.05$) for DYD than all other dietary treatments (103.9 μmol/g vs. average 46.2 μmol/g). Fecal microbial communities showed *Firmicutes*, *Bacteroidetes*, *Fusobacteria*, and *Proteobacteria* as abundant phyla. There was greater β-diversity for dogs fed DYD which differed from all other diets in both weighted and unweighted UNIFRAC analyses. Inclusion of these novel, plant-based, protein sources showed no detrimental effects on nutrient digestibility or fecal characteristics and represent viable protein sources in canine diets that can produce beneficial shifts in fecal metabolites.

Keywords: dog, digestibility, legume, microbiota, pulse, yeast

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INTRODUCTION

Recent trends in the pet food industry have centered on providing high-protein diets that contain novel protein sources. Legumes, pulses, and yeast, have been identified as alternatives to traditional protein sources (i.e., animal-derived protein) to satisfy consumer demand for non-traditional diets, such as grain-free, while simultaneously meeting the nutritional requirements of dogs.

The inclusion of legumes and pulses (crops in the *Leguminosae* family harvested for the dry grain) can negatively impact digestibility and fecal quality due to presence of anti-nutritional factors and oligosaccharides (1, 2). However, they are protein-rich ingredients that are easily incorporated into pet diets because of their low lipid content (3, 4). Yeast (*Saccharomyces cerevisiae*) products have shown beneficial health effects in several animal models including the modulatory effects of colonic microbiota in dogs (5–7).

Minimal research has been conducted as regards to the effects on acceptability, digestibility, and fecal characteristics of legumes and yeast when used as main protein sources in extruded canine diets. Therefore, the objectives of this study were to evaluate the effects of extruded canine diets containing green lentils (GLD), garbanzo beans (GBD), peanut flour (PFD), or dried yeast (DYD) on ATTD, gastrointestinal tolerance, fermentative end-products, and fecal microbiota populations. It was hypothesized that extruded diets containing legumes or yeast would result in similar macronutrient digestibility to poultry by-product meal (CON), as most of the anti-nutritional factors would be deactivated during thermal processing. Greater saccharolytic fermentation was expected in dogs fed the legume or yeast containing-diets due to their intrinsically greater dietary fiber content and composition.

MATERIALS AND METHODS

Experimental Design

All animal care protocols used in this study were approved by the Institutional Animal Care and Use Committee at the University of Illinois at Urbana-Champaign. All methods were performed in accordance with the United States Public Health Service Policy on Humane Care and Use of Laboratory Animals.

Ten adult, intact, female Beagles (average age: 5.0 ± 1.2 yr, average weight: 11.9 ± 1.3 kg, average body condition score: 5.9 on a 9-point scale) were used in a replicated 5 × 5 Latin square design. Each 14 d experimental period consisted of a 10 d diet adaptation phase, followed by a 4 d total fecal and urine collection phase. On day 14 of each experimental period, a 5 ml, fasted, blood sample was collected from all dogs for a serum chemistry and complete blood count (**CBC**) analyses conducted at the University of Illinois Veterinary Medicine Diagnostics Laboratory (Urbana, IL). The dogs were housed in a temperature-controlled room at the Veterinary Medicine Basic Sciences Building with a 14 h light/10 h dark schedule. The dogs were individually housed in kennels (1.2 m wide by 1.8 m long) during the diet adaptation phases which permitted nose to nose contact with dogs in adjacent runs and visual contact with all dogs in the room. During collection phases, the dogs were individually housed in metabolic crates. The dogs were fed twice daily (8:00 and 16:00) with *ad libitum* access to water throughout the study.

Dogs were randomly assigned to one of five diets formulated with either garbanzo beans (GBD), green lentils (GLD), peanut flour (PFD), a dried yeast product (DYD), or poultry by-product meal (CON) as the primary protein source (**Table 1**). The legumes and yeast were included at the expense of poultry by-product meal and rice to provide test diets with similar nutrient composition and energy content. All diets were formulated to be complete and balanced according to AAFCO (8) recommended values for adult dogs at maintenance. Diets were extruded by Wenger Manufacturing (Wenger Manufacturing, Inc., Sabetha, KS). Extrusion processing parameters (**Supplementary Table 1**) were adjusted as needed to ensure uniformity of the final product characteristics (e.g., density, texture, and kibble size). Food intake was individually calculated to maintain body weight based on metabolizable energy requirements. Any food refusals were measured after each meal throughout the duration of the study. Body weight and body condition were measured weekly and food intake was adjusted accordingly during the adaptation phase to maintain body weight, if necessary.

Sample Collection and Preparation

A fresh fecal sample was collected from each dog within 15 min of defecation and analyzed for pH, dry matter (DM), short-chain fatty acids (SCFA), branched-chain fatty acids (BCFA), ammonia, phenols, indoles, and microbiota. All fecal samples were assessed for fecal quality using 5-point scoring system: 1 = hard, dry pellets; 2 = hard formed, remains firm and soft, 3 = soft, formed and moist stool; 4 = soft, unformed stool; or 5 = watery, liquid that can be poured. Dry matter was analyzed in 2 g duplicates and dried for 48 h in a 105°C forced-air oven. For analysis of BCFA, SCFA, and ammonia, 5 g of feces were collected in a 30 ml Nalgene bottle containing 5 ml of 2 N hydrochloric acid. Phenols/indoles were collected by weighing 2 g of feces into duplicate plastic tubes and covered in Parafilm. Short-chain fatty acid and phenol/indole samples were stored at −20°C until analysis. Fecal sample allocated for microbiota were stored in 2 ml cryovials and stored at −80°C until analysis.

Total feces and urine were collected simultaneously during the 4 d collection phase of the study. All fecal samples were weighed, quality scored, and stored at −20°C until analyzed to determine macronutrient apparent total tract digestibility (ATTD). Total urine was collected into Nalgene bottles containing 10 ml of 2 N hydrochloric acid and weighed. A 25% subsample of the total weight was collected and stored at −20°C until analysis.

A 5 ml, fasted, blood sample was collected via jugular venipuncture from all dogs as a health check at the end of each experimental period. Serum metabolites were analyzed using 4 ml of blood collected in a serum separator vacutainer tube and a CBC was analyzed using the remaining 1 ml of blood collected in an EDTA vacutainer tube (Becton, Dickinson and Company, Franklin Lakes, NJ).

TABLE 1 | Ingredient composition of canine diets containing legumes or yeast.

Ingredient	Dietary treatment				
	Control	Garbanzo bean	Green lentils	Peanut flour	Dried yeast
Garbanzo bean	–	43.56	–	–	–
Green lentil	–	–	44.65	–	–
Peanut flour	–	–	–	28.08	–
Dried yeast	–	–	–	–	29.88
Poultry by-product meal	33.5	22.26	19.15	11.22	10.00
Rice	42.96	10.00	10.00	38.46	33.87
Poultry fat	8.47	8.74	10.14	4.43	8.26
Corn	10.00	10.00	10.00	10.00	10.00
Dried beet pulp	2.50	2.50	2.50	2.50	2.50
Palatant	1.00	1.00	1.00	1.00	1.00
Ca carbonate	0.78	0.66	0.57	1.51	1.89
Dical. phosphate	–	0.49	1.20	2.00	1.80
Salt	0.30	0.30	0.30	0.30	0.30
Vitamin premix[1]	0.18	0.18	0.18	0.18	0.18
Mineral premix[2]	0.18	0.18	0.18	0.18	0.18
Choline chloride	0.12	0.12	0.12	0.12	0.12
BHT (antioxidant)	0.02	0.02	0.02	0.02	0.02

[1]*Provided per kg diet: 17.4 mg manganese ($MnSO_4$), 284.3 mg iron ($FeSO_4$), 17.2 mg copper ($CuSO_4$), 2.2 mg cobalt ($CoSO_4$), 166.3 mg zinc ($ZnSO_4$), 7.5 mg iodine (KI), and 0.2 mg selenium ($Na2SeO_3$).*

[2]*Provided per kg diet: 10.8 mg copper ($CuSO_4$), 0.36 mg selenium (Na_2SeO_3), 150 mg zinc ($ZnSO_4$, ZnO), 2,562.8 IU vitamin A, 254 IU vitamin D3, 32.1 IU vitamin E.*

Maillard Reaction Product Analysis

A sample of each diet was analyzed for the presence of Maillard reaction products (MRP). The analyzed MRP were hydroxymethylfurfural (HMF), furosine (FS), carboxymethyllysine (CML), and fructoselysine (FL). Reactive lysine content of the dietary treatments were calculated using the furosine procedure (9).

The samples were analyzed for HMF using the HPLC procedure with modifications (10). Dried sample (100 mg) was homogenized for 30 min with 1.3 ml of 1.2% (w/v) glacial acetic acid solution in water and 50 µl of Carraz I and 50 µl of Carraz II reagents (Carrez Clarification Kit, Sigma-Aldrich, St. Louis, MO). The mixture then was centrifuged (model 5416C Eppendorf Centrifuge, Brinkman Instruments, Inc., Westbury, NY) at a rate of 10,000 x *g* for 30 min. The supernatant was filtered through 0.2 µm PTFE filter. An isocratic HPLC system was used with Alliance 2695 separation module (Waters Corporation, Milford, MA), an Inertsil ODS-3 column (25 cm × 0.46 cm i.d. × 5 µm df; MetaChem Technologies, Inc., Torrance, CA), and a 1050 Diode Array Detector (DAD, Agilent Technologies, Inc., Palo Alto, CA). HPLC grade water and methanol, added in a ratio of 90:10 (v/v), was used as a mobile phase at 1 ml/min flow rate for separations. The wavelength for HMF was detected at 284 nm using a UV detector.

Samples were analyzed for FS and CML using gas chromatography-mass spectrometry (11). The defatting step was modified by adding 50 mg of the dried sample and 5 ml pentane to a 15 ml screw cap, glass tube with a PTFE-lined cap. The samples then were vortexed for 5 min, centrifuged to separate the particulate, and the excess pentane was removed. A 50 µl internal standard solution, composed of 1.15 mg/ml of cycloleucine in water, was added. The defatted sample then was hydrolyzed and derivatized (11). Concentration of FL was calculated from FS (12).

Chemical Analyses

Fecal samples were composited by dog and experimental period and dried in a 57°C forced-air oven. Diet and fecal samples were ground through a 2 mm screen using a Wiley mill (model 4, Thomas Scientific, Swedesboro, NJ) and were analyzed for DM, ash, organic matter (OM), acid hydrolyzed fat (AHF), crude protein (CP), gross energy (GE), and total dietary fiber (TDF). Dry matter, ash, and OM were determined according to AOAC (13) methods 934.01 and 942.05. Total nitrogen values were determined according to AOAC (13) method 992.15 with CP calculated from Leco (TruMac N, Leco Corporation, St. Joseph, MI). Acid hydrolyzed fat, used to measure total fat content (14, 15). Gross energy was analyzed with bomb calorimetry (Model 6200, Parr Instruments Co., Moline, IL). Total dietary fiber was analyzed (16).

Fecal concentrations of SCFA and BCFA were measured using gas chromatography (17). Fecal ammonia concentrations were determined (18). Fecal phenol and indole concentrations were analyzed through gas chromatography (19).

Anti-nutritional Factors and Oligosaccharides

Processing samples were collected for all diets throughout the extrusion process. Samples were taken of the base mix, after the preconditioner, after the extruder, and the coated final diet

at multiple time-points. Processing samples were analyzed for trypsin inhibitors according to AACC (14) methods 22–40 s and urease activity according to AACC (14) methods 22–90. Free sugar profiles of substrates were determined (20, 21).

DNA Extraction, Amplification, Sequencing, and Bioinformatics

Total DNA was extracted from fresh fecal samples using a Mo-Bio PowerSoil kit (MO BIO Laboratories, Inc., Carlsbad, CA). Quantification of DNA concentration was completed using a Qubit® 2.0 Fluorometer (Life technologies, Grand Island, NY). A Fluidigm Access Array (Fluidigm Corporation, South San Francisco, CA), in combination with Roche High Fidelity Fast Start Kit (Roche, Indianapolis, IN), were used for amplification of the 16S rRNA gene. The primers 515F (5′-GTGCCAGCMGCCGCGGTAA-3′) and 806R (5′-GGACTACHVGGGTWTCTAAT-3′), targeting a 292 bp-fragment of V4 region, were used for amplification (primers synthesized by IDT Corp., Coralville, IA) (22). Fluidigm specific primer, forward and reverse tags, were added in accordance with the Fluidigm protocol. The quality of amplicons' regions and sizes were confirmed by Fragment Analyzer (Advanced Analytics, Ames, IA). A DNA pool was generated through the combination of equimolar amounts of the amplicons from each sample. The pooled samples were selected by size on a 2% agarose E-gel (Life Technologies, Grand Island, NY) and extracted using a Qiagen gel purification kit (Qiagen, Valencia, CA). The pooled, size-selected, and cleaned products were then analyzed on an Agilent Bioanalyzer to confirm appropriate profile and mean size. The Roy J. Carver Biotechnology Center at the University of Illinois performed Illumina sequencing on a MiSeq using v3 reagents (Illumina Inc., San Diego, CA). A FASTX-Toolkit (version 0.0.14) removed the Fluidigm tags. Analysis of sequences was completed using QIIME 2.0 (22) and DADA2 (version 1.14) (23). The high quality (quality value $\geq$ 20) sequence data, derived from the sequencing process, were de-multiplexed. An opened-reference OTU clustered the sequences into operational taxonomic units (OTU), choosing against the SILVA 138 reference OTU database with a 97% similarity threshold (24). The OTUs observed fewer than 2 times (singletons), as well as OTUs with <0.01% of the total observation were discarded. An average of 56,012 reads were obtained, with a total of 2,800,636 reads. The number of reads ranged from 37,603 to 79,027 per sample. Rarefaction curves based on observed species, Chao1, and the plateaus observed in the phylogenetic distance whole tree measures suggest sufficient sequencing depth. To analyze for diversity and species richness, the dataset was rarified to 37,600 reads. Weighted and unweighted unique fraction metric (UniFrac) distances were performed by principal coordinates analysis (PCoA). This measured the phylogenetic distance between sets of taxa in a phylogenetic tree as a fraction of the branch length of the tree, based on the 97% OTU composition and abundance matrix (25).

Statistical Analyses

All data were analyzed in SAS (SAS Institute, Inc., version 9.4, Cary, NC) using the MIXED models procedure with the exception of fecal score data, which were analyzed using the GLIMMIX procedure. The model was run with a fixed effect of diet and a random effect of dog. The differences among treatments were reported using a Fisher-protected least significance test with a Tukey adjustment to control for the Type 1 experiment-wise error. Means were considered to be statistically significant using a probability of $P < 0.05$. The reported standard errors of the means (SEM) were determined from the MIXED models procedure in SAS. Statistical analysis could not be performed on the MRP, anti-nutritional factor, or oligosaccharide data because the procedures were only performed using technical replicates.

RESULTS

Diet Proximate Analysis, Food Intake, and Fecal Characteristics

Ingredient composition (**Table 1**) of all five diets was targeted to be similar with the exception of the test ingredient. The percent inclusion of the test ingredients varied in order for the diets to have similar macronutrient composition (**Table 2**). Dry matter content of the diets ranged from 91.8 to 93.9%. Macronutrient composition of the dietary treatments is reported on a dry matter basis (DMB). Control had the highest CP content (31.2%) compared with the other dietary treatments, which ranged from 26.6 (PFD) to 29.1% (DYD).

Food intake (g/d, DMB) was significantly lower ($P < 0.05$) for GLD, with an intake of 169.1 g/d, compared with CON, with an intake of 175.6 g/d (**Table 3**). Other dietary treatments were not different from each other ($P > 0.05$).

Fecal output (g/d, DMB) was highest ($P < 0.05$) for DYD at 33.6 g/d, but not different ($P > 0.05$) from GLD which was 30.8 g/d (**Table 3**). Control had the lowest ($P < 0.05$) fecal output at 24.1 g/d but did not differ ($P > 0.05$) from GBD (28.1 g/d) or PFD (24.8 g/d). Fecal output on an as-is basis followed the same pattern with DYD as the highest ($P < 0.05$) at 93.6 g/d and CON as the lowest ($P < 0.05$) at 58.2 g/d. Despite the differences in fecal output, no significant differences ($P > 0.05$) were observed in fecal scores among all treatments (**Table 3**). All fecal scores were within the ideal range of 2–3 with an average value of 2.5. No significant differences ($P > 0.05$) were observed in total urine output (**Table 3**) among treatments.

Maillard Reaction Products, Anti-nutritional Factors, and Oligosaccharides

The concentration of MRP (**Figure 1**) present in each of the dietary treatments used in this study were measured. The concentration of hydroxymethylfurfural (HMF) was highest in PFD (41.4 μg/g, DMB) compared with the other treatments, which had an average HMF concentration of 18.1 μg/g, DMB. In the current study, DYD had the highest concentrations of fructoselysine (FL), furosine (FS), and carboxymethyllysine (CML) at 39.7, 12.6, and 69.7 μg/g, respectively. For FL, the remaining dietary treatments ranged from 11.2 μg/g (GLD) to 21.9 μg/g (PFD). The range of FS concentration for the

TABLE 2 | Analyzed chemical composition and energy content of canine diets containing legumes or yeast.

	Dietary treatment[1]				
Item	**CON**	**GBD**	**GLD**	**PFD**	**DYD**
Dry matter (%)	91.8	92.5	92.2	93.9	91.8
	Dry matter basis				
Crude protein (%)	31.2	26.9	27.4	26.6	29.1
Acid hydrolyzed fat (%)	15.9	17.3	14.5	15.1	15.6
Total dietary fiber (%)	11.2	11.9	11.2	10.5	17.7
Soluble (%)	5.6	3.9	4.2	4.6	6.8
Insoluble (%)	5.6	8.1	7.0	5.9	10.9
Ash (%)	7.2	7.0	7.1	7.0	7.6
Gross energy (kcal/g)	5.1	5.0	5.0	5.1	5.1

[1]*CON, Poultry by-product meal control; GBD, Garbanzo bean; GLD, Green lentil; PFD, Peanut flour; DYD, Dried yeast.*

TABLE 3 | Food intake, fecal scores, and fecal output of dogs fed diets containing legumes or yeast as the primary protein source.

	Dietary treatment[1]					
Item	**CON**	**GBD**	**GLD**	**PFD**	**DYD**	**SEM[2]**
Food intake (g/d, as-is)	191.2^{a}	186.9^{ab}	183.3^{b}	185.1^{b}	186.1^{ab}	4.235
Food intake (g/d, DMB[3])	175.6^{a}	172.9^{ab}	169.1^{b}	173.7^{ab}	170.8^{ab}	3.918
Fecal score	2.2	2.5	2.8	2.5	2.4	0.231
Fecal output (g/d, as-is)	58.2^{c}	89.6^{ab}	93.7^{a}	73.2^{bc}	93.6^{a}	6.021
Fecal output (g/d, DMB[3])	24.1^{c}	28.1^{bc}	30.8^{ab}	24.8^{c}	33.6^{a}	1.541
Urine output (ml/d)	161.8	172.7	152.1	159.8	135.9	16.707

[1]*CON, Poultry by-product meal control; GBD, Garbanzo bean; GLD, Green lentil; PFD, Peanut flour; DYD, dried yeast.*
[2]*SEM, Standard error of the mean.*
[3]*DMB, Dry matter basis.*
$^{a-c}$*Means within a row with different superscripts are different ($P < 0.05$).*

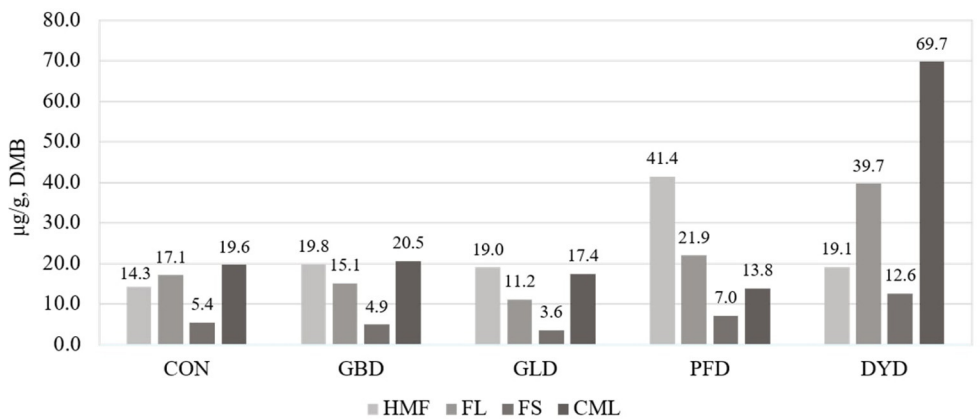

FIGURE 1 | Presence of Maillard reaction products (MRPs) in canine diets containing legumes or yeast as the primary protein source. DMB, Dry matter basis; CON, Poultry by-product meal control; GBD, Garbanzo bean; GLD, Green lentil; PFD, Peanut flour; DYD, Dried yeast; HMF, Hydroxymethylfurfural; FL, Fructoselysine; FS, Furosine; CML, Carboxymethyllysine. Statistical analysis could not performed due to use of technical replicates.

remaining dietary treatments was narrower from 3.6 µg/g (GLD) to 7.0 µg/g (PFD). Lastly, for CML, the remaining diets ranged from 13.8 (PFD) to 20.5 µg/g (GBD). The dietary treatments were determined to have minimal heat damage (**Figure 2**), with

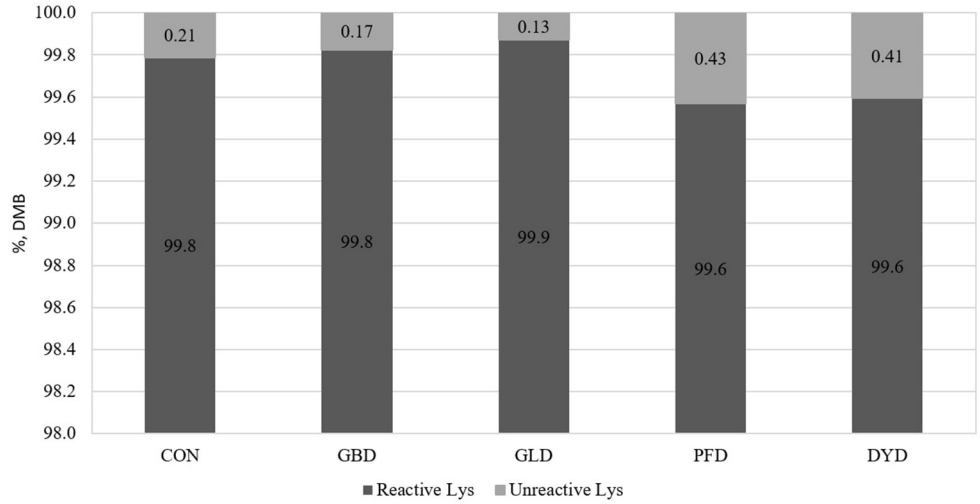

FIGURE 2 | Reactive lysine content in canine diets containing legumes or yeast as the primary protein source. DMB, Dry matter basis; CON, Poultry by-product meal control; GBD, Garbanzo bean; GLD, Green lentil; PFD, Peanut flour; DYD, Dried yeast.

TABLE 4 | Anti-nutritional factors and oligosaccharide content of legume-based canine diets at various processing stages.

	Anti-nutritional factors (DMB[1])		Oligosaccharides (%, DMB[1])		
Diet stage of processing[2]	**Trypsin inhibitor (TIU/g)**	**Urease activity (Δ pH units)**	**Raffinose**	**Stachyose**	**Verbascose**
Base mix					
CON	0	0	0.04	0.03	0
GBD	4,385.0	0	0.27	0.61	0.01
GLD	731.3	0	0.14	1.15	0.39
After preconditioner					
CON	0	0	0.03	0.05	0
GBD	4,486.9	0	0.28	0.26	0.01
GLD	1,533.7	0	0.15	0.56	0.08
After extruder					
CON	0	0	0.05	0.04	0
GBD	1,182.8	0	0.24	0.66	0.03
GLD	495.9	0	0.17	1.22	0.31
Final diet					
CON	0	0	0.03	0.03	0.01
GBD	0	0	0.27	0.72	0.02
GLD	0	0	0.15	1.17	0.43

[1]*DMB, Dry matter basis.*
[2]*CON, Poultry by-product meal control; GBD, Garbanzo bean diet; GLD, Green lentil diet.*

reactive lysine contents ranging from 99.6% (DMB) in PFD and DYD to 99.9% in GLD.

Anti-nutritional factor content for the dietary treatments at various stages of processing (**Table 4**) showed trypsin inhibitor activity present in GBD and GLD at all stages of processing until the final diet. The GBD base mix had trypsin inhibitor activity measured at 4,385.0 TIU/g. After the preconditioner, the activity was similar at 4,486.9 TIU/g, and then decreased after the extruder to 1,182.8 TIU/g (DMB). There was no urease activity detectable for any of the diets at any processing stage.

Oligosaccharide content of the diets was measured at various processing stages (**Table 4**). Raffinose content was numerically higher for GBD at all processing stages, ranging from 0.24 to 0.28% (DMB). Stachyose content was higher for GLD at each processing stage, ranging from 0.56% (after preconditioner) to 1.22% (after extruder). Similarly, verbascose content was highest in GLD at each processing stage (0.39% base mix, 0.08% after

TABLE 5 | Apparent total tract macronutrient and energy digestibility of dogs fed diets containing legumes or yeast as the primary protein source.

	Dietary treatment[1]					
Nutrient digestibility	**CON**	**GBD**	**GLD**	**PFD**	**DYD**	**SEM[2]**
Dry matter (%)	86.3^{a}	83.7^{ab}	82.1^{bc}	85.6^{a}	80.1^{c}	0.793
Organic matter (%)	91.2^{a}	87.5^{b}	85.8^{bc}	90.3^{a}	84.3^{c}	0.617
Crude protein (%)	86.6^{a}	83.2^{bc}	81.5^{c}	85.0^{ab}	83.7^{abc}	0.863
Acid hydrolyzed fat (%)	94.7^{a}	94.2^{ab}	94.1^{b}	95.5^{a}	87.9^{c}	0.327
Total dietary fiber (%)	50.8^{a}	44.2^{a}	26.7^{b}	52.9^{a}	42.2^{a}	3.132
Digestible energy (kcal/g)	4.69^{a}	4.46^{bc}	4.37^{cd}	4.49^{b}	4.29^{d}	0.029
Metabolizable energy (kcal/g)	4.44^{a}	4.26^{b}	4.13^{bc}	4.27^{b}	4.04^{c}	0.041

[1]*CON, Poultry by-product meal control; GBD, Garbanzo bean; GLD, Green lentil; PFD, Peanut flour; DYD, dried yeast.*
[2]*SEM, Standard error of the mean.*
$^{a-d}$*Means within a row with different superscripts are different ($P < 0.05$).*

preconditioner, 0.31% after extruder, and 0.43% in the final diet) in contrast with other dietary treatments.

Apparent Total Tract Macronutrient and Energy Digestibility

All diets were well-digested with macronutrient ATTD at or above 80% (**Table 5**). In terms of DM, OM, CP, and AHF, PFD and CON diets consistently had the highest ($P < 0.05$) ATTD. Dogs fed DYD consistently had the lowest ($P < 0.05$) ATTD for DM (80.1%), OM (84.3%), CP (83.7%), and AHF (87.9%). The TDF digestibility of the diets did not differ ($P > 0.05$), with the exception of GLD which was significantly lower ($P < 0.05$) at 26.7% than all other dietary treatments. Digestible energy content (**Table 5**) were highest for CON (4.7 kcal/g) and lowest ($P < 0.05$) for DYD with values of 4.3 kcal/g (DMB) but was not different ($P > 0.05$) from GLD with values of 4.4 kcal/g. Metabolizable energy content (**Table 5**) followed the same pattern as DE, with dogs fed CON having the highest ($P < 0.05$) ME of 4.4 kcal/g and dogs fed DYD and GLD having the lowest ($P < 0.05$) at 4.0 and 4.1 kcal/g, respectively.

Fecal Fermentative End-Products and Serum Chemistry

Total fecal concentrations of SCFA (μmol/g, DMB) were lowest ($P < 0.05$) for CON at 381.1 μmol/g (**Table 6**). Total fecal SCFA concentrations in the other dietary treatments were not significantly different from each other ($P > 0.05$) with GBD being the highest at 711.0 μmol/g. Acetate and propionate concentrations were highest ($P < 0.05$) for GBD at 459.5 μmol/g and 207.2 μmol/g, respectively. At 103.9 μmol/g, DYD had the highest ($P < 0.05$) butyrate concentration. This is more than twice the butyrate concentrations measured in the other dietary treatments, which were not significantly different from each other ($P > 0.05$) with an average of 46.2 μmol/g.

Total fecal BCFA concentrations (μmol/g, DMB) were highest ($P < 0.05$) for DYD at 23.4 μmol/g and lowest ($P < 0.05$) for GLD and PFD at 16.1 μmol/g and 16.0 μmol/g, respectively (**Table 6**). No significant differences were observed in isobutyrate concentrations among the dietary treatments. Isovalerate concentrations were higher ($P < 0.05$) for DYD at 12.3 μmol/g than GBD at 8.3 μmol/g. Valerate concentrations were also higher ($P < 0.05$) for DYD at 3.6 μmol/g than PFD (1.1 μmol/g) and CON (1.0 μmol/g).

Total fecal phenol and indole concentrations (μmol/g, DMB) were highest ($P < 0.05$) in dogs fed CON at 2.21 μmol/g (**Table 6**). Dogs fed GLD had the lowest ($P < 0.05$) total phenol and indole concentration with a value of 1.18 μmol/g. Phenols were lowest ($P < 0.05$) for GLD (0.2 μmol/g) and DYD (0.3 μmol/g) and highest ($P < 0.05$) for PFD, GBD and CON, both of which had a phenol concentration of 0.5 μmol/g. Indoles were highest ($P < 0.05$) in DYD and CON with values of 1.6 μmol/g and 1.7 μmol/g, respectively. The ammonia concentration (**Table 6**) was lower ($P < 0.05$) for PFD (1.6 mg/g) than CON (2.3 mg/g), with the rest of the treatments not different ($P > 0.05$) from each other.

Serum chemistry and a CBC were analyzed throughout the study to monitor health of the dogs and to ensure dietary treatments did not cause any negative health effects. All serum metabolites (**Table 7**) were within normal ranges for all dietary treatments. Additionally, the analyzed CBC components were determined to be normal for healthy adult dogs (data not shown).

Fecal Microbiota

The microbial composition at the phylum level is shown in **Table 8**. The most abundant phyla included Firmicutes (ranging from 47% of the sequences for dogs fed PFD to 62% for dogs fed GBD), Bacteroidetes (16% for DYD to 25% PFD), and Fusobacteria (13% for GBD to 21% for CON). Proteobacteria corresponded to 7% or less of the sequences among dietary treatments. Actinobacteria comprised ∼1% of the sequences overall, while Deferribacteres and Epsilonbacteraeota corresponded to <1% of the total sequences among dietary treaments.

The microbial composition at the family level is shown in **Table 9**, identifying over 30 different families. The relative abundance at the family level varied among different treatments. The most abundant families included *Fusobacteriaceae, Lachnospiraceae, Bacteroidaceae, Erysipelotrichaceae,* and *Veillonellaceae*. At the genus level, an increased relative abundance of the genus *Megamonas* was observed in dogs fed

TABLE 6 | Fecal fermentative end-product concentrations of dogs fed diets containing legumes or yeast as the primary protein source.

	Dietary treatment[1]					
Item (DMB[2])	**CON**	**GBD**	**GLD**	**PFD**	**DYD**	**SEM[3]**
Fecal pH	6.57[a]	6.05[ab]	6.03[b]	6.37[ab]	6.05[ab]	0.135
Ammonia (mg/g)	2.3[a]	1.9[ab]	1.9[ab]	1.6[b]	2.1[ab]	0.124
SCFA[4] (μmol/g)						
Acetate	221.1[c]	459.5[a]	368.1[b]	361.6[b]	349.7[b]	19.803
Propionate	114.8[b]	207.2[a]	198.5[ab]	172.3[ab]	150.4[ab]	21.637
Butyrate	45.2[b]	44.4[b]	49.7[b]	45.4[b]	103.9[a]	7.604
Total SCFA	381.1[b]	711.0[a]	616.2[a]	579.3[a]	604.0[a]	37.575
BCFA[4] (μmol/g)						
Isobutyrate	7.8	6.5	5.6	6.3	7.4	0.653
Isovalerate	11.3[ab]	8.3[b]	8.6[ab]	8.6[ab]	12.3[a]	0.931
Valerate	1.0[b]	1.8[ab]	2.0[ab]	1.1[b]	3.6[a]	0.469
Total BCFA	20.1[ab]	16.7[ab]	16.1[b]	16.0[b]	23.4[a]	1.659
Phenols/indoles (μmol/g)						
Phenols	0.5[a]	0.5[a]	0.2[b]	0.5[a]	0.3[b]	0.101
Indoles	1.7[a]	0.9[b]	1.1[b]	1.1[b]	1.6[a]	0.121
Total Phenol/Indoles	2.2[a]	1.5[bc]	1.2[c]	1.7[bc]	1.9[b]	0.158

[1]*CON, Poultry by-product meal control; GBD, Garbanzo bean; GLD, Green lentil; PFD, Peanut flour; DYD, dried yeast.*
[2]*DMB, Dry matter basis; SEM, Standard error of the mean.*
[3]*SEM, Standard error of the mean.*
[4]*SCFA, Short chain fatty acids; BCFA, Branched chain fatty acids.*
[a–c]*Means within a row with different superscript letters are different ($P < 0.05$).*

TABLE 7 | Serum metabolites of dogs fed diets containing legumes or yeast as primary protein sources.

		Dietary treatment[1]					
Item	**Reference range[2]**	**CON**	**GBD**	**GLD**	**PFD**	**DYD**	**SEM[3]**
Creatinine (mg/dL)	0.5–1.5	0.6	0.6	0.5	0.6	0.5	0.022
BUN (mg/dL)[3]	6.0–30.0	12.8	11.1	11.4	12.2	13.3	0.496
Total protein (g/dL)	5.1–7.0	6.1	5.9	5.9	5.8	6.2	0.125
Albumin (g/dL)	2.5–3.8	3.3	3.3	3.3	3.4	3.3	0.064
Globulin (g/dL)	2.7–4.4	2.7	2.6	2.6	2.6	2.7	0.097
Ca (mg/dL)	7.6–11.4	10.4	10.4	10.4	10.4	10.6	0.087
P (mg/dL)	2.7–5.2	4.3	4.4	4.8	4.3	4.2	0.146
Na (mmol/L)	141–152	144.6	144	144.5	145.1	144.9	0.464
K (mmol/L)	3.9–5.5	4.3	4.4	4.4	4.4	4.3	0.076
Na:K ratio	28–36	34.0	33.0	32.8	33.2	33.6	0.639
Cl (mmol/L)	107–118	109.7	109.9	109.5	109.9	109.9	0.672
Glucose (mg/dL)	68–126	97.0	95.7	91.1	96.0	95.3	2.511
Total Bilirubin (mg/dL)	0.1–0.3	0.2	0.2	0.2	0.2	0.2	0.011
Cholesterol (mg/dL)	129–297	235.9	237	216.2	226.8	213.8	13.741
Triglycerides (mg/dL)	32–154	76.0	72.2	73.5	84.4	67.0	7.093
Bicarbonate (mmol/L)	16–24	22.0	22.0	22.5	23.0	21.9	0.409

[1]*CON, Poultry by-product meal control; GBD, Garbanzo bean; GLD, Green lentil; PFD, Peanut flour; DYD, dried yeast.*
[2]*Reference ranges were provided by the University of Illinois Veterinary Diagnostics Laboratory.*
[3]*SEM, Standard error of the mean; BUN, Blood urea nitrogen.*

GBD and GLD compared to the remaining treatment groups. Additionally, dogs fed DYD had higher relative abundances of several genera belonging to the Frimicutes phylum compared with dogs fed the remaining dietary treatments, including *Clostridium Sensu Stricto 1, Ruminococcus Gauvreauii Group, Romboutsia, Erysipelatoclostridium,* and *Erysipelotrichaceae*

TABLE 8 | Relative abundance of bacterial phyla and families of dogs fed diets containing legumes or yeast as primary protein sources.

		Dietary treatment[1]					
Phylum	**Family**	**CON**	**GBD**	**GLD**	**PFD**	**DYD**	**SEM[2]**
Actinobacteria		0.9^{ab}	1.9^{a}	1.5^{ab}	0.6^{b}	0.9^{ab}	0.308
	Bifidobacteriaceae	0.5^{ab}	1.1^{ab}	1.3^{a}	0.3^{b}	0.7^{ab}	0.308
	Coriobacteriaceae	0.4^{ab}	0.8^{a}	0.2^{b}	0.4^{ab}	0.2^{b}	0.133
Bacteroidetes		22.8^{ab}	17.2^{b}	20.2^{ab}	25.4^{a}	16.1^{b}	1.988
	Bacteriodaceae	14.1	12.2	13.9	17.0	12.3	1.473
	Prevotellaceae	7.5^{a}	4.7^{b}	5.5^{ab}	7.6^{a}	3.8^{b}	0.981
Firmicutes		48.2^{b}	62.0^{a}	55.3^{ab}	46.7^{b}	61.4^{a}	3.298
	Clostridiaceae	0.2^{b}	0.2^{b}	0.1^{b}	0.4^{b}	3.5^{a}	0.506
	Lachnospiraceae	18.9	16.6	14.6	18.1	15.1	1.718
	Peptococcaceae	0.9^{a}	0.8^{ab}	0.5^{ab}	0.6^{ab}	0.3^{b}	0.222
	Peptostreptococcaceae	6.5^{ab}	5.6^{ab}	4.3^{b}	4.7^{b}	7.8^{a}	0.751
	Ruminococcaceae	8.6^{ab}	5.2^{b}	6.1^{b}	9.5^{a}	5.6^{b}	0.942
	Erysipelotrichaceae	6.1^{c}	11.8^{b}	10.6^{bc}	5.4^{c}	18.7^{a}	2.027
	Acidaminococcaceae	1.6^{ab}	1.3^{bc}	0.7^{c}	2.1^{a}	0.9^{bc}	0.244
	Veillonellaceae	2.0^{c}	17.9^{a}	12.8^{ab}	5.5^{bc}	4.8^{c}	2.023
Fusobacteria		21.1^{a}	13.4^{b}	17.1^{ab}	20.2^{ab}	17.3^{ab}	1.705
	Fusobacteriaceae	21.1^{a}	13.4^{b}	17.1^{ab}	20.2^{ab}	17.3^{ab}	1.705
Proteobacteria		7.1^{ab}	5.5^{ab}	5.8^{ab}	7.1^{a}	4.2^{b}	0.767
	Succinivibrionaceae	2.4^{ab}	2.8^{a}	3.0^{a}	2.3^{ab}	0.8^{b}	0.525
	Burkholderiaceae	4.2^{ab}	2.5^{b}	2.8^{b}	4.6^{a}	3.3^{ab}	0.422

[1]*CON, Poultry by-product meal control; GBD, Garbanzo bean; GLD, Green lentil; PFD, Peanut flour; DYD, dried yeast.*

[2]*SEM, Standard error of the mean.*

$^{a-c}$*Means within a row with different superscript letters are different ($P < 0.05$).*

UCG-003. However, due to the complexity of the hindgut microbial composition, the changes in microbial diversity for each dietary treatment were also determined.

The β-diversity was determined based on Bray-Curtis Dissimilarity Analysis (**Figure 3**). Differences in microbial diversity among dietary treatments were based on a combination of *p*- and *q*-values <0.01. Dogs fed CON and PFD had similar fecal microbial communities in contrast with dogs fed DYD, GBD, or GLD. While GBD and GLD did not differ from each other, DYD resulted in a different microbial composition. The β-diversity based on weighted UniFrac analysis (**Figure 4A**), showed fecal microbial community abundance was similar between dogs fed CON and PFD. Additionally, dogs fed GBD and GLD had similar microbial abundance. Dogs fed DYD differed from all other dietary treatments. The β-diversity based on unweighted UniFrac analysis (**Figure 4B**) showed an identical pattern as the weighted UniFrac analysis. The α-diversity (**Figure 5**), measured using Faith's phylogenetic diversity (PD), indicated that species evenness within a sample was higher ($P < 0.05$) in dogs fed CON than dogs fed GLD.

The taxonomic differences were then characterized through Linear Discriminant Analysis (LDA) Effect Size (LEfSe) according to specific dietary treatments and protein sources (**Figure 6**). According to the LDA scores, Lachnospiraceae was a family featured in dogs fed PFD. Dogs fed GLD had the Actinobacteria phylum featured, particularly taxonomic groups related to bifidobacterium. Similar to GLD, dogs fed GBD showed Actinobacteria as a discriminant feature, along with *Megamonas*, a genus from the Firmicutes phylum, and *Negativicutes* class. Firmicutes, along with several taxonomic groups within this phylum, and Erysipelotrichales were featured in dogs fed DYD. Dogs fed CON featured taxa mostly from Firmicutes and Bacteroidetes phyla.

DISCUSSION

Diet, Food Intake, and Fecal Characteristics

All 5 diets were formulated to target similar nutrient and ingredient composition, with the exception of the primary protein source, which was included at the expense of poultry by-product meal and rice. The inclusion level of the legumes and yeast ingredients varied to make the diets as isocaloric as possible. The difference in food intake (DMB) observed in dogs fed GLD and CON can be attributed to the individualized preference of the dogs or the lowered AHF content of GLD. Overall, diets were well-accepted and did not result in inadequate daily food intake or body weight loss during the study.

The large numerical differences between fecal output on an as-is basis and fecal output on a DMB are indicative of the substantial water-holding capacity of legumes. Legumes and pulses typically contain ∼30% dietary fiber (2) which are responsible for the water-holding capacity of the legumes

TABLE 9 | Relative abundance of bacterial genera of dogs fed diets containing legumes or yeast as the primary protein source.

		Dietary treatment[1]					
Phylum	**Genus**	**CON**	**GBD**	**GLD**	**PFD**	**DYD**	**SEM[2]**
Actinobacteria	*Collinsella*	0.4^{ab}	0.8^{a}	0.2^{b}	0.4^{ab}	0.2^{b}	0.133
Bacteroidetes	*Alloprevotella*	3.3^{a}	1.6^{bc}	2.3^{ab}	3.1^{a}	0.5^{c}	0.422
	Prevotellaceae Ga6A1 Group	2.0^{a}	1.1^{bc}	0.2^{c}	1.5^{ab}	0.8^{bc}	0.257
Firmicutes	*Clostridium Sensu Stricto 1*	0.2^{b}	0.2^{b}	0.1^{b}	0.4^{b}	3.5^{a}	0.506
	Ruminococcus Gauvreauii Group	0.1^{b}	0.3^{b}	0.3^{b}	0.1^{b}	1.6^{a}	0.132
	Ruminococcus Torques Group	3.9^{a}	1.5^{b}	1.4^{b}	3.1^{ab}	1.9^{b}	0.464
	Peptococcus	0.9^{a}	0.8^{ab}	0.5^{ab}	0.6^{ab}	0.3^{b}	0.222
	Romboutsia	1.2^{b}	1.7^{b}	1.5^{b}	1.1^{b}	3.3^{a}	0.343
	Butyricicoccus	0.1^{b}	0.7^{a}	0.6^{a}	0.2^{ab}	0.1^{b}	0.109
	Faecalibacterium	6.1^{ab}	2.9^{c}	3.9^{bc}	7.3^{a}	4.7^{abc}	0.812
	Negativibacillus	0.5^{a}	0.1^{bc}	0.1^{bc}	0.5^{ab}	0^{c}	0.117
	Erysipelatoclostridium	0.4^{b}	0.0^{b}	0.2^{b}	0.3^{b}	3.4^{a}	0.214
	Erysipelotrichaceae UCG-003	0.1^{b}	0.4^{b}	0.6^{ab}	0.2^{b}	1.4^{a}	0.271
	Faecalitalea	0.1^{b}	0^{b}	0.2^{b}	0.1^{b}	0.6^{a}	0.134
	Phascolarctobacterium	1.6^{ab}	1.3^{bc}	0.7^{c}	2.1^{a}	0.9^{bc}	0.244
	Megamonas	1.9^{c}	17.6^{a}	12.1^{ab}	5.4^{bc}	4.5^{c}	2.099
Fusobacteria	*Fusobacterium*	21.1^{a}	13.4^{b}	17.1^{ab}	20.2^{ab}	17.3^{ab}	1.705
Proteobacteria	*Anaerobiospirillum*	2.4^{a}	2.8^{a}	3.0^{a}	2.2^{ab}	0.8^{b}	0.525
	Sutterella	2.7^{ab}	2.1^{ab}	1.8^{b}	3.4^{a}	2.8^{ab}	0.363

[1]*CON, Poultry by-product meal control; GBD, Garbanzo bean; GLD, Green lentil; PFD, Peanut flour; DYD, dried yeast.*
[2]*SEM, Standard error of the mean.*
$^{a-c}$*Means within a row with different superscript letters are different ($P < 0.05$).*

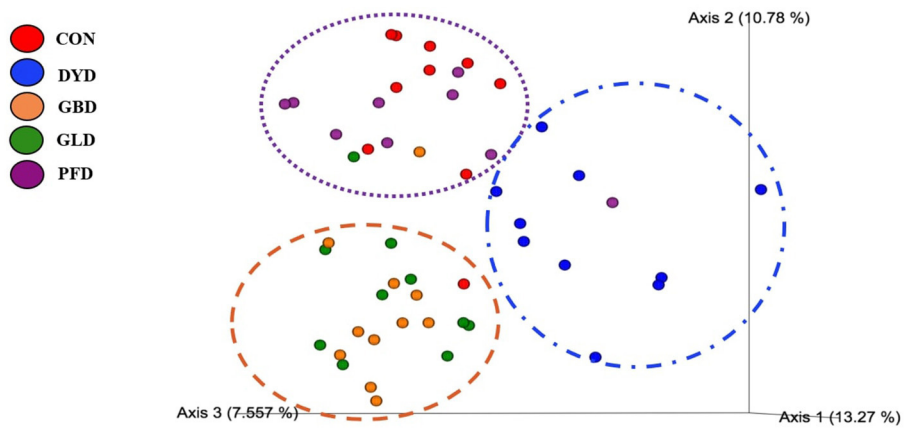

FIGURE 3 | Principal coordinated plots of Bray Curtis Dissimilarity analysis of fecal microbial communities of dogs fed diets containing legumes or yeast as the primary protein source. CON, Poultry by-product meal control; GBD, Garbanzo bean; GLD, Green lentil; PFD, Peanut flour; DYD, Dried yeast.

included in these diets. The water-holding capacity of legumes have been shown to increase after cooking processes (26) such as extrusion. A previous study corroborated these findings, demonstrating that the fecal moisture content of dogs fed either lentils or peas was higher than dogs fed a rice diet (27). Despite the differences in fecal output in the current study, this did not impact the fecal quality among dogs fed the different treatments.

Maillard Reaction Products

Maillard reactions are spontaneous, non-enzymatic, browning reactions that occur between a reducing sugar and a free amino group in a protein when exposed to heat. While Maillard reactions can improve palatability, advanced stages can negatively impact protein quality (28, 29). Lysine is particularly reactive in Maillard reactions due to the presence of an epsilon amino group, reducing its availability (30). Because lysine is

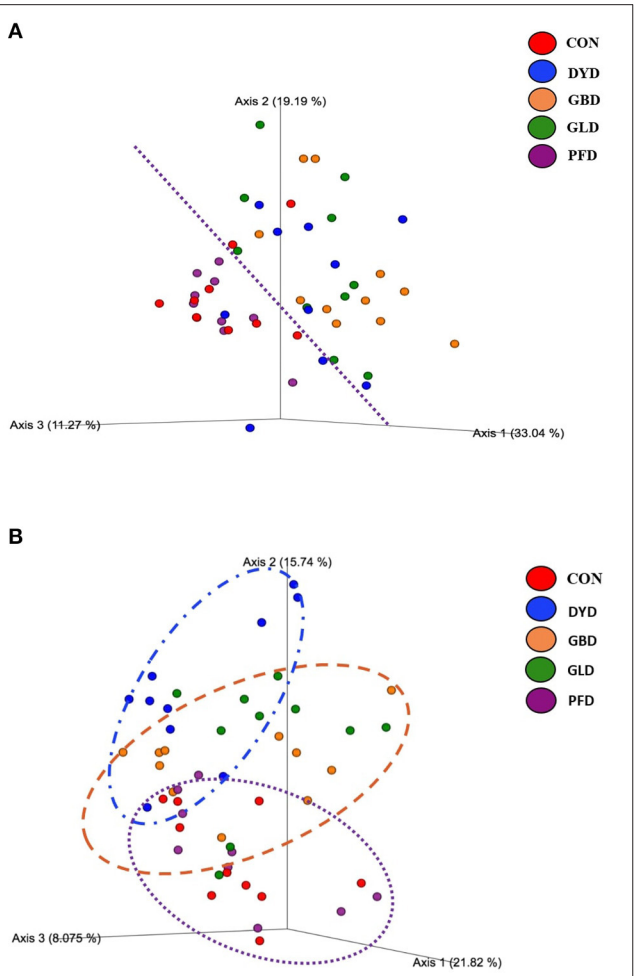

FIGURE 4 | Principal coordinated plots of weighted **(A)**, and unweighted **(B)** UNIFRAC distances of fecal microbial communities of dogs fed diets containing legumes or yeast as the primary protein source. CON, Poultry by-product meal control; GBD, Garbanzo bean; GLD, Green lentil; PFD, Peanut flour; DYD, Dried yeast.

commonly the first or second-limiting amino acid in commercial pet foods, a reduction in lysine availability can result in decreased protein quality of the diet (31).

Hydroxymethylfurfural is a common intermediate product and a common marker of advanced stages of the Maillard reaction (12, 32). The higher concentration of HMF observed in PFD could be attributed to using a dark roasted peanut flour in the diet. While Maillard reactions that occur during the roasting process are responsible for color and flavor development of peanut flour, it can also negatively impact the protein quality of the ingredient. One study showed that as roast color darkened, the amount of available lysine present in peanut flour decreased (33). With an inclusion of peanut flour at nearly 30% in the current study, the exposure of the peanut flour to dark roasting conditions and the added exposure to extrusion temperatures could contribute to the higher concentration of HMF in the overall diet. One study estimated that a 70 kg adult human has an average daily intake of 0.28 mg HMF/kg $BW^{0.75}$ compared with a 20 kg adult dog, fed a commercial extruded diet, which ingests 34.5 mg HMF/kg $BW^{0.75}$ per day (12). This 122-fold difference is possibly attributed to the fact that dogs are monophagous and consistently consume higher protein commercial diets that often undergo heat processing.

The acid hydrolysis of FL causes a chemical conversion to FS, regenerated lysine, and pyridosine (34). The formation of FS is considered to be constant at about 30–34% yield (34), allowing it to be used as an indirect measure of FL. A large amount of variation has been previously reported in the FL and FS contents of canine diets based on processing or storage conditions (12, 35). Extruded, canned, and pelleted canine diets and found FL contents of 0.71, 4.46, and 0.21 g/kg (DMB), respectively (12). Additionally, the FS contents of the diets were 14.1, 21.7, and 11.5 g/kg (DMB), respectively. A different study measured the FS content of a single extruded dog diet to be 0.9 mg/g (as-is) but increased to 1.5 mg/g after 12 weeks of storage at 22.2°C or 3.2 mg/g after 12 weeks of storage at 37.8°C (35). The FL or FS contents in these previous studies are higher than the FS and FL measured in the current study, potentially due to differences in extrusion parameters or storage conditions of the diets.

The FS content of the diets was also used to calculate the concentration of reactive lysine, or the lysine available for protein synthesis (9). One of the limitations of using the furosine method to determine reactive lysine is the assumption that furosine comprises 32% of Amadori products, a value derived from the Amadori products present in milk (9, 36). Some variation in reactive lysine content has been observed in extruded canine diets, potentially due to the differences in processing parameters. A previous study has measured the reactive to total lysine content of extruded commercial canine maintenance and growth diets to be 0.85 and 0.75, respectively (37, 38). Similarly, the reactive to total lysine ratio was analyzed in commercially-available extruded, canned, and pelleted canine diets to be 0.90, 0.98, and 0.84, respectively (37, 38).

The formation of CML in foods is often used as a marker for advanced glycation end-products (AGE) that have been linked to negative health effects in humans, rats, and dogs, such as increased inflammatory and oxidative stress or insulin resistance (39–42). Therefore, CML is commonly measured in human foods to help prevent the formation of AGE (41), but CML concentrations have not been extensively measured in canine diets. One study measured the CML concentration to be 15.4 g/kg (DMB) in extruded adult canine diets. In canned adult canine diets, the CML concentration was 36.5 g/kg (12).

Anti-nutritional Factors and Oligosaccharides

Anti-nutritional factors are protective metabolites in plants that deter consumption of the plant by birds or pests (43). Trypsin inhibitors, classified into either Kunitz or Bowman-Birk families, reduce digestion and absorption of protein in legumes through modulation of trypsin, an important protease (44, 45). However, heat processing methods have been proven to be effective in reducing or eliminating trypsin inhibitors (45, 46) increasing digestion and improving palatability of legumes (47). Extrusion

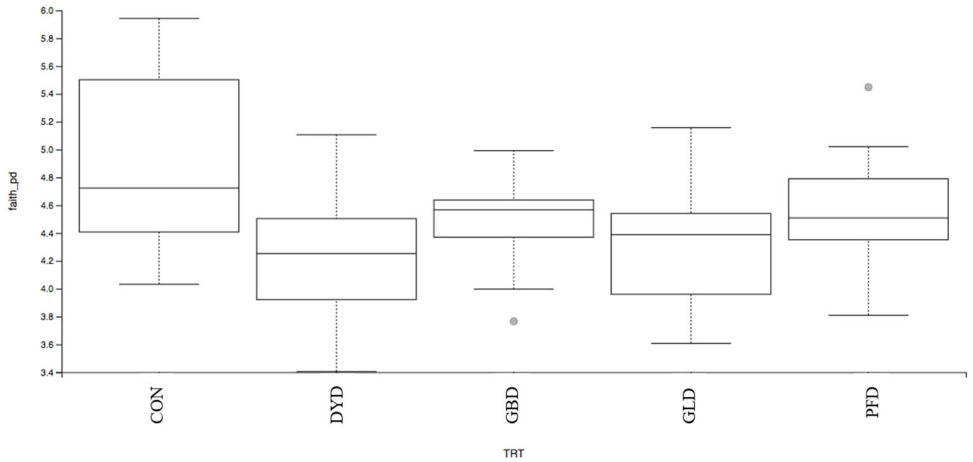

FIGURE 5 | Alpha-diversity analysis of fecal microbial communities, measured by Faith's phylogenetic diversity (PD), of dogs fed diets containing legumes or yeast as the primary protein source. CON, Poultry by-product meal control; GBD, Garbanzo bean; GLD, Green lentil; PFD, Peanut flour; DYD, Dried yeast.

has been shown to decrease trypsin inhibitor activity in extruded soybean meal diets from 2.7 mg/g to 0.6 mg/g (48). Additionally, extrusion of raw lentil flour demonstrated a 98.3% to 99.5% reduction in trypsin inhibitor activity at extrusion temperatures ranging from 140 to 180°C (49). The extrusion temperatures of the diets used in the current study were sufficient in eliminating the trypsin inhibitor activity in the final test diets and, therefore, did not negatively impact protein digestibility.

Urease is an enzyme present in many legumes that is responsible for catalyzing the hydrolysis reaction of urea to ammonia and carbon dioxide (50). Urease activity is measured through the increase in pH caused by the accumulation of ammonia (51), which can be used as an indirect marker of the presence of anti-nutritional factors. Traditionally, urease activity of 0.05 Δ pH units or less is indicative of over-processing of soybeans, while 0.25 ΔpH units or higher indicates over-processing (52, 53). However, this range corresponds to the processing of the soybean ingredient only and may not be applicable to a pet diet matrix where many ingredients have been processed prior to inclusion which explain the lack of urease activity measured in the dietary treatments used in the current study. Additionally, urease is more susceptible to heat treatment than trypsin inhibitors (54). Therefore, it would be expected that there would be negligible urease activity when trypsin inhibitors were also inactivated in the final test diets. One previous study reported similar urease activities in extruded canine diets containing traditional or defatted soybean meal or micronized, toasted, or raw soybeans (55). The urease activity of all of the dietary treatments ranged from 0 to 0.04 Δ pH units after extrusion (55). A different study analyzed the urease activity of extruded canine diets containing either 30% poultry by-product meal or soybean meal and observed slightly higher urease activity (Δ pH of 0.05 and 0.08, respectively) than that measured in the current study (56).

The inclusion of legumes in companion animal diets have been negatively associated with increased gas production and gastrointestinal discomfort (57, 58). Stemming from the rapid fermentation of α-galactooligosaccharides in the hind gut (59). These negative effects have been shown to be mitigated through heat treatment, such as extrusion (60), which may explain the lack of gastrointestinal distress observed in the dogs in the current study. However, inconsistencies in heat treatment impacting oligosaccharide concentrations have been reported (61). The raffinose and stachyose content of dried pea flour in a previous study decreased from 1.6 and 2.0%, respectively, in the raw flour to 0.8 and 1.5%, respectively, after extrusion (62). However, the oligosaccharide content in the diets from the current study did not consistently decrease throughout the stages of extrusion, as was hypothesized. A different study only noted a consistent decrease in sucrose concentration as a result of extruding black bean flour, with variable results measured for stachyose content (63). Pre-treatment of the ingredients, such as soaking or pre-cooking, could aid in decreasing the oligosaccharide content in these ingredients compared with raw ingredients (60). It has also been suggested that both cultivar and processing conditions can impact the ability of the oligosaccharides to be reduced during extrusion (61, 62).

Apparent Total Tract Macronutrient and Energy Digestibility

Nutrient digestibility is an important factor to consider during diet formulation, as the digestibility of nutrients does not occur individually, but results from associative effects in the diet matrix (64). In the current study, all dietary treatments were considered to be well-digested by dogs, with macronutrient ATTD higher than 80% digestible. The only exception was TDF.

The macronutrient ATTD in dogs fed lentils or garbanzo beans has demonstrated variable responses in previous studies. A previous study analyzed a diet containing 69.5% lentils had apparent total tract DM, OM, CP, and fat digestibility at 74.5, 79.3, 79.9, and 89.4%, respectively (27). However, the ATTD of TDF reported for the lentil diet in this study was higher (33.4%)

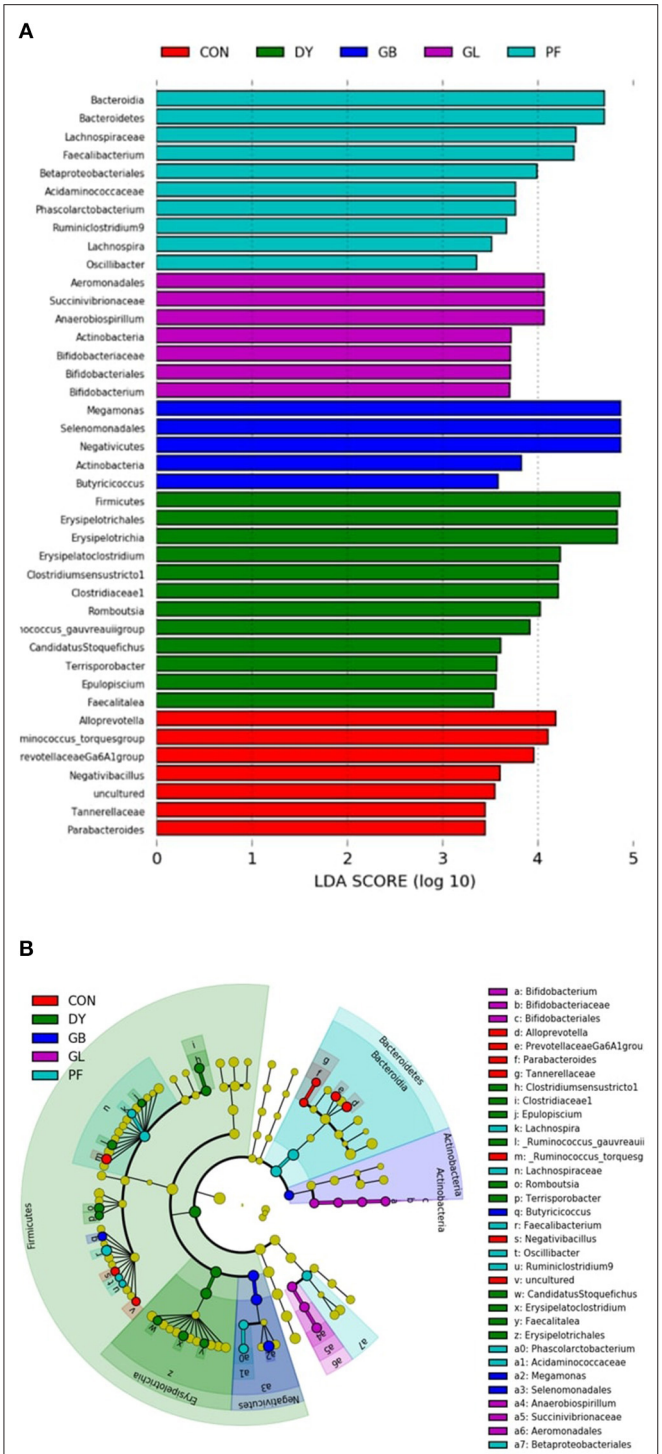

FIGURE 6 | Linear discriminant analysis (LDA; **A**) effect size (LEfSe; **B**) Analysis of fecal microbiota of dogs fed legumes or yeast as the primary protein sources. CON, Poultry by-product meal control; GBD, Garbanzo bean; GLD, Green lentil; PFD, Peanut flour; DYD, Dried yeast.

than the current study (26.7%). A different study measured higher ATTD of DM, OM, and CP in dogs fed a combination of 120 g/kg garbanzo beans and lentils to be 90.7, 92.3, and 92.3%, respectively (64). In dogs fed garbanzo beans and lentils, the ME content was calculated at 3.5 kcal/g (65).

Few studies have focused on evaluating the digestibility of peanut flour. One study analyzed the apparent ileal CP digestibility of a diet containing 31% peanut flour in broiler chickens and growing pigs (66). The apparent ileal CP digestibility of peanut flour was 77% when fed to broiler chickens and 79% when fed to pigs (66). Similarly, in a separate study, broiler chickens fed either 100 or 200 g/kg peanut flour meal had DM ATTD of 72 and 70%, respectively (67). In that study, the ME content of broiler chickens fed 100 or 200 g/kg peanut flour meal were estimated to be 3.77 and 3.91 kcal/g, respectively (67). As mentioned previously, the lowered CP digestibility could be a result of the roasting process of the peanuts which can damage lysine making it unavailable for protein synthesis. In one of the few studies available that evaluated peanut flour in canine diets, dogs were fed a diet containing 7% peanut flour and reported true nitrogen digestibility to be 93% compared with casein (98%), defatted whole egg powder (96%), dried egg albumin (84%), and dried beef muscle (98%) (68).

The digestibility of DYD is consistent with previous studies that evaluated various yeast products in dogs. The effects of a live yeast supplement on macronutrient ATTD in young Beagles was previously analyzed (6). The ATTD of DM (68.4%), ash (23.8%), and CP (66.5%) reported were lower than the digestibility measurements in the current study (6). The ATTD of crude fat (87.7%) measured in that study was similar to the AHF digestibility in the current study (87.9%). However, differences in fat extraction methods should be taken into account when comparing these results as crude fat often underestimates total lipid content and while creating more variability (69). Another study analyzed the effects of supplementing a *Saccharomyces cerevisiae* fermentation product on macronutrient ATTD in adult dogs (7). The apparent total DM, OM, and CP digestibilities of dogs supplemented with 125 mg/d were similar to the current study at 87.0, 89.0, and 87.7%, respectively. Dogs supplemented with 500 mg/d of the fermented yeast product had apparent total DM, OM, and CP digestibilities of 86.5, 88.6, and 86.8%, respectively (7). Lastly, a study measured the macronutrient ATTD of dogs fed 15% brewer's yeast, 15% autolyzed sugarcane yeast, or 15% integral sugarcane yeast (70). The dogs fed 15% brewer's yeast had apparent DM, OM, CP, and AHF digestibilities of 82, 86, 86, and 88%, respectively. Similarly, the DM, OM, CP, and AHF digestibilities of dogs fed integral sugarcane yeast were 82, 86, 83, and 86%, respectively, while the autolyzed sugarcane yeast was slightly lower at 79, 84, 80, and 84%, respectively (70).

Fecal Fermentative End-Products

The production of SCFA is a result of dietary fiber entering the large intestine and being degraded by saccharolytic bacteria (71). In the current study, the fecal concentrations of SCFA from dogs fed the legume- and yeast-based diets are indicative of increased saccharolytic fermentation compared with CON. It has been reported the total SCFA fecal concentrations of dogs fed a diet containing 15% garbanzo beans to be 163 μmol/g compared with a diet containing 15% pea flour (179 μmol/g)

and a commercial diet (182 μmol/g) (72). Acetate, propionate, and butyrate concentrations for the 15% garbanzo bean diet were 68, 47 g, and 15.2 μmol/g, respectively (72). The lower fecal SCFA concentrations reported compared with the current study could be due to the lower inclusion of the garbanzo beans in their diet, resulting in less saccharolytic fermentation in the hindgut.

Acetate is typically the most abundant SCFA produced (73), which is reflected in the acetate concentrations measured for all five dietary treatments in the current study. Fecal concentrations of butyrate in dogs fed DYD more than doubled that of dogs fed the remaining diets, potentially due to the β-glucan content of yeast. Previous studies did not observe the same pattern in butyrate concentrations of dogs supplemented with 125, 250, or 500 mg/d of a fermented yeast product (48.2, 35.6, and 42.8 μmol/g (DMB), respectively) compared with dogs not supplemented with yeast (38.6 μmol/g) (7). The differences may be due to the use of a fermented yeast product rather than whole-body dried yeast, as well as the lower dietary inclusion of the yeast product compared to the level of dried yeast in the current study.

Proteolytic fermentation takes place mainly in the distal large intestine, with BCFA as the primary end-products (74). Few studies are available evaluating fecal BCFA concentrations in dogs fed legume-based sources. However, similar total fecal BCFA concentrations as the current study in dogs fed 15% garbanzo beans at 13.1 μmol/g comprised of 3.8 μmol/g isobutyrate, 7.8 μmol/g isovalerate, and 1.5 μmol/g valerate have been reported (72).

Fecal Microbiota

It has been well-established there is a definitive link between the metabolic roles of the gastrointestinal microbiota and canine health (75). Dietary modulation of the gastrointestinal microbiota can impact gut health and physiology, host immunity, and alter metabolic pathways (76). Previous studies have exemplified the use of legumes and yeast to modify the gastrointestinal microbiome.

In dogs fed 15% chickpea or peas, Firmicutes, Bacteroidetes, and Fusobacterium were the most abundant phyla represented (72), which corroborates the findings in the current study. At the genus level, dogs fed diets containing chickpeas had a lower relative abundance of *Prevotella*, *Alloprevotella*, and *Sutterella* than dogs fed a raw meat diet (72). In that same study, dogs fed a pea-based diet had a higher relative abundance of *Megamonas* compared to dogs fed a raw meat diet. A similar pattern was observed in the current study, with dogs fed GLD and GBD having higher relative abundances of *Megamonas* than dogs fed the remaining treatments. In addition to being a major propionate producer, *Megamonas* expresses α-galactosidase, a key enzyme in the degradation of galacto-oligosaccharides (77). Legume plants, including oilseeds like soybeans and pulses, contain galacto-oligosaccharides (i.e., raffinose, stachyose, and verbascose). These can be hydrolyzed to D-galactose and sucrose by the enzyme α-galactosidase, an enzyme not found in the canine digestive tract. Therefore, the increased relative abundance of *Megamonas* demonstrates an adaptation of the gut microbiota of dogs fed the GLD and GBD treatments to the consumption of oligosaccharides.

One study examined the influence of 25% navy beans on the canine gut microbiota using pyrosequencing of 16S rRNA and determined no changes in relative abundances at the phylum or family level compared with a negative control (a meat and bone meal and rice diet with 0% inclusion of navy bean powder) (78). In mice fed graded levels of lentils (5, 10, 20%), the relative abundance of Firmicutes accounted for 50% of the total phyla for mice fed 10 and 20% lentils but lower (42%) for mice fed 5% lentils compared with the basal diet, indicating the change may be dose-dependent (79). Increased relative abundance of *Bifidobacterium* have been reported when a bean extract diet was fed to mice (80). In this study, Actinobacteria and *Bifidobacteriaceae* were generally increased in dogs fed GLD and GBD in contrast with other treatments groups.

Previous studies have demonstrated that live *Saccharomyces cerevisiae* supplementation in dogs has decreased pathogenic bacteria, such as *Escherichia coli* (6). At the phylum level, dogs supplemented with a yeast fermentative product had a higher relative abundance of Actinobacteria and Firmicutes (7). Additionally, the fecal microbial composition of dogs supplemented with 1.4% yeast cell wall in raw chicken or beef diets. The yeast cell wall inclusion in both chicken and beef diets resulted in an increase of *Megamonas* within the Firmicutes phyla (2.4% in beef and 3.6% in chicken) compared with the 1.7% in dogs fed the control beef diet and 0.6% in dogs fed the control chicken diet (81). Saccharolytic bacteria produce SCFA through a complex cross-feeding system within the gastrointestinal microbiota (73, 82). Firmicutes has been shown to be primarily a butyrate producer in humans (83, 84), which may be an explanation for the increased fecal butyrate concentration measured in the current study.

Although diet is the primary factor determining microbial composition in the gastrointestinal tract (85), individual variation within a population can influence the dietary effect on the gut microbiota. Factors such as life stage, sex, breed, and health status can impact how the microbiota are modulated and are important considerations (86, 87).

CONCLUSIONS

The high inclusion of legumes and yeast in extruded diets was well-accepted by dogs throughout the study. The analyzed serum chemistry and CBC were all within normal ranges for healthy adult dogs for the duration of the study. No negative effects were observed in fecal quality and all diets were highly digestible for all macronutrients. Dogs fed the experimental diets had greater SCFA concentrations than dogs fed CON. In particular, dogs fed DYD had high butyrate concentrations. Therefore, it can be concluded that these proteins are viable novel sources that can safely be included in canine diets, with inclusion levels over 40% for garbanzo beans and green lentils, and near 30% inclusion levels for peanut flour and dry yeast.

DATA AVAILABILITY STATEMENT

The data presented in the study are deposited in the Illinois Data Bank repository, accession number; doi: 10.13012/B2IDB-4677176_V1.

ETHICS STATEMENT

The animal study was reviewed and approved by IACUC.

AUTHOR CONTRIBUTIONS

GD, JH, and MG designed the experiment. LR performed the animal trials. LR and FH performed the laboratory analyses. LR and MG performed the statistical analyses, with fecal microbiota data analyzed by BS and SR-Z. LR wrote the manuscript. All authors provided intellectual input and reviewed this manuscript.

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SUPPLEMENTARY MATERIAL

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REFERENCES

1. Yamka RM, Harmon DL, Schoenherr WD, Khoo C, Gross KL, Davidson SJ, et al. *In vivo* measurement of flatulence and nutrient digestibility in dogs fed poultry by-product meal, conventional soybean meal, and low-oligosaccharide low-phytate soybean meal. *Am J Vet Res.* (2006) 67:88–94. doi: 10.2460/ajvr.67.1.88
2. McCrory MA, Hamaker BR, Lovejoy JC, Eichelsdoerfer PE. Pulse consumption, satiety, weight management. *Adv Nutr.* (2010) 1:17–30. doi: 10.3945/an.110.1006
3. Boye J, Zare F, Pletch A. Pulse proteins: Processing, characterization, functional properties and applications in food and feed. *Food Res Int.* (2010) 43:414–31. doi: 10.1016/j.foodres.2009.09.003
4. Hall C, Hillen C, Robinson JG. Composition, nutritional value, and health benefits of pulses. *Cereal Chem.* (2017) 94:11–31. doi: 10.1094/CCHEM-03-16-0069-FI
5. Moyad MA, Robinson LE, Zawada ET Jr, Kittelsrud JM, Chen DG, et al. Effects of a modified yeast supplement on cold/flu symptoms. *Urol Nurs.* (2008) 28:50–5.
6. Stercova E, Kumprechtova D, Auclair E, Novakova J. Effects of live yeast dietary supplementation on nutrient digestibility and fecal microflora in beagle dogs. *J Anim Sci.* (2016) 94:2909–18. doi: 10.2527/jas.2016-0584
7. Lin C-Y. Alexander C, Steelman AJ, Warzecha CM, de Godoy MRC, et al. Effects of a *Saccharomyces cerevisiae* fermentation product on fecal characteristics, nutrient digestibility, fecal fermentative end-products, fecal microbial populations, immune function, and diet palatability in adult dogs. *J Anim Sci.* (2019) 97:1586–99. doi: 10.1093/jas/skz064
8. Association of American Feed Control Officials. *Official Publication*. Champaign, IL: Association of American Feed Control Officials (2018).
9. Pahm AA, Pedersen C, Stein HH. Application of the reactive lysine procedure to estimate lysine digestibility in distillers dried grains with solubles fed to growing pigs. *J Agric Food Chem.* (2008) 56:9441–6. doi: 10.1021/jf801618g
10. Vorlová L, Borkovcová I, Kalábová K, Večerek V. Hydroxymethylfurfural contents in foodstuffs determined by HPLC method. *J Food Nutr Res.* (2006) 45:34–8.
11. Charissou A, Ait-Ameur L, Birlouez-Aragon I. Kinetics of formation of three indicators of the Maillard reaction in model cookies: influence of baking temperature and type of sugar. *J Ag Food Chem.* (2007) 55:4532–9. doi: 10.1021/jf063024j
12. van Rooijen C, Bosch GA, van der Poel FB, Wierenga PA, Alexander LA, Hendriks WH. Quantitation of Maillard reaction products in commercially available pet foods. *J Agric Food Chem.* (2014) 62:8883–91. doi: 10.1021/jf502064h
13. Association of Official Analytical Chemists. *Official Methods of Analysis*. 17th ed. Gaithersburg, MD: Association of Official Analytical Chemists (2006).
14. American Association of Cereal Chemists. *Approved Methods*. 11th ed. St. Paul, MN: American Association of Cereal Chemists (2006).
15. Budde EF. The determination of fat in baked biscuit type of cat foods. *J Assoc Off Agric Chem.* (1952) 35:799–805. doi: 10.1093/jaoac/35.3.799
16. Prosky L, Asp NG, Schweizer TF, Devries JW, Furda I. Determination of insoluble and soluble dietary fiber in foods and food products: collaborative study. *J AOAC.* (1992) 75:360–7. doi: 10.1093/jaoac/75.2.360
17. Erwin ES, Marco GJ, Emery EM. Volatile fatty acid analysis of blood and rumen fluid by gas chromatography. *J Dairy Sci.* (1961) 44:1768–71. doi: 10.3168/jds.S0022-0302(61)89956-6
18. Chaney AL, Marbach EP. Modified reagents for determination of urea and ammonia. *Clin Chem.* (1962) 8:130–2. doi: 10.1093/clinchem/8.2.130
19. Flickinger EA, Schreijen EMWC, Patil AR, Hussein HS, Grieshop CM, Merchen NR, et al. Nutrient digestibilities, microbial populations, protein catabolites as affected by fructan supplementation of cat diets. *J Anim Sci.* (2003) 81:2008–18. doi: 10.2527/2003.8182008x
20. Churms SC. Carbohydrates. In: Zweig G, Sherma J, editors. *Handbook of Chromatography*. Boca Raton, FL: CRC Press (1982).
21. Kakehi K, Honda S. Silyl ethers of carbohydrates. In: Biermann CJ. and McGinnis GD, editors. *Analysis of Carbohydrates*. Boca Raton, FL: CRC Press (1989). p. 43–5.
22. Caporaso JG, Lauber CL, Walters WA, Berg-Lyons D, Huntley J, Fierer N, et al. Ultra-high-throughput microbial community analysis on the Illumina HiSeq and MiSeq platforms. *ISME J.* (2012) 6:1621–4. doi: 10.1038/ismej.2012.8
23. Callahan BJ, McMurdie PJ, Rosen MJ, Han AW, Johnson AJA, Holmes SP. DADA2: high-resolution sample inference from Illumina amplicon data. *Nat Methods.* (2016) 13:581–3. doi: 10.1038/nmeth.3869
24. Quast C, Pruesse E, Yilmaz P, Gerken J, Schweer T, Yarza P, et al. The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. *Nucleic Acids Res.* (2013) 41:D590–6. doi: 10.1093/nar/gks1219
25. Lozupone C, Knight R. UniFrac: a new phylogenetic method for comparing microbial communities. *Appl Environ Microbiol.* (2005) 71:8228–35. doi: 10.1128/AEM.71.12.8228-8235.2005
26. Elhardallou SB, Walker AF. The water-holding capacity of three starchy legumes in the raw, cooked and fibre-rich fraction forms. *Plant Food Hum Nutr.* (1993) 44:171–9. doi: 10.1007/BF01088382
27. Carciofi AC. Takakura, de-Oliveira SLD F, Teshima E, Jeremias JT, Brunetto MA, et al. Effects of six carbohydrate sources on dog diet digestibility and post-prandial glucose and insulin response. *J Anim Physiol Anim Nutr.* (2008) 92:326–63. doi: 10.1111/j.1439-0396.2007.00794.x
28. Lee Y-Y, Tang T-K, Phuah E-T, Alitheen NBM, Tan C-P, Lai O-M. New functionalities of Maillard reaction products as emulsifiers and encapsulating agents, and the processing parameters: a brief review. *J Sci Food Agric.* (2016) 97:1379–85. doi: 10.1002/jsfa.8124
29. Seiquer I, Diaz-Alguacil J, Delgado-Andrade C, López-Frías M, Hoyos AM, Galdó G, et al. Diets rich in Maillard reaction products affect protein digestibility in adolescent males aged 11-14 y. *Am J Clin Nutr.* (2006) 83:1082–8. doi: 10.1093/ajcn/83.5.1082

30. Hemmler D, Roullier-Gall C, Marshall JW, Rychlik M, Taylor AJ, Schmitt-Kopplin P. Insights into the chemistry of non-enzymatic browning reactions in different ribose-amino acid model systems. *Sci Rep.* (2018) 8:1–10. doi: 10.1038/s41598-018-34335-5
31. van Rooijen C, Bosch G, van der Poel AF, Wierenga PA, Alexander L, Hendriks WH. The Maillard reaction and pet food processing: effects on nutritive value and pet health. *Nutr Res Rev.* (2013) 26:130–48. doi: 10.1017/S0954422413000103
32. Surh YJ, Tannenbaum SR. Activation of the Maillard reaction product 5-(hydroxymethyl)furfural to strong mutagens via allyclic sulfonation and chlorination. *Chem Res Toxicol.* (1994) 7:313–8. doi: 10.1021/tx00039a007
33. McDaniel KA, White BL, Dean LL, Sanders TH, Davis JP. Compositional and mechanical properties of peanuts roasted to equivalent colors using different time/temperature combinations. *J Food Sci.* (2012) 77:C1293–9. doi: 10.1111/j.1750-3841.2012.02979.x
34. Krause R, Knoll K, Henle T. Studies on the formation of furosine and pyridosine during acid hydrolysis of different Amadori products of lysine. *Eur Food Res Technol.* (2003) 216:277–83. doi: 10.1007/s00217-002-0649-0
35. Chiang CH. A simple and rapid high-performance liquid chromatographic procedure for determination of furosine, a lysine-reducing sugar derivative. *J Agric Food Chem.* (1983) 31:1373–4. doi: 10.1021/jf00120a060
36. Bujard E, Finot PA, Madelaine R, van Kiet AL, Deutch R, Isely A. Measure of availability and blocked lysine in industrial milks. *Ann Nutr Alim.* (1978) 32:291–305.
37. van Rooijen C, Bosch G, van der Poel AFB, Wierenga PA, Alexander L, Hendriks WH. Reactive lysine content in commercially available pet foods. *J Nutr Sci.* (2014) 3:1–6. doi: 10.1017/jns.2014.29
38. Williams PA, Hodgkinson SM, Rutherfurd SM, Hendriks WH. Lysine content in canine diets can be severely heat damaged. *J Nutr.* (2006) 136:1998S—2000. doi: 10.1093/jn/136.7.1998S
39. Ames JM. Determination of N epsilon-(carboxymethyl)lysine in foods and related systems. *Ann N Y Acad Sci.* (2008) 1126:20–4. doi: 10.1196/annals.1433.030
40. Teodorowicz M, Hendriks WH, Wichers HJ, Savelkoul HFJ. Immunomodulation by processed animal feed: the role of Maillard reaction products and advanced glycation end-products (AGEs). *Front Immunol.* (2018) 9:2088. doi: 10.3389/fimmu.2018.02088
41. Prosser CG, Carpenter EA, Hodgkinson AJ. Nε-carboxymethyllysine in nutritional milk formulas for infants. *Food Chem.* (2019) 274:886–90. doi: 10.1016/j.foodchem.2018.09.069
42. Palaseweenun, A. Hagen-Plantinga PE, Schonewille JT, Koop G, Butre C, Jonathan M, et al. Urinary excretion of advanced glycation end products in dogs and cats. *J Anim Physiol Anim Nutr.* (2020) 105:1–8. doi: 10.1111/jpn.13347
43. Avilés-Gaxiola S, Chuck-Hernández C, Saldívar SOS. Inactivation methods of trypsin inhibitor in legumes: a review. *J Food Sci.* (2018) 83:17–29. doi: 10.1111/1750-3841.13985
44. Batt AR, St Germain CP, Gokey T, Guliaev AB, Baird T Jr. Engineering trypsin for inhibitor resistance. *Protein Sci.* (2015) 24:1463–74. doi: 10.1002/pro.2732
45. Khattab RY, Arntfield SD. Nutritional quality of legume seeds as affected by some physical treatments 2. Antinutritional factors. *Food Sci Technol.* (2009) 42:1113–8. doi: 10.1016/j.lwt.2009.02.004
46. Kozlowska H, Elkowicz K, Rutkowski A. Thermal inactivation of trypsin inhibitors of soybean prepearations added to meat. *Meat Sci.* (1980) 4:95–102. doi: 10.1016/0309-1740(80)90035-2
47. Hamid H, Thakur NS, Kumar P. Anti-nutritional factors, their adverse effects and need for adequate processing to reduce them in food. *Agric Int.* (2017) 4:56–60. doi: 10.5958/2454-8634.2017.00013.4
48. Romarheim OH, Aslaksen MA, Storebakken T, Krogdahl Å, Skrede A. Effect of extrusion on trypsin inhibitor activity and nutrient digestibility of diets based on fish mal, soybean meal, white flakes. *Arch Anim Nutr.* (2005) 59:365–75. doi: 10.1080/17450390500352897
49. Rathod RP, Annapure US. Effect of extrusion process on antinutritional factors and protein and start digestibility of lentil splits. *Food Sci Tech.* (2016) 66:114–23. doi: 10.1016/j.lwt.2015.10.028
50. Sirko A, Brodzik R. Plant ureases: roles and regulation. *Acta Biochim Pol.* (2000) 47:1189–95. doi: 10.18388/abp.2000_3972
51. Baker EC, Mustakas GC. Heat inactivation of trypsin inhibitor, lipoxygenase and urease in soybeans: effect of acid and base additives. *J Am Oil Chem Soc.* (1973) 50:137–41. doi: 10.1007/BF02640466
52. Silva LMC, Salgado AM, Coelho MAZ. Urease activity. In: Vermelho AB, Couri S, editors. *Methods to Determine Enzymatic Activity.* (2013). p. 292–319. doi: 10.2174/978160805301113010017
53. White CE, Campbell DR, McDowell LR. Effects of dry matter content on trypsin inhibitors and urease activity in heat treated soya beans fed to weaned piglets. *Anim Feed Sci Tech.* (2000) 87:105–15. doi: 10.1016/S0377-8401(00)00168-1
54. Purushotham B, Radhakrishna PM, Sherigara BS. Effects of steam conditioning and extrusion temperature on some anti-nutritional factors of soybabean (Glycine max) for pet food applications. *Am J Anim Vet Sci.* (2007) 2:1–5. doi: 10.3844/ajavsp.'2007.1.5
55. Félix AP, Zanatta CP, Brito CBM, Sá Fortes CML, Oliveira SG, Maiorka A. Digestibility and metabolizable energy of raw soybeans manufactured with different processing treatments and fed to adult dogs and puppies. *J Anim Sci.* (2013) 91:2794–801. doi: 10.2527/jas.2011-4662
56. Tortola L, Souza NG, Zaine L, Gomes MOS, Matheus LFO, R.Vasconcellos S, et al. Enzyme effects on extruded diets for dogs with soybean meal as substitute for poultry by-product meal. *J Anim Physiol Anim Nutr.* (2013) 97:39–50. doi: 10.1111/jpn.12009
57. Abdel-Gawad AS. Effect of domestic processing on oligosaccharide content of some dry legume seeds. *J Food Chem.* (1993) 46:25–31. doi: 10.1016/0308-8146(93)90070-V
58. Rupérez P. Oligosaccharides in raw and processed legumes. *Z Lebensm Unters Forsch A.* (1998) 206:130–3. doi: 10.1007/s002170050228
59. Sosulski FW, Elkowicz L, Reichert RD. Oligosaccharides in eleven legumes and their air-classified protein and starch fractions. *J Food Sci.* (1982) 47:498–502. doi: 10.1111/j.1365-2621.1982.tb10111.x
60. Kelkar S, Siddiq M, Harte JB, Dolan KD, Nyombaire G, Suniaga H. Use of low-temperature extrusion for reducing phytohemagglutinin activity (PHA) and oligosaccharides in beans (*Phaseolus vulgaris* L) cv navy and pinto. *J Food Chem.* (2012) 133:1636–9. doi: 10.1016/j.foodchem.2012.02.044
61. Ai Y, Cichy KA, Harte JB, Kelly JD, Ng PKW. Effects of extrusion cooking on the chemical composition and functional properties of dry common bean powders. *J Food Chem.* (2016) 211:538–45. doi: 10.1016/j.foodchem.2016.05.095
62. Berrios JJ, Morales P, Cámara M, Sánchez-Mata MC. Carbohydrate composition of raw and extruded pulse flours. *J Food Res Int.* (2010) 43:531–6. doi: 10.1016/j.foodres.2009.09.035
63. Berrios JJ, Cámara M, Torija ME, Alonso M. Effect of extrusion cooking and sodium bicarbonate addition on the carbohydrate composition of black bean flours. *J Food Process Preserv.* (2002) 26:113–28. doi: 10.1111/j.1745-4549.2002.tb00856.x
64. Brown RG. Digestibility of pet foods. *Can Vet J.* (1987) 28:314–5.
65. Cargo-Froom CL, Fan MZ, Pfeuti G, Pendlebury C, Shoveller AK. Apparent and true digestibility of macro and micro nutrients in adult maintenance dog foods containing either a majority of animal or vegetable proteins. *J Anim Sci.* (2019) 97:1010–9. doi: 10.1093/jas/skz001
66. Park CS, Helmbrecht A, Htoo JK, Adeola O. Comparison of amino acid digestibility in full-fat soybean meal, two soybean meals, and peanut flour between broiler chickens and growing pigs. *J Anim Sci.* (2017) 95:3110–9. doi: 10.2527/jas.2017.1404
67. Zhang F, Adeola O. Energy values of canola meal, cottonseed meal, bakery meal, and peanut flour meal for broiler chickens determined using the regression method. *Poult Sci.* (2017) 96:397–404. doi: 10.3382/ps/pew239
68. Morgan AF, Hunt CN, Arnrich L, Lewis E. Evaluation of five partially purified protein by nitrogen balance in mature dogs, including a study of the antitryptic activity of egg white. *J Nutr.* (1950) 43:63–75. doi: 10.1093/jn/43.1.63
69. Thiex N. Evaluation of analytical methods for the determination of moisture, crude protein, crude fat, and crude fiber in distillers dried grains with solubles. *J AOAC Int.* (2009) 92:61–3. doi: 10.1093/jaoac/92.1.61
70. Martins MS, Sakomura NK, Souza DF, Filho FOR, Gomes MOS, Rasconcellos RS, et al. Brewer's yeast and sugarcane yeast as protein sources for dogs. *J Anim Physiol Anim Nutr.* (2014) 98:948–57. doi: 10.1111/jpn.12145

71. Morrison DJ, Preston T. Formation of short chain fatty acids by the gut microbiota and their impact on human metabolism. *Gut Microbes.* (2016) 7:189–200. doi: 10.1080/19490976.2015.1134082
72. Sandri M, Sgorlon S, Conte G, Serra A, Monego SD, Stefanon B. Substituion of a commercial diet with raw meat complemented with vegetable foods containing chickpeas or peas affects faecal microbiome in healthy dogs. *Ital J Anim Sci.* (2019) 18:1205–14. doi: 10.1080/1828051X.2019.1645624
73. Zhang LS, Davies SS. Microbial metabolism of dietary components to bioactive metabolites: opportunities for new therapeutic interventions. *Genome Med.* (2016) 8:1–18. doi: 10.1186/s13073-016-0296-x
74. Tiwari UP, Singh AK, Jha R. Fermentation characteristics of resistant starch, arabinoxylan, and β-glucan and their effects on the gut microbial ecology of pigs: a review. *Anim Nutr.* (2019) 5:217–26. doi: 10.1016/j.aninu.2019.04.003
75. Wernimont SM, Radosevich J, Jackson MI, Ephraim E, Badri DV, Macleay JM, et al. The effects of nutrition on the gastrointestinal microbiome of cats and dogs: impact on health and disease. *Front Immunol.* (2020) 11:1266. doi: 10.3389/fmicb.2020.01266
76. Rajoka MSR, Shi J, Mehwish HM, Zhu J, Li Q, Shao D, et al. Interaction between diet composition and gut microbiota and its impact on gastrointestinal tract health. *Food Sci Hum Well.* (2017) 6:121–30. doi: 10.1016/j.fshw.2017.07.003
77. Polansky O, Sekelova Z, Faldynova M, Sebkova A, Sisak F, Rychlik I. Important metabolic pathways and biological processes expressed by chicken cecal microbiota. *Appl Envrion Microbiol.* (2016) 82:1569–76. doi: 10.1128/AEM.03473-15
78. Kerr KR, Forster G, Dowd SE, Ryan EP, Swanson KS. Effects of dietary cooked navy bean on the fecal microbiome of healthy companion dogs. *PLoS ONE.* (2013) 8:e74998. doi: 10.1371/journal.pone.0074998
79. Graf D, Monk JM, Lepp D, Wu W, McGillis L, Roberton K, et al. Cooked red lentils dose-dependently modulate the colonic microenvironment in healthy C57B1/6 male mice. *Nutrients.* (2019) 11:1853–74. doi: 10.3390/nu11081853
80. Song H, Han W, Yan F, Xu D, Chu Q, Zheng X. Dietary *Phaseolus vulgaris* extract alleviated diet-induced obesity, insulin resistance and hepatic steatosis and alters gut microbiota composition in mice. *J Funct Foods.* (2016) 20:236–44. doi: 10.1016/j.jff.2015.10.022
81. Beloshapka AN, Dowd SE, Suchodolski JS, Steiner JM, Duclos L, Swanson KS. Fecal microbial communities of healthy adult dogs fed raw meat-based diets with or without inulin or yeast cell wall extracts as assessed by 454 pyrosequencing. *FEMS Microbiol Ecol.* (2013) 84:532–41. doi: 10.1111/1574-6941.12081
82. den Besten G, van Eunen K, Groen AK, Venema K, Reijngoud DJ, Bakker BM. The role of short-chain fatty acids in the interplay between diet, gut microbiota, and host energy metabolism. *J Lipid Res.* (2013) 54:2325–40. doi: 10.1194/jlr.R036012
83. Louis P, Flint HJ. Diversity, metabolism and microbial ecology of butyrate-producing bacteria from the human large intestine. *FEMS Microbiol Lett.* (2009) 294:1–8. doi: 10.1111/j.1574-6968.2009.01514.x
84. Parada Venegas D, De la Fuente MK, Landskron G, González MJ, Quera R, Dijkstra G, et al. Short chain fatty acids (SCFAs)-mediated gut epithelial and immune regulation and its relevance for inflammatory bowel diseases. *Front Immunol.* (2019) 10:277. doi: 10.3389/fimmu.2019.01486
85. Fan Y, Zhang J. Dietary modulation of intestinal microbiota: future opportunities in experimental autoimmune encephalomyelitis and multiple sclerosis. *Front Microbiol.* (2019) 10:740. doi: 10.3389/fmicb.2019.00740
86. Hang I, Rinttila T, Zentek J, Kettunen A, Alaja S, Apajalahti J, et al. Effect of high contents of dietary animal-derived protein or carbohydrates on canine faecal microbiota. *BMC Vet Res.* (2012) 8:90. doi: 10.1186/1746-6148-8-90
87. Deng P, Swanson KS. Gut microbiota of humans, dogs and cats: current knowledge and future opportunities and challenges. *Br J Nutr.* (2015) 113:S6–17. doi: 10.1017/S0007114514002943

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Miscanthus Grass as a Novel Functional Fiber Source in Extruded Feline Diets

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Although dietary fiber is not considered an essential nutrient in a complete and balanced diet for felines, it provides a substrate for fermentation by gut microbiota, thus promoting gastrointestinal health through the production of fermentative metabolites, as well as improving laxation. The aim of this research was to evaluate the novel fiber source, *Miscanthus* grass (*Miscanthus giganteus*), in comparison with traditional fiber sources and their effects on fecal quality, apparent total tract digestibility (ATTD), fecal fermentative end products, and microbiota of healthy adult cats. Four dietary treatments were evaluated, differing in dietary fiber source. The diets were formulated to meet or exceed the AAFCO (2018) nutritional profile for adult cats and contained either cellulose (CO), *Miscanthus* grass fiber (MF), a blend of *Miscanthus* fiber and tomato pomace (MF + TP), or beet pulp (BP). The study was conducted using a completely randomized design with 28 neutered adult, domesticated shorthair cats (19 females and 9 males, mean age 2.2 ± 0.03 years; mean body weight 4.6 ± 0.7 kg, mean body condition score 5.6 ± 0.6). The experimental period comprised 21 days, and a fresh fecal and a total fecal collection were performed during the last 4 days of the trial period. Daily food intake (DM basis) was similar across all groups ($P > 0.05$). Additionally, treatment did not affect fecal output (as-is or DM basis), fecal score, or fecal pH ($P > 0.05$). Cats fed BP had significantly higher total dietary fiber ATTD than all the other treatments ($P < 0.05$) and the highest concentrations of total short-chain fatty acid, acetate, and propionate ($P < 0.05$), while butyrate concentrations were similar for all treatments ($P > 0.05$). Inclusion of dietary fibers was effective in modulating gut microbiota. Cats fed diets containing *Miscanthus* grass had greater α-diversity than cats fed BP. As no adverse effects on health, fecal quality, or ATTD of macronutrients were observed with the inclusion of 9% *Miscanthus* grass fiber or fiber blend, the data suggest that *Miscanthus* grass fiber and fiber blends are viable alternatives to the traditional dietary fiber sources used in commercial extruded feline diets, being most comparable to cellulose.

Keywords: cats, dietary fiber, fecal microbiota, *Miscanthus* grass, nutrient digestibility, post-biotics

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INTRODUCTION

Since the 1950s, when the term "dietary fiber" was first introduced, several attempts have been made by the scientific community and regulatory bodies to provide a clearer and more encompassing definition of this term (1). This is because a diverse group of substances and ingredients fall under this umbrella term, which differ in many aspects including origin, physicochemical properties, and physiological effects. In 2016, the Food and Drug Administration issued a final ruling on the definition of "dietary fiber." They defined it as "non-digestible soluble and insoluble carbohydrates (with 3 or more monomeric units), and lignin that are intrinsic and intact in plants; isolated or synthetic non-digestible carbohydrates determined by the Food and Drug Administration to have physiological effects that are beneficial to human health" (2). Many studies have shown the broad range of health benefits associated with dietary fiber intake by humans, with a major focus on gut health (3). This interest has spread to companion animal nutrition as well.

Miscanthus grass (*Miscanthus giganteus*) is an ingredient that has the potential to act as a novel dietary fiber source in companion animal diets. This grass is largely composed of insoluble fibers, making it compositionally similar to cellulose, and a potential base for blends with more fermentable fiber sources to develop desirable fiber matrices. It also contains naturally occurring xylooligosaccharides that may provide a prebiotic-like effect; however, more research is required to determine this. As an ingredient, *Miscanthus* grass offers many positive marketing attributes such as "natural," non-GMO, non-by-product, and potentially organic. Limited data are available on the use of *Miscanthus* grass in monogastric diets, especially in regard to its effects on gastrointestinal health. Therefore, the goal of this research was to compare the effects of *Miscanthus* grass fiber to traditional dietary fiber sources and their effects on gastrointestinal tolerance, apparent total tract macronutrient digestibility, and fecal fermentative end products in adult cats fed extruded diets.

The most traditionally used sources of dietary fiber in companion animal diets are cellulose and beet pulp (4). These ingredients, like most fiber sources, have distinct fiber profiles that allow them to provide different physiological effects. Fiber characteristics such as viscosity, solubility, and fermentability determine the functional effects of various dietary fiber sources. Cellulose is made up almost entirely of insoluble, non-viscous fiber. Purified sources of cellulose are highly uniform in composition, while other commercially available sources may be by-products of other industries resulting in more variable compositions. Beet pulp has a mixed composition, consisting of viscous, non-viscous, soluble, and insoluble fibers. The ratios of these fiber portions can be inconsistent, leading to more variability in this product (4). It was hypothesized that the diet containing *Miscanthus* grass would produce results similar to the diet containing cellulose, with the addition of tomato pomace to the *Miscanthus* fiber blend leading to results that are intermediate between cellulose and beet pulp diets.

TABLE 1 | Ingredient composition of treatments containing traditional and novel fiber sources for adult felines.

Ingredient, % as is	Treatment[1]			
	CO	MF	MF + TP	BP
Poultry by-product meal	40.31	40.00	38.31	37.81
Brewers rice	32.00	30.00	32.00	30.00
Poultry fat	8.50	8.81	8.50	9.00
Yellow corn	5.00	5.00	5.00	5.00
Corn gluten meal 60%	5.00	5.00	5.00	5.00
AFB palatant	1.00	1.00	1.00	1.00
Salt	0.50	0.50	0.50	0.50
Choline chloride	0.13	0.13	0.13	0.13
Potassium chloride	0.10	0.10	0.10	0.10
BHT antioxidant	0.10	0.10	0.10	0.10
Mineral premix[2]	0.18	0.18	0.18	0.18
Vitamin premix[3]	0.18	0.18	0.18	0.18
Cellulose	**7.00**	0.00	0.00	0.00
Miscanthus grass	0.00	**9.00**	**7.00**	0.00
Beet pulp	0.00	0.00	0.00	**11.00**
Tomato pomace	0.00	0.00	**2.00**	0.00

[1]*CO, cellulose; MF, M-fiber; MF + TP, M-fiber + tomato pomace; BP, beet pulp.*
[2]*Provided per kg diet: 17.4 mg manganese ($MnSO_4$), 284.3 mg iron ($FeSO_4$), 17.2 mg copper ($CuSO_4$), 2.2 mg cobalt ($CoSO_4$), 166.3 mg zinc ($ZnSO_4$), 7.5 mg iodine (KI), and 0.2 mg selenium (Na_2SeO_3).*
[3]*Provided per kg diet: 11,000 IU vitamin A, 900 IU vitamin D_3, 57.5 IU vitamin E, 0.6 mg vitamin K, 7.6 mg thiamin, 11.9 mg riboflavin, 18.5 mg pantothenic acid, 93.2 mg niacin, 6.6 mg pyridoxine, 12.4 mg biotin, 1,142.1 μg folic acid, and 164.9 μg vitamin B_{12}. The bold values represent the formulated inclusion levels of the traditional and novel dietary fiber ingredients that were evaluated in this study.*

MATERIALS AND METHODS

Diets, Animals, and Experimental Design

Four diets were formulated to meet or exceed the AAFCO nutrient profile for adult cats ($n = 7$ cats/treatment) (5). They were formulated with similar ingredient composition, except for the dietary fiber sources being tested, and to have similar nutrient composition and a targeted total dietary fiber (TDF) content of 15%. To achieve this target, the diets were formulated to contain 7% cellulose (CO), 9% *Miscanthus* grass fiber (MF), 7% *Miscanthus* grass fiber plus 2% tomato pomace (MF + TP), or 11% beet pulp (BP) (**Table 1**).

All animal procedures were approved by the University of Illinois Institutional Animal Care and Use Committee. Twenty-eight neutered, adult domesticated shorthair cats were used in a completely randomized design. At the start of the experiment, all cats were adapted to the CO diet for 7 days. After this control adaptation period, all cats were randomly assigned to one of the four treatment diets and were fed for 21 days to maintain body weight. The CO group consisted of two males and five females [age 2.2 ± 0.3; body weight (BW) 4.3 ± 0.4; body conditions score (BCS) 5.4 ± 0.2]; the BP group, two males and five females (age 2.2 ± 0.3; BW 4.4 ± 0.6; BCS 5.4 ± 0.6); the MF group, three males and four females (age 2.2 ± 0.2; BW 4.9 ± 0.6; BCS 5.9 ± 0.6); and the MF + TP group, two males and five females (age 2.2 ± 0.3; BW 4.7 ± 1.0; BCS 5.6 ± 0.9). During the last 4 days of this period, a total fecal and a fresh fecal collection were performed.

Cats were group housed for 20 h of the day and individually housed in stainless steel cages for 4 h per day for feeding. Feeding occurred twice a day from 08:00 to 10:00 and 15:00 to 17:00. Cats had free access to water at all times. Food refusals were weighed and recorded after each feeding. Body weights and body condition scores were measured and recorded weekly. Cats were housed in the Edward R. Madigan Laboratory in a climate-controlled room with a 14-h light and 10-h dark cycle. Human socialization periods took place at a minimum of two times per week, and cats had access to behavioral enrichments such as scratching posts.

Sample Collection and Preparation

For the duration of the 4-day fecal collection period, cats were housed individually. All feces were collected during this time and composited by cat to determine total fecal output. Each sample also was evaluated for fecal score on a five-point scale (1 = hard, dry pellets; 2 = hard formed, remains firm and soft; 3 = soft, formed and moist stool; 4 = soft, unformed stool; or 5 = watery, liquid that can be poured), and then samples were stored at −20°C for later analysis to determine the apparent total tract digestibility (ATTD) of macronutrients.

A fresh fecal sample was collected from each cat within 15 min of defecation during the 4-day fecal collection period. These samples were also evaluated for fecal pH and score and dry matter. Then, they were aliquoted to determine ammonia, short-chain fatty acid (SCFA), branched-chain fatty acid (BCFA), phenol, and indole concentrations. To determine dry matter (DM) content, duplicates of ∼2 g of feces were dried in a forced air oven at 105°C. For determination of fecal ammonia, SCFA, and BCFA concentrations, 3 g of each fresh sample was placed in a Nalgene bottle and mixed with 3 ml of 2 N hydrochloric acid and stored at −20°C for later analysis. Duplicates of 2 g of each fresh sample were placed into plastic test tubes, covered with parafilm, and stored at −20°C for later analysis of phenols and indoles. Fecal samples allocated for microbiota analysis were stored in 2 ml cryovials and stored at −80°C until analysis.

On 0 and 21 days of the experimental period, cats were fasted overnight, and a blood sample was collected to evaluate blood metabolites and health status. Cats were sedated before collecting 5 ml of blood *via* jugular venipuncture. For complete blood count analysis, 1 ml of blood from each cat was placed in EDTA vacutainer tubes and 4 ml was placed in serum separator tubes (Becton, Dickinson and Company, Franklin Lakes, NJ). Blood analyses were completed by the Clinical Pathology Laboratory at the University of Illinois College of Veterinary Medicine (Urbana, IL).

Experimental diets were subsampled and ground in a Wiley mill (model 4; Thomas Scientific, Swedesboro, NJ) using a size 10-mesh screen resulting in 2 mm average particle size used for proximate laboratory analysis. Total fecal samples were composited for each animal and partially dried in a forced air oven at 57°C. After drying, they were also ground in a Wiley mill to a 2-mm particle size.

Chemical Analyses

After the diet and feces samples were prepared, DM and ash content were determined following the AOAC procedures [(6); methods 934.01 and 942.05). Crude protein concentration was evaluated using the Official Method of AOAC International by measuring total nitrogen with a LECO TruMac (model 630-300-300, Leco Corporation, St. Joseph, MI) (6). Fat content of the diet and fecal samples was determined using acid-hydrolysis and ether extraction following the methods of Budde and the American Association of Cereal Chemists (7, 8). Bomb calorimetry was utilized to determine the gross energy of the samples using a Parr 6200 calorimeter (Parr Instruments Co., Moline, IL). Further analysis of the fecal samples was completed to determine TDF content according to Prosky et al. and the Official Method of AOAC International (methods 985.29 and 991.43) (6, 9). Diet samples were analyzed using the same methods to determine TDF as well as soluble dietary fiber (SDF) and insoluble dietary fiber (IDF) contents.

Gas chromatography was used to measure SCFA and BCFA concentrations in the fresh fecal samples using a modified method of Sunvold et al. (10). These analyses were completed using a Hewlett-Packard gas chromatograph (Model 5890A Hewlett Packard, Avondale, PA) equipped with a flame ionization detector on a column (1.8 m × 4 mm i.d.) packed with GP 10% SP-1200/1% H_3PO_4 on 80/100 Chromosorb W AW (Supelco, Bellefonte, PA). Nitrogen was used as the carrier gas at a flow rate of 45 ml/min. Oven temperature was set at 125°C, the injection port at 175°C, and the detector port at 180°C. Fecal phenol and indole concentrations were measured using a Thermo Scientific TRACE 1300 Gas Chromatograph coupled with a FID in duplicate according to the modified procedure of Flickinger et al. (11). The internal standard used was 5-methylinodle. Following this method, 1-μl sample was injected at 220°C in splitless mode. A Nukol Supelco column (60 m length, 0.32 mm diameter) with a film thickness of 0.25 μm was used to separate the phenolic compounds. The oven temperature was held at 150°C for 1 min and then increased at 25°C per min until reaching 200°C and held at this temperature for 35 min. Ammonia concentration was measured according to the procedures of Chaney and Marbach (12).

Microbial Analysis

Total DNA extraction from fresh fecal samples was completed using a Mo Bio PowerSoil kit (MO BIO Laboratories, Inc., Carlsbad, CA). A Qubit® 3.0 fluorometer (Life Technologies, Grand Island, NY) was used to quantify DNA concentration prior to amplification and sequencing. A Fluidigm Access Array (Fluidigm Corporation, South San Francisco, CA), in combination with Roche High Fidelity Fast Start Kit (Roche, Indianapolis, IN), was used for the amplification of the 16S rRNA gene. The primers 515F (5′-GTGYCAGCMGCCGCGGTAA-3′) and 806R (5′-GGACTACNVGGGTWTCTAAT-3′), targeting a 292-bp fragment of V4 region, were used for amplification (primers synthesized by IDT Corp., Coralville, IA) (13). A Fluidigm-specific primer, forward and reverse tags, was added in accordance with the Fluidigm protocol. A Fragment Analyzer

(Advanced Analytics, Ames, IA) was used to verify the quality of amplicon region and size. A DNA pool was generated through a combination of equimolar amounts of the amplicons from each sample. The pooled samples were selected by size on a 2% agarose E-gel (Life Technologies, Grand Island, NY) and extracted using a Qiagen gel purification kit (Qiagen, Valencia, CA). The pooled, size-selected, and cleaned products were then run on an Agilent Bioanalyzer in order to confirm appropriate profile and mean size. The Roy J. Carver Biotechnology Center at the University of Illinois performed Illumina sequencing on a MiSeq using v3 reagent (Illumina Inc., San Diego, CA). A FASTX-Toolkit (version 0.0.14) removed the Fluidigm tags. Analysis of sequences was completed using QIIME 2.0 and DADA2 (version 1.14) (14, 15). The high-quality (quality value $\geq$ 20) sequence data, derived from the sequencing process, were demultiplexed. An open reference OTU clustered the sequences into operational taxonomic units (OTU), choosing against the SILVA 138 reference OTU database with a 97% similarity threshold (16). The OTUs observed fewer than two times (singletons), as well as OTUs with <0.01% of the total observations, were discarded. An average of 47,315 reads were obtained, with a total of 1,324,844 reads. The number of reads ranged from 39,416 to 56,474 per sample. To analyze for diversity and species richness, the dataset was rarified to 39,415 reads. Weighted and unweighted unique fraction metric (UniFrac) distances were performed by principal coordinate analysis (PCoA) (17).

Statistical Analysis

Data were analyzed using the MIXED Model procedures of SAS version® 9.4 (SAS Institute Inc., Cary, NC). Animal was used as the random effect, and treatment diet was used as the fixed effect in the statistical model. Data normality was checked using the UNIVARIATE procedure, comparing all treatment least-square means. Experiment-wise error was controlled for using Tukey adjustment. The significance level was set at a probability of $P < 0.05$. Pooled standard errors of the mean also were obtained using the MIXED model procedure.

RESULTS

Food Intake and Apparent Total Tract Macronutrient and Energy Digestibilities

The four treatment diets were formulated to contain similar nutrient composition (**Table 1**). This was confirmed through the chemical analysis of the diets (**Table 2**). Treatment did not have a significant effect on daily food intake (DM), wet fecal output (g/day), fecal DM output (g/day), fecal score, or fecal pH ($P > 0.05$) (**Table 3**). Additionally, the ATTD of DM, organic matter (OM), and crude protein (CP) were similar across treatments ($P > 0.05$). Dry matter digestibility ranged from 78.3 to 82.7%. Acid-hydrolyzed fat digestibility of CO (94.5%) was significantly higher compared with that of MF and MF + TP (91.7 and 91.2%, respectively) ($P < 0.05$) with BP being intermediate (92.6%). The BP diet had the highest TDF digestibility (54.2%) when compared with all the other treatments (average 22.1%) ($P < 0.05$). Digestible energy (kcal/g), which was calculated by subtracting fecal gross energy from diet gross energy, was higher for CO (3.94 kcal/g) than MF+TP (3.72 kcal/g) ($P < 0.05$), with MF and BP being intermediate.

TABLE 2 | Chemical composition of treatments containing traditional and novel fiber sources for adult felines.

Item	Treatment[1]			
	CO	MF	MF + TP	BP
Dry matter, %	94.2	93.1	92.4	91.9
			% DM basis	
Organic matter	93.9	93.5	93.8	93.1
Ash	6.1	6.5	6.2	6.9
Acid hydrolyzed fat	17.6	16.7	16.2	16.2
Crude protein	31.3	30.9	29.6	30.3
Total dietary fiber	15.1	15.0	15.7	15.7
Soluble dietary fiber	3.4	3.8	3.2	6.1
Insoluble dietary fiber	11.7	11.2	12.5	9.6
Gross energy, kcal/g	4.7	4.6	4.6	4.5

[1]*CO, cellulose; MF, M-fiber; MF + TP, M-fiber + tomato pomace; BP, beet pulp.*

TABLE 3 | Food intake, fecal characteristics, and total tract apparent macronutrient digestibility of adult felines fed dietary treatments containing traditional and novel fiber sources.

Item	Treatment[1]				
	CO	MF	MF + TP	BP	SEM[2]
Food intake, as is	57.3	57.1	62.9	64.9	4.83
Dry matter, g/day	54.0	53.1	58.1	59.7	4.49
Fecal output, g/day (as is)	27.5	24.6	29.5	37.5	3.97
Fecal output, g/day (DM basis)	11.6	11.3	12.4	10.4	1.21
Fecal score	2.0	1.8	2.0	2.2	0.10
Fecal pH	7.7	7.5	7.3	7.1	0.21
Digestibility, %					
Dry matter	79.1	78.3	78.7	82.7	1.40
			% DM basis		
Organic matter	82.5	81.8	82.1	86.3	1.19
Acid hydrolyzed fat	94.5^{a}	91.7^{b}	91.2^{b}	92.6^{ab}	0.57
Crude protein	84.1	84.6	83.7	83.1	1.29
Total dietary fiber	21.8^{b}	19.1^{b}	25.5^{b}	54.2^{a}	5.44
Digestible energy, kcal/g	3.94^{a}	3.74^{ab}	3.72^{b}	3.85^{ab}	0.05

[1]*CO, cellulose; MF, M-fiber; MF + TP, M-fiber + tomato pomace; BP, beet pulp.*
[2]*Standard error of the mean.*
[a,b]*Means in the same row with different superscript letters are different ($P < 0.05$).*

Fecal Fermentative End Products

Cats fed the MF + TP and MF diets had significantly higher total fecal phenol and indole concentrations than CO- and BP-fed cats ($P < 0.05$) (**Table 4**). Fecal indole concentration followed the same pattern as total phenol and indole ($P < 0.05$), while fecal phenol concentration was not significantly affected ($P > 0.05$). Fecal ammonia concentration was also similar across all treatments ($P > 0.05$). BP resulted in the highest concentrations

of total SCFA, acetate, and propionate ($P < 0.05$), while butyrate concentrations were similar for all treatments ($P > 0.05$). Total BCFA, isobutyrate, and isovalerate concentrations were higher in the MF + TP group than in the CO and BP groups ($P < 0.05$), with MF being intermediate. A similar trend for valerate concentration was observed with the MF + TP group being higher than the BP group ($P < 0.05$), with CO and MF groups being intermediate.

Fecal Microbiota

The fecal microbiota composition for cats fed different dietary fibers was comprised of seven phyla (**Figure 1**) with Firmicutes, Bacteriodota, Proteobacteria, and Actinobacteria corresponding to more than 90% of the total sequences. Cats fed MF and MF + TP treatments had greater ($P < 0.05$) relative abundances of Firmicutes in relation to the BP treatment. Bacteriodota phylum was increased ($P < 0.05$) in cats fed BP in contrast with MF. Proteobacteria was increased ($P < 0.05$) in cats fed BP in contrast with all other dietary groups. A total of 38 families, 102 genera, and 66 species were identified; within those, over 10 and 20 taxonomic groups for family (**Table 5**) and genera (**Table 6**)

TABLE 4 | Fecal fermentative end products for adult felines fed treatments containing traditional and novel fiber sources.

Item (μmol/g DM basis)	Treatment[1]				
	CO	MF	MF + TP	BP	SEM[2]
Total phenols/indoles	1.0^{b}	1.8^{a}	1.8^{a}	0.6^{b}	0.18
Phenols	0.0	0.1	0.1	0.1	0.01
Indoles	1.0^{b}	1.7^{a}	1.7^{a}	0.5^{b}	0.17
Total short-chain fatty acids	168.0^{b}	189.2^{b}	256.5^{b}	583.7^{a}	42.87
Acetate	99.1^{c}	121.3^{bc}	161.4^{b}	390.2^{a}	27.13
Propionate	44.5^{b}	42.4^{b}	61.0^{b}	161.5^{a}	13.66
Butyrate	24.4	25.5	34.2	32.0	3.54
Total branched-chain fatty acids	13.1^{b}	17.2^{ab}	22.7^{a}	12.1^{b}	2.08
Isobutyrate	2.9^{b}	3.6^{ab}	4.6^{a}	3.1^{b}	0.35
Isovalerate	4.5^{b}	5.3^{ab}	7.4^{a}	4.2^{b}	0.65
Valerate	5.7^{ab}	8.3^{ab}	10.2^{a}	4.8^{b}	1.29
Ammonia, mg/g DM	1.2	1.4	1.7	1.4	0.15

[1]*CO, cellulose; MF, M-fiber; MF + TP, M-fiber + tomato pomace; BP, beet pulp.*
[2]*Standard error of the mean.*
[a–c]*Means in the same row with different superscript letters are different (P < 0.05).*

TABLE 5 | Fecal microbial composition (%) at the family level for cats fed treatments containing traditional and novel fiber sources.

Phylum	Family	Treatment[1]				
		CO	MF	MF + TP	BP	SEM[2]
Actinobacteria	*Bifidobacteriaceae*	0.75^{a}	0.02^{b}	0.26^{ab}	0.53^{ab}	0.178
	Coriobacteriaceae	3.97^{a}	3.53^{ab}	2.98^{ab}	2.38^{b}	0.431
Bacteroidota	*Bacteroidaceae*	9.40^{b}	10.39^{ab}	12.26^{ab}	13.80^{a}	1.280
	Prevotellaceae	12.33^{b}	7.99^{b}	9.50^{b}	17.19^{a}	1.536
	Rikenellaceae	0.40	0.62	0.60	0.02	0.207
	Tannerellaceae	1.54^{ab}	1.88^{ab}	2.10^{a}	1.29^{b}	0.232
Firmicutes	*Erysipelotrichaceae*	5.58^{ab}	7.86^{a}	7.14^{ab}	4.97^{b}	0.929
	RF39	0.03^{b}	0.30^{a}	0.00^{b}	0.00^{b}	0.084
	Clostridiaceae	1.13^{ab}	0.41^{b}	0.72^{ab}	1.63^{a}	0.355
	Butyricicoccaceae	1.53^{a}	1.48^{a}	1.38^{a}	0.45^{b}	0.241
	Oscillospiraceae	3.13^{a}	3.21^{a}	3.06^{a}	0.93^{b}	0.373
	Eubacterium coprostanoligenes group	0.68^{a}	0.52^{a}	0.51^{a}	0.01^{b}	0.171
	Anaerovoracaceae	2.13^{a}	2.80^{a}	2.44^{a}	0.49^{b}	0.469
Proteobacteria	*Succinivibrionaceae*	1.76^{b}	0.84^{c}	1.02^{bc}	4.25^{a}	0.282

[1]*CO, cellulose; MF, M-fiber; MF + TP, M-fiber + tomato pomace; BP, beet pulp.*
[2]*Standard error of the mean.*
[a–c]*Means in the same row with different superscript letters are different (P < 0.05).*

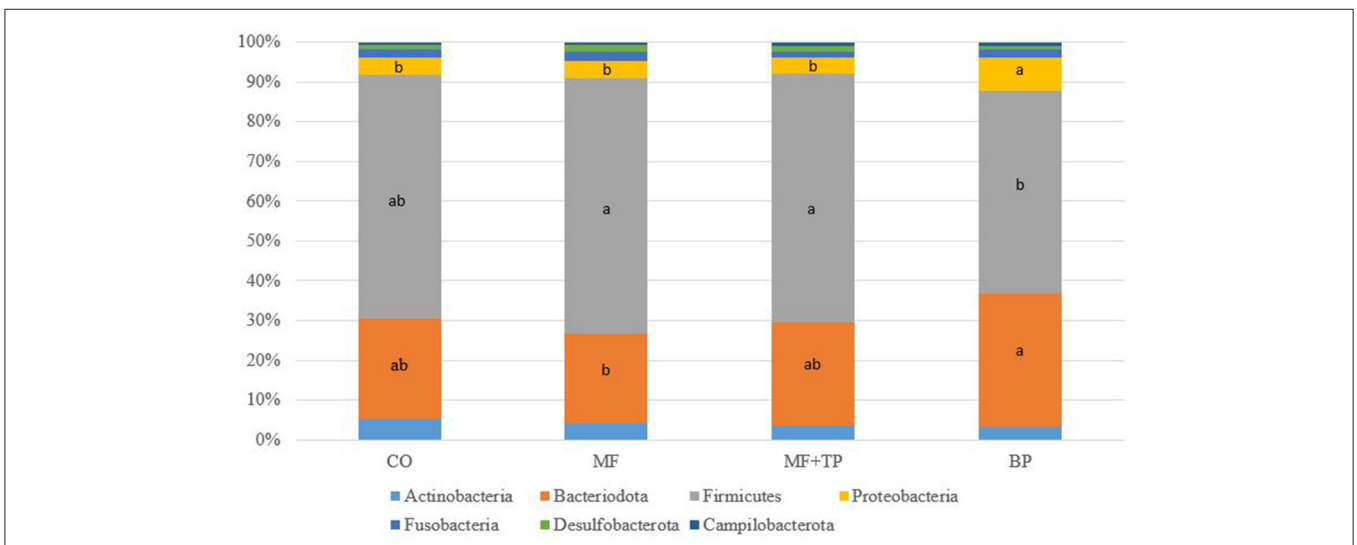

FIGURE 1 | Fecal microbial composition at the phyla level for cats fed diets containing traditional and novel fiber sources.

TABLE 6 | Fecal microbial composition (%) at the genus level for cats fed treatments containing traditional and novel fiber sources.

Phylum	Genus	Treatment[1]				
		CO	MF	MF + TP	BP	SEM[2]
Actinobacteria	*Bifidobacterium*	0.75^{a}	0.02^{b}	0.26^{ab}	0.53^{ab}	0.178
	Collinsella	3.97^{a}	3.53^{ab}	2.95^{ab}	2.38^{b}	0.431
Bacteroidota	*Bacteroides*	9.40^{b}	10.39^{ab}	12.26^{ab}	13.80^{a}	1.280
	Paraprevotella	0.01^{b}	0.12^{ab}	0.20^{a}	0.00^{b}	0.043
	Prevotella	9.22^{b}	5.42^{b}	6.03^{b}	14.80^{a}	1.460
	Alistipes	0.40^{ab}	0.47^{ab}	0.54^{a}	0.02^{b}	0.173
	Parabacteroides	1.54^{ab}	1.88^{ab}	2.10^{a}	1.29^{b}	0.232
	Allobaculum	0.46^{ab}	0.75^{a}	0.31^{ab}	0.08^{b}	0.193
Firmicutes	*RF39*	0.03^{b}	0.30^{a}	0.00^{b}	0.00^{b}	0.084
	Clostridia UCG-014	2.07^{ab}	2.26^{ab}	2.55^{a}	1.42^{b}	0.363
	Butyricicoccus	0.73^{a}	0.56^{ab}	0.45^{ab}	0.19^{b}	0.152
	Butyricicoccaceae UCG-009	0.74^{a}	0.88^{a}	0.82^{a}	0.22^{b}	0.177
	Colidextribacter	1.23^{a}	1.16^{a}	1.22^{a}	0.58^{b}	0.156
	Oscillibacter	0.61^{a}	0.50^{a}	0.44^{a}	0.13^{b}	0.103
	Candidatus soleaferrea	0.26^{ab}	0.33^{a}	0.30^{a}	0.11^{b}	0.065
	Incertae sedis	0.18^{ab}	0.52^{a}	0.29^{a}	0.00^{b}	0.087
	Phocea	0.22^{a}	0.16^{ab}	0.09^{ab}	0.03^{b}	0.056
	Eubacterium coprostanoligenes group	0.68^{a}	0.52^{a}	0.51^{a}	0.01^{b}	0.171
	Mogibacterium	0.24^{ab}	0.29^{ab}	0.43^{a}	0.00^{b}	0.138
	Eubacterium brachy group	0.39^{a}	0.36^{a}	0.22^{ab}	0.08^{b}	0.066
	Eubacterium nodatum group	0.78^{ab}	0.90^{a}	0.89^{a}	0.32^{b}	0.182
	Megasphaera	1.15^{a}	0.77^{ab}	1.26^{a}	0.28^{b}	0.290
Proteobacteria	*Succinivibrio*	1.76^{b}	0.84^{c}	1.02^{bc}	4.25^{a}	0.282

[1]*CO, cellulose; MF, M-fiber; MF + TP, M-fiber + tomato pomace; BP, beet pulp.*
[2]*Standard error of the mean.*
$^{a-c}$*Means in the same row with different superscript letters are different ($P < 0.05$).*

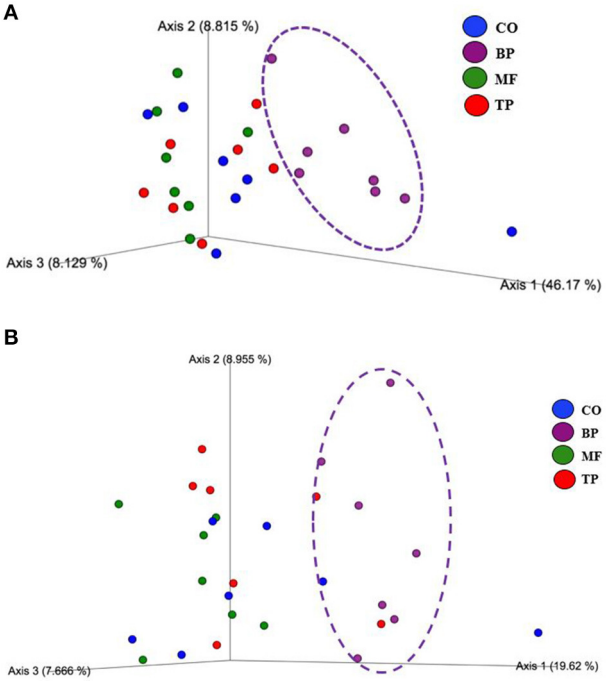

FIGURE 2 | Principal coordinated plots of weighted **(A)** and unweighted **(B)** UniFrac distances of fecal microbial communities of cats fed diets containing traditional and novel fiber sources.

differed ($P < 0.05$) among treatments, respectively. The relative abundance of Bacteroidaceae was greater ($P < 0.05$) for cats fed BP in comparison with those fed CO, but it did not differ for cats fed MF and MF + TP treatments. Cats fed BP also had greater ($P < 0.05$) relative abundance of Prevotellaceae in contrast with cats fed all the other dietary treatments. The relative abundance of Oscillospiraceae, Butyricicoccaceae, and Anaerovoracaceae were consistently higher ($P < 0.05$) in cats fed CO, MF, and MF + TP treatments in contrast with the BP treatment, whereas Succinivibrionaceae was consistently lower ($P < 0.05$) in cats fed those dietary treatments when compared with the BP treatment (**Table 5**).

The relative abundance of Collinsella, a genus within the family Coriobacteriaceae and the phylum Actinobacteria, was greater ($P < 0.05$) in cats fed the CO treatment (4.0%) in contrast with BP (2.4%), and intermediate in cats fed the MF or MF + TP treatments (3.5 and 3.0%, respectively). In contrast, the relative abundance of Prevotella was greater in cats fed BP when compared with cats fed CO (9.2%), MF (5.4%), and MF + TP (6.0%). In addition, the relative abundance of several genera were consistently higher in cats fed CO, MF, and MF + TP, in contrast with BP including Clostridia UCG-014, Butyricicoccaceae UCG-009, Colidextrobacter, Oscillibacter, and Megasphaera. Cats fed BP (4.3%) had greater relative abundance of Succinivibrio than cats fed CO (1.8%) and MF + TP (1.0%), with MF (0.8%) being lowest (**Table 6**).

Beta-diversity based on weighted (**Figure 2A**) and unweighted (**Figure 2B**) UniFrac analysis showed that fecal microbial community composition of cats fed the BP treatment differed (P and q value <0.05) in comparison with cats fed CO, MF, and MF + TP treatments. Alpha-diversity was measured as Pielou evenness, Faith's phylogenetic diversity, and Shannon entropy (**Figure 3**). Fecal microbial diversity and richness based on the Pielou evenness index (**Figure 3A**) revealed that cats fed the BP treatment had lower α-diversity than cats fed the MF + TP treatment ($P < 0.05$ and q value < 0.1). Similarly, the α-diversity of cats fed BP was lower than cats fed other dietary treatments based on Faith's phylogenetic diversity (P and q value < 0.05; **Figure 3B**) and was also lower (P and q value < 0.05) than cats fed MF and MF + TP based on Shannon entropy index (**Figure 3C**).

Serum Chemistry

Blood analysis was performed to determine the health status of the cats during the experimental period. Serum metabolites (**Supplementary Material**) were within normal ranges observed

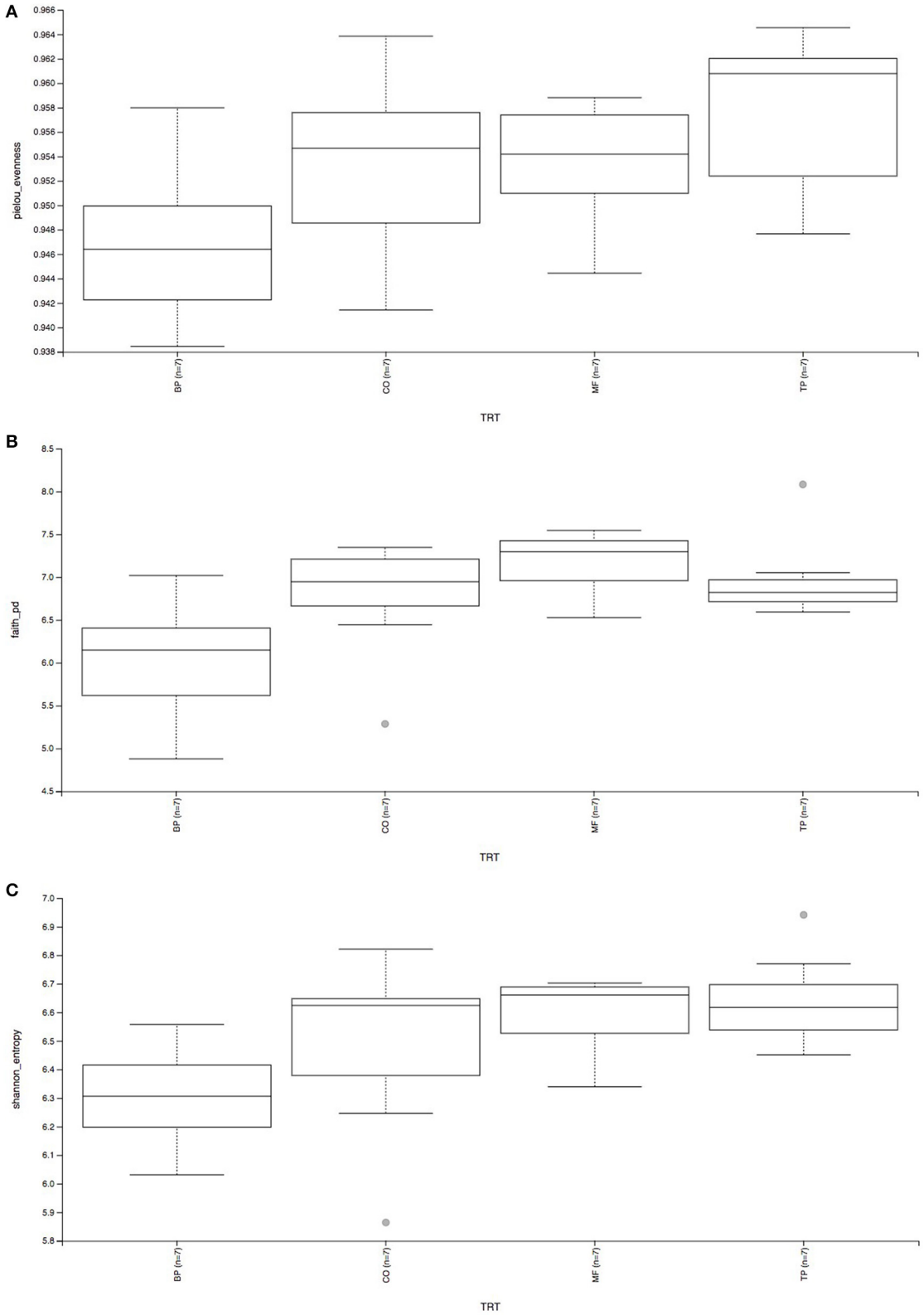

FIGURE 3 | Alpha-diversity analysis of fecal microbial communities, measured by Pielou evenness **(A)**, Faith's phylogenetic diversity **(B)**, and Shannon entropy **(C)** of cats fed diets containing traditional and novel fiber sources.

in healthy adult cats, and no treatment × time interaction or main treatment effect was observed. At baseline, however, the creatinine levels of cats assigned to the MF (1.7 mg/dl) and BP (1.6 mg/dl) treatments were slightly above the reference values. Similarly, cats fed CO (1.6 mg/dl), MF (1.8 mg/dl), and BP (1.6 mg/dl) diets, on 21 days of the experimental period, had slightly higher creatinine levels than the reference range (0.5–1.5 mg/dl).

DISCUSSION

Diet, Food Intake, and Fecal Characteristics

The experimental diets all were formulated to maintain similar ingredients and nutrient composition, differing only in dietary fiber source (cellulose, beet pulp, *Miscanthus* grass fiber, and *Miscanthus* grass fiber and tomato pomace blend). Minimal variations in ingredient inclusion rates were necessary to obtain the targeted TDF content of 15%. This level of TDF was chosen to reflect the higher levels of commercial dietary fiber inclusion in order to emphasize any physiological responses to the various fiber ingredients. The chemical composition of the diets fell within a relatively narrow range, with the CO treatment having a slightly higher acid-hydrolyzed fat (AHF) and CP concentration, as well as a gross energy value. This could be due to the slightly higher inclusion level of poultry by-product meal in this diet. Overall, diets were very close to their target TDF content of 15%. Small variations are expected as TDF content can be affected by the plant's growing conditions, time of harvest, and plant maturity, among other things. The BP diet had higher levels of SDF and lower IDF as was expected based on the typical fiber profile of this ingredient (4). The CO, MF, and MF+TP diets were similar in SDF and IDF contents, which was also expected due to the similar fiber profile of cellulose and *Miscanthus* grass (18). It was predicted that the MF + TP blend might have slightly lower TDF, SDF, and IDF contents than MF as 2% tomato pomace was added at the expense of 2% of the *Miscanthus* grass fiber. The level of tomato pomace in the fiber blend was chosen to reflect the practical commercial inclusion of this ingredient in fiber matrices to increase SDF content. The composition of tomato pomace was reported by Swanson et al. as 56.9% TDF, 4.2% SDF, and 52.7% IDF (DM basis) in comparison with *Miscanthus* grass composition reported by Donadelli and Aldrich as 90% TDF, 7.3% SDF, and 82.7% IDF (DM basis) (18, 19). However, only lower SDF values were observed for the MF + TP blend compared with MF alone (3.2 and 3.8%, respectively), possibly due to the expected TDF variability of plant by-products that was previously mentioned or the low inclusion level of the tomato pomace.

Food intake (g/day) on an as-fed and DM bases did not differ among the treatment groups. Similarly, Donadelli and Aldrich saw no effect on food intake when cellulose, beet pulp, and *Miscanthus* grass were added to the formula at a 10% inclusion rate (20). Detweiler et al. evaluated diets including either 15.5% beet pulp (17.1% TDF), 9.6% cellulose (15.1% TDF), or 14% soybean hulls (16.6% TDF) and observed that cats fed the diet containing beet pulp had lower intake than the diet containing soybean hulls due to feed refusals (21). This indicates that although no effect on palatability was observed in this study with an inclusion level up to 9% *Miscanthus* grass (15.0% TDF), or up to 10% inclusion (13.8% TDF), higher inclusion levels and, subsequently, higher TDF contents may impact palatability and must be considered in the practical utilization of this ingredient (20).

Similar fecal scores were observed for all treatments ranging from 1.81 to 2.18 on a five-point scale. Previous research reported similar fecal scores with 8% inclusion of cellulose (11.2% TDF) and 12.5% inclusion of beet pulp (10.6% TDF), 1.8 and 2.3, respectively (10). Fecal output (g/day) on an as-is basis, as well as on a DM basis, were not significantly different among treatments. However, numerically, cats fed BP had the highest fecal output on an as-is basis and the lowest on a DM basis. This is due to the higher soluble fiber content of beet pulp that has a higher water-holding capacity, therefore increasing fecal water content and overall fecal mass. A similar effect was reported in felines by Detweiler et al. and in other studies across multiple species including canines and swine (21–23).

Apparent Total Tract Macronutrient and Energy Digestibilities

Many studies have reported that dietary fiber sources can impact the digestibility of other macronutrients depending on their level of inclusion and fiber profile. However, DM and OM digestibility did not vary among dietary treatments in this study, and all diets were well-digested by adult cats. The DM and OM digestibility coefficients in the current research were reported to be just a few percentage units higher than similar treatments evaluated by Donadelli and Aldrich who compared diets with 10% inclusions of cellulose, beet pulp, and *Miscanthus* grass and observed that cats fed beet pulp (DM: 81.1%; OM: 85.9%) had significantly higher DM and OM coefficients than cellulose (DM: 75.5%; OM: 79.4%) and *Miscanthus* grass (DM: 76.2%; OM: 80.5%) (20). Kienzle et al. reported that the addition of dietary fiber significantly decreased OM digestibility by cats, and Sunvold et al. reported decreased OM and DM digestibility by cats when compared with a diet with no added fiber source (10, 24). The DM and OM digestibility coefficients reported by Sunvold et al. for the diet containing 12.5% beet pulp (DM: 80.4%; OM: 83.8%) and 8.1% cellulose (DM: 81.0%; OM: 83.5%) were similar to the values obtained for the diets in this study containing 11% beet pulp (DM: 82.7%; OM: 86.3%) and 7% cellulose (DM: 79.1%; OM: 82.5%) (10).

No difference in CP digestibility was detected among treatments in this study with a range of 83.1–84.6%. However, Donadelli and Aldrich observed the cellulose treatment to have a significantly higher CP digestibility than the beet pulp treatment with *Miscanthus* grass being intermediate (86.1, 85.8, and 84.2%, respectively) (20). In contrast, in this study, while not significant, the CP digestibility of the BP treatment was reported to be numerically lower than for all the other treatments. Many similar effects have been reported due to beet pulp's moderate level of fermentability compared with cellulose and other fiber sources with greater concentrations of insoluble fiber (10, 21,

25). Providing greater amounts of substrate for saccharolytic fermentation may result in increased microbial proliferation causing more microbial protein to be present in the feces. The quantification of this microbial nitrogen during analysis can lead to underestimations of actual crude protein digestibility.

The CO treatment resulted in higher AHF digestibility than the treatments containing *Miscanthus* grass fiber (MF and MF + TP). A similar effect was reported by Donadelli and Aldrich (20). The lipid content of the cellulose diet was slightly higher compared with the other treatments in both of these studies, which could have contributed to the higher digestibility. Another possible factor could be the higher lignin content of *Miscanthus* grass compared with cellulose or beet pulp, measured by Donadelli and Aldrich to be 13.68, 0.73, and 6.38%, respectively (18). Lignin has been reported to bind bile acids, inhibiting their action during lipid digestion, and potentially lowering fat digestibility (26). Digestible energy (kcal/g) followed the same pattern as AHF digestibility. The lower fat digestibility and DE can be beneficial tools in the development of diets for overweight and obese cats, which is a serious clinical condition in the pet population. According to these data, *Miscanthus* grass fiber may avoid further reductions in dietary fat content, which may assist maintaining palatability of weight management diets. This is important since weight management or loss diets tend to be formulated with higher concentrations of dietary fiber and lower fat content, resulting in poor acceptance, especially by cats. However, further studies should evaluate the impact of the utilization of *Miscanthus* grass fiber on fecal bile acid concentrations of cats. Since lignin can bind with bile acids in the gastrointestinal tract, it is possible that greater amounts of bile acids will be excreted in the feces, lowering their ability to recycle *via* enterohepatic circulation. This could lead to increased requirements of dietary taurine for cats, since taurine conjugates bile acids to form water-soluble bile salts.

Total dietary fiber ATTD was the highest for the cats fed BP than for all the other treatments. This was expected as beet pulp has been shown to be moderately fermented in the feline intestinal tract in comparison with cellulose, which has a low fermentative potential (10). Both treatments including *Miscanthus* grass fiber (MF: 19.1% and MF + TP: 25.5%) were similar to cellulose (21.8%) in this regard, as they had greater IDF content, which is poorly fermented and, therefore, excreted in higher quantities in the feces. Donadelli and Aldrich reported a similar TDF digestibility coefficient (20.8%) with the inclusion of 10% *Miscanthus* grass (20). While not significantly different, the MF + TP treatment had a numerically higher TDF digestibility than did MF and CO treatments. This could be due to the inclusion of tomato pomace in the fiber blend that was reported by Swanson et al. to have a higher fermentation potential than cellulose using an *in vitro* model with canine fecal inoculum (19).

Fecal Fermentative End Products and Microbiota

Short-chain fatty acids are the major organic end products of saccharolytic fermentation, with increased concentration indicating increased fermentative processes. While not an entirely accurate representation of complete SCFA production in the large intestine, fecal SCFA concentration has been utilized by researchers as a non-invasive method of estimating the production of these fermentative end products by the gut microbiota (27). Total fecal SCFA concentration was the highest in the BP group (583.7 μmol/g, DM basis) compared with all the other treatments. Detweiler et al. evaluated higher levels of beet pulp (15.5% inclusion; 17.1% TDF) that resulted in higher levels of total SCFA (699.7 μmol/g, DM basis), and also observed the beet pulp treatment to produce the highest total SCFA compared with cellulose and soybean hulls (21). Fischer et al. reported similar results when evaluating a diet including 15.5% beet pulp (26% TDF) in overweight cats (28). This increased production of SCFA indicates that beet pulp has higher fermentability compared with the other fiber substrates evaluated, which is supported by the findings of Sunvold et al. who observed that beet pulp had a higher OM disappearance and total SCFA production than did cellulose in an *in vitro* assay using feline fecal inoculum (10). A decrease in gut lumen and fecal pH also is associated with higher fermentative activity as the buildup of these metabolites starts to acidify the environment. However, no difference in fecal pH was observed, with values ranging from 7.1 (BP) to 7.7 (CO).

When evaluating the fecal SCFA on an individual basis, the same trend was observed for fecal acetate and propionate concentrations, being highest for the BP group. Our findings also are supported by Detweiler et al. who reported that cats fed beet pulp had significantly higher fecal concentrations of acetate (459.2 μmol/g, DM basis) and propionate (139.0 μmol/g) compared with cats fed diets with no additional fiber, cellulose, or soybean hulls (average acetate 219.6 μmol/g; average propionate 62.0 μmol/g) ($P < 0.05$) (21). Fischer et al. also observed that when compared with diets containing wheat bran and sugarcane fiber and a diet with no added fiber source (average acetate 217 mM/kg DM; average propionate 95.7 mM/kg), cats fed beet pulp had significantly higher fecal concentrations of acetate (427 mM/kg) and propionate (214 mM/kg) ($P < 0.05$) (28). No statistical differences were observed in fecal butyrate concentration across treatments. However, CO and MF treatments had numerical values (24.4 and 25.5 μmol/g, respectively) that grouped closer together, while MF + TP and BP treatments also were more closely grouped (34.2 and 32.0 μmol/g). It is well-established that SCFAs play a significant role in maintaining gastrointestinal health as they provide energy to colonocytes, reduce inflammation, and have been implicated in the inhibition of cancer (29). While the available substrate is an important factor affecting SCFA production, complex factors such as the removal of fermentative wastes and microbial population composition also play a critical role and are important to consider when evaluating the relationships between dietary components and fermentative metabolites (29).

The fermentation of protein by microbiota in the large intestine results in end products such as ammonia, phenols, indoles, and BCFA. Increases in these compounds often are seen as a negative outcome as they are considered putrefactive compounds that can lead to unwanted fecal malodor (30). No difference was observed in fecal ammonia (1.4–1.7 mg/g DM) or phenol concentration (0.037–0.053 μmol/g, DM) among

treatments. Detweiler et al. also reported no significant difference in these concentrations among treatments including beet pulp, cellulose, and soybean hulls as fiber sources and a treatment with no added fiber source (21). Total phenol and indole and individual indole concentrations followed similar patterns of being higher in the treatment groups containing *Miscanthus* grass fiber (MF and MF + TP). While the indole compound can help to maintain gut homeostasis by promoting barrier functions, regulating inflammation, and possibly aid in satiety, it can also be metabolized into indoxyl sulfate, which is a uremic toxin that has been associated with negative health outcomes in humans such as cardiovascular disease and chronic kidney disease (29). Barry et al. reported higher indole concentrations (1.4 μmol/g, DM) with lower inclusion levels of cellulose (4% inclusion; 7.9% TDF), while Detweiler et al. reported lower indole concentrations (0.7 μmol/g, DM) with higher inclusion levels of cellulose (9.6%; 15.1% TDF) (21, 31). The inclusion level of cellulose and the indole concentration in the current study (7% inclusion; 0.97 μmol/g, DM) were intermediate to the values reported in previous studies. In contrast, Detweiler et al. observed a higher level of indole (1.4 μmol/g, DM) with a higher inclusion rate of beet pulp (15.5% inclusion; 17.1% TDF) (21). Overall, the range of indole concentration observed across treatments in this study was similar to the ranges reported in other studies evaluating healthy adult cats (21, 31).

Increased BCFA concentration indicates that higher levels of peptides and amino acids are present in the large intestine and are available for fermentation. Cats fed the MF + TP diet had greater total BCFA concentrations than cats fed CO and BP treatments. Previous research by Barry et al. indicated that the addition of rapidly fermentable fibers (fructooligosaccharides and pectin) increased total BCFA concentrations compared with cellulose (31). This could explain the effect reported with the addition of tomato pomace in the MF + TP fiber blend, as higher levels of rapidly fermented pectin are generally observed in fruit by-products (4). In contrast, the total BCFA concentrations reported by Barry et al. were much higher (44.0–63.9 μmol/g, DM) with low inclusions (4%) of cellulose, fructooligosaccharides (FOS), and pectin than those observed in the current study (12.06–22.68 μmol/g, DM) (31).

The use of dietary fiber has been an effective strategy in the modulation of gut microbiota to support gastrointestinal and systemic host health. Metabolites produced by gut microbiota (e.g., SCFA) are involved in the beneficial health effects on the host. These metabolites have been described as post-biotics, a term that is ill-defined by the scientific community (32, 33). The domestic cat, despite being a strict carnivore and having a short and unsacculated colon, has considerable capacity for hindgut microbial fermentation and production of fermentative end products (34). In companion animal nutrition, a few studies have evaluated the effects of dietary fiber in the modulation of gut microbiota in cats (35–41); however, most of those studies evaluated the effects of soluble and (or) prebiotic sources [e.g., FOS, lactosucrose, pectin, xylooligosaccharides (XOS)] in contrast to cellulose or a no-added fiber diet. Thus, the effect of *Miscanthus* grass fiber and tomato pomace on fecal microbiota has not been evaluated previously. Characterization of the feline gut microbiota has shown that Firmicutes, Bacteroidetes, Proteobacteria, and Actinobacteria are dominant phyla in adult healthy cats (38, 39, 42, 43). Our findings agree with current literature, even though the relative abundance of each phylum may differ among individuals and based on experimental methods used.

The phyla Firmicutes, Bacteroidetes (Bacteroidota), and Actinobacteria are considered important producers of metabolites that have direct beneficial effect on gut and host health (44). A recent study evaluating the effects of dietary XOS supplementation on the fecal microbiota of healthy cats reported that diets containing either 0.04 or 0.4% of XOS, at the expense of cellulose, resulted in increased relative abundance of Collinsella (2.6–4.4%) and decreased abundance of Megasphaera (0.80–0.82%) in contrast with cats fed a control diet containing 1% cellulose (1.7 and 1.3%, respectively) (41). A similar relative abundance of Megasphaera was observed in cats fed CO, MF, and MF + TP diets (range: 0.8–1.3%) in this study. Lyu et al. also reported increased relative abundance of Ruminococcaceae, Erysipelotrichaceae, and Lachnospiraceae in contrast to cats fed the control diet (41). In the current study, cats fed CO, MF, and MF + TP treatments had increased relative abundance of Allobaculum, a genus within the Erysipleotrichaceae family, and a few genera within the family Ruminococcaceae (i.e., Candidatus Soleaferrea, Incertae Sedis, and Phocea). Garcia-Mazcorro et al. reported increased relative abundance of Veilonellaceae and decreased relative abundance of Gammaproteobacteria in fecal samples of healthy cats during FOS and inulin supplementation (40). In the current study, a greater relative abundance of Megasphaera, a genus within the family Veilonellaceae, and a lower relative abundance of Succinivibrio, a genus within the family Succinivibrionaceae and class Gammaproteobacteria, were observed in fecal samples of cats fed CO, MF, or MF + TP treatments in contrast with cats fed the BP treatment. More recently, Butowski et al. evaluated the fecal microbial communities of cats fed kibble, raw, and raw + fiber diets (45). The fiber sources included in the kibble diet were beet pulp and inulin (% inclusion not provided), and in the raw + fiber diet 2% of inulin and 2% of cellulose were included (as-is basis). In general, lower relative abundance of Collinsella (0.03%) and Bacteroides (0.2%) and greater relative abundance of Prevotella were reported for cats fed the kibble diet in comparison with our findings. The relative abundance of Succinivibrio of cats fed BP (4.3%) was greater than the relative abundance of cats fed the kibble diet (1.2%) as reported by Butowski et al. (45). Differences in the relative abundance of microbial taxa among studies can be affected by many variables including animal variation and differences in methods including DNA extraction, variable region and primers used for sequencing, bioinformatic procedures, and the reference database utilized.

From this study, it is clear that different dietary fibers exert distinctive effects on the modulation of the feline gut microbiota. Cats fed CO, MF, and MF + TP treatments had greater microbial taxa similarities among them, in contrast with cats fed the BP treatment. This effect was evident based on the presence and absence of particular taxa (unweighted UniFrac), as well as their relative abundance (weighted UniFrac). Microbial diversity has been used as an indicator of gut health, as lower microbial diversity has been associated with clinical conditions

including irritable bowel disease and small cell lymphoma in cats (46). Therefore, increased α-diversity observed in cats fed diets containing *Miscanthus* grass fiber may support gut health by maintaining microbial richness and evenness in adult cats. However, healthy cats supplemented with either FOS or apple pomace had decreased α-diversity when compared with baseline values (47). Overall, there were apparent microbial benefits across all dietary treatments. In addition, all cats in this study were healthy, and therefore, microbial shifts should be evaluated on different dietary fiber sources and amounts may be used to modulate gut microbiota and their metabolites in the hindgut of felines.

Serum Chemistry

Serum chemistry and complete blood count analysis were within reference ranges for healthy adult cats. Creatinine was an exception and observed to be slightly higher than the reference range for MF and BP treatments at baseline and for CO, MF, and BP treatments at the end of the trial period. These deviations from the reference range were small and could be due to individual variation among cats. No effect of treatment was observed. Additionally, glucose concentrations were above the normal range. However, this has been observed as a side effect of the sedation used during the blood collection. The results of the serum chemistry and complete blood cell count (data not provided) and the lack of clinical symptoms indicate that the treatments did not result in any negative health outcomes.

Implications

The findings from this research indicate that *Miscanthus* grass fiber is an advisable dietary fiber for adult felines. The addition of *Miscanthus* grass fiber and the MF + TP blend had no detrimental effects on animal health, fecal quality, or macronutrient digestibility. Diet inclusion of *Miscanthus* grass fiber up to 9% (15% TDF) had no negative effect on voluntary food intake, indicating that it had acceptable palatability to the cats. The resulting concentrations of fecal fermentative end products were more similar to those observed in the CO group than in the BP group, as expected from the similarities in the fiber profile of these ingredients. In conclusion, *Miscanthus* grass can be utilized by the pet food industry as an economical and environmentally conscious ingredient that can provide flexibility in the formulation of diets that aim to maximize the health benefits of dietary fiber. *Miscanthus* grass fiber can be effectively used as a base ingredient to develop fiber blends in combination with more soluble and fermentable dietary fiber, including prebiotic sources, which might be beneficial to improve SCFA production and modulate gut microbiota. In this study, inclusion of 2% TP in combination with MF resulted in small numerical increases in fecal SCFA concentrations, suggesting that fiber blends can be used to support gut and host health. Inclusion of dietary fibers was an effective strategy to modulate feline gut microbiota. Cats fed diets containing *Miscanthus* grass fiber had greater α-diversity than cats fed BP. Future studies should further evaluate nutraceutical uses, additional fiber blends, and diet formats, as *Miscanthus* grass fiber can be a functional ingredient in multiple dietary platforms, including weight management, gut health, and hairball control.

DATA AVAILABILITY STATEMENT

The data generated for the study are deposited in the Illinois Data Bank repository, accession number (doi: 10.13012/B2IDB-3595148_V1).

ETHICS STATEMENT

The animal study was reviewed and approved by University of Illinois Animal Care and Use Committee.

AUTHOR CONTRIBUTIONS

MdG designed the experiment. SF and FH performed the laboratory analyses. SF and MdG performed the statistical analyses and wrote the manuscript. BS and SR-Z performed the bioinformatics analysis for fecal microbial analysis. All authors revised and provided intellectual input on this manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2021.668288/full#supplementary-material

REFERENCES

1. Dai FJ, Chau CF. Classification and regulatory perspectives of dietary fiber. *J Food Drug Anal.* (2016) 25:37–42. doi: 10.1016/j.jfda.2016.09.006
2. Food and Drug Administration. Food labeling: revision of the nutrition and supplement facts labels. *Fed Regist.* (2016) 81:33741–999.
3. Anderson JW, Baird P, Davis RH, Ferreri S, Knudtson M, Koraym, et al. Health benefits of dietary fiber. *Nutr Rev.* (2009) 67:188–205. doi: 10.1111/j.1753-4887.2009.00189.x
4. de Godoy MRC, Kerr KR, Fahey GC Jr. Alternative dietary fiber sources in companion animal nutrition. *Nutrients.* (2013) 5:3099–117. doi: 10.3390/nu5083099

5. Association of American Feed Control Officials (AAFCO). *Official Publication*. Champaign, Illinois: AAFCO (2018).
6. Association of Official Analytical Chemists International (AOAC). *Official Methods of Analysis*, 17th ed. Arlington, Virginia: AOAC (2006).
7. Budde EF. The determination of fat in baked biscuit type of dog foods. *J AOAC.* (1952) 35:799–805. doi: 10.1093/jaoac/35.3.799
8. American Association of Cereal Chemists (AACC). *Approved Methods*, 8th ed. St. Paul, Minnesota: AACC (1983).
9. Prosky L, Asp NG, Schwizer TF, De Vries JW, Furda I. Determination of insoluble and soluble dietary fiber in foods and food products: collaborative study. *J Assoc Off Anal Chem.* (1992) 75:360–7. doi: 10.1093/jaoac/75.2.360
10. Sunvold GD, Fahey GC Jr, Merchen NR, Bourquin LD, Titgemeyer EC, Bauer LL, et al. Dietary fiber for cats: *in vitro* fermentation of selected fiber sources by cat fecal inoculum and in vivo utilization of diets containing selected fiber sources and their blends. *J Anim Sci.* (1995) 73:2329–39. doi: 10.2527/1995.7382329x
11. Flickinger EA, Schreijen EM, Patil AR, Hussein HS, Grieshop CM, Merchen NR, et al. Nutrient digestibilities, microbial populations, and protein catabolites as affected by fructan supplementation of dog diets. *J Anim Sci.* (2003) 81:2008–18. doi: 10.2527/2003.8182008x
12. Chaney AL, Marbach EP. Modified reagents for determination of urea and ammonia. *Clin Chem.* (1962) 8:130–2. doi: 10.1093/clinchem/8.2.130
13. Caporaso JG, Lauber CL, Walters WA, Berg-Lyons D, Huntley J, Fierer N, et al. Ultra-high-throughput microbial community analysis on the Illumina HiSeq and MiSeq platforms. *ISME J.* (2012) 6:1621–4. doi: 10.1038/ismej.2012.8
14. Caporaso JG, Kuczynski, Stombaugh J, Bittinger K, Bushman FD, Costello EK, et al. QIIME allows analysis of high-throughput community sequencing data. *Nat Methods.* (2010) 7:335–6. doi: 10.1038/nmeth.f.303
15. Callahan BJ, McMurdie PJ, Rosen MJ, Han AW, Johnson AJA, Holmes SP. DADA2: high-resolution sample inference from Illumina amplicon data. *Nat Methods.* (2016) 13:581–3. doi: 10.1038/nmeth.3869
16. Quast C, Pruesse E, Yilmaz P, Gerken T, Schweer P, Yarza J, et al. The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. *Nucleic Acids Res.* (2013) 41:D590–6. doi: 10.1093/nar/gks1219
17. Lozupone C, Knight R. UniFrac: a new phylogenetic method for comparing microbial communities. *Appl Environ Microbiol.* (2005) 71:8228–35. doi: 10.1128/AEM.71.12.8228-8235.2005
18. Donadelli RA, Aldrich CG. The effects on nutrient utilization and stool quality of beagle dogs fed diets with beet pulp, cellulose, and Miscanthus grass. *J Anim Sci.* (2019) 97:4134–9. doi: 10.1093/jas/skz265
19. Swanson KS, Grieshop CM, Clapper GM, Shields RG Jr, Belay T, Merchen NR, et al. Fruit and vegetable fiber fermentation by gut microflora from canines. *J Anim Sci.* (2001) 79:919–26. doi: 10.2527/2001.794919x
20. Donadelli RA, Aldrich CG. The effects of diets varying in fibre sources on nutrient utilization, stool quality and hairball management in cats. *J Anim Physiol Anim Nutr.* (2020) 104:715–24. doi: 10.1111/jpn.13289
21. Detweiler KB, He F, Mangian HF, Davenport GM, de Godoy MRC. Extruded feline diets formulated with high inclusion of soybean hulls: effects on apparent total tract macronutrient digestibility, and fecal quality and metabolites. *J Anim Sci.* (2019) 97:1042–51. doi: 10.1093/jas/skz014
22. Burkhalter TM, Nerchen NR, Bauer LL, Murray SM, Patil AR, Brent JL, et al. The ratio of insoluble to soluble fiber components in soybean hulls affects ileal and total-tract nutrient digestibilities and fecal characteristics of dogs. *J Nutr.* (2001) 131:1978–85 doi: 10.1093/jn/131.7.1978
23. Serena A, Jørgensen H, Bach Knudsen E. Digestion of carbohydrates and utilization of energy in sows fed diets with contrasting levels and physicochemical properties of dietary fiber. *J Anim Sci.* (2008) 86:2208–16. doi: 10.2527/jas.2006-060
24. Kienzle E, Meyer H, Schneider R. Investigations on palatability, digestibility and tolerance of low digestible food components in cats. *J Nutr.* (1991) 121:S56–7. doi: 10.1093/jn/121.suppl_11.S56
25. Rossoni Serão MC, Fahey GC Jr. Companion animal nutrition as affected by dietary fibre inclusion. In: Delcour JA, Poutanen K, editors. *Fibre-Rich and Wholegrain Foods*. Cambridge, UK: Woodhead Publishing (2013). p. 407–20.
26. Rodriguez-Gutierrez G, Rubio-Senent F, Lama-Munoz A, Garcia A, Fernandez-Bolanos J. Properties of lignin, cellulose, and hemicelluloses isolated from olive cake and olive stones: binding of water, oil, bile acids, and glucose. *J Agric Food Chem.* (2014) 62:8973–81. doi: 10.1021/jf502062b
27. Den Besten G, van Eunen K, Groen AK, Venema K, Reijngoud D-J, Bakker BM. The role of short-chain fatty acids in the interplay between diet, gut microbiota, and host energy metabolism. *J Lipid Res.* (2013) 54:2325–40. doi: 10.1194/jlr.R036012
28. Fischer MM, Kessler AM, de Sá LRM, Vasconcellos RS, Roberti Filho FO, Nogueria SP, et al. Fiber fermentability effects on energy and macronutrient digestibility, fecal traits, postprandial metabolite responses, and colon histology of overweight cats. *J Anim Sci.* (2012) 90:2233–45. doi: 10.2527/jas.2011-4334
29. Zhang, LS, Davies SS. Microbial metabolism of dietary components to bioactive metabolites: opportunities for new therapeutic interventions. *Genome Med.* (2016) 8:1–18. doi: 10.1186/s13073-016-0296-x
30. O'Neill DH, Phillips VR. A review of the control of odour nuisance from livestock buildings: part 3. Properties of the odorous substances which have been identified in livestock wastes or in the air around them. *J Agric Eng Res.* (1992) 53:23–50. doi: 10.1016/0021-8634(92)80072-Z
31. Barry KA, Wojcicki BJ, Middelbos IS, Vester BM, Swanson KS, Fahey GC Jr. Dietary cellulose, fructooligosaccharides, and pectin modify fecal protein catabolites and microbial populations in adult cats. *J Anim Sci.* (2010) 88:2978–87. doi: 10.2527/jas.2009-2464
32. Tsilingiri K, Rescigno M. Postbiotics: what else? *Benef Microbes.* (2013) 4:101–7. doi: 10.3920/BM2012.0046
33. Zółkiewicz J, Marzec A, Ruszczyński, M, Feleszko W. Postbiotics—A step beyond pre- and probiotics. *Nutrients.* (2020) 12:2189. doi: 10.3390/nu12082189
34. Brosey BP, Hill RC, Scott KC. Gastrointestinal volatile fatty acid concentrations and pH in cats. *Am J Vet Res.* (2000) 61:359–61. doi: 10.2460/ajvr.2000.61.359
35. Terada A, Hara H, Kato S, Kimura T, Fujimori I, Hara K, et al. Effect of lactosucrose (4G-B-d-galactosylsucrose) on faecal flora and faecal putrefactive products of cats. *J Vet Med Sci.* (1993) 55:291–5. doi: 10.1292/jvms.55.291
36. Sparkes, AH, Papasouliotis K, Sunvold G, Werrett G, Gruydd-Jones G, et al. Effect of dietary supplementation with fructooligosaccharides on faecal flora of healthy cats. *Am J Vet Res.* (1998) 59:436–40.
37. Kanakupt K, Vester Boler BM, Dunsford BR, Fahey GC Jr. Effects of short-chain fructooligosaccharides and galactooli- gosaccharides, individually and in combination, on nutrient digestibility, faecal fermentative metabolite concentrations, and large bowel microbial ecology of healthy adult cats. *J Anim Sci.* (2011) 89:1376–84. doi: 10.2527/jas.2010-3201
38. Barry KA, Middelbos IS, Vester Boler BM, Swanson KS, Fahey GC Jr. Effects of dietary fiber on the feline gastrointestinal metagenome. *J Proteome Res.* (2012) 11:5924–33. doi: 10.1021/pr3006809
39. Barry KA, Hernot DC, Van Loo J, Fahey GC Jr, de Godoy MRC. Fructan supplementation of senior cats affects stool metabolite concentrations and faecal microbiota concentrations, but not nitrogen partitioning in excreta. *J Anim Sci.* (2014) 92:4964–71. doi: 10.2527/jas.2013-7510
40. Garcia Mazcorro JF, Barcenas-Walls JR, Suchodolski JS, Steiner JM. Molecular assessment of the fecal microbiota in healthy cats and dogs before and during supplementation with fructo-oligosaccharides (FOS) and inulin using high-throughput 454-pyrosequencing. *Peer J.* (2017) 8:e3184. doi: 10.7717/peerj.3184
41. Lyu Y, Debevere S, Bourgeois H, Ran M, Broeckx BJG, Vanhaecke L, et al. Dose-dependent effects of dietary xylooligosaccharides supplementation on microbiota, fermentation and metabolism in healthy adult cats. *Molecules.* (2020) 25:50030. doi: 10.3390/molecules25215030
42. Desai AR, Musil KM, Carr AP, Hill JE. Characterization and quantification of feline faecal microbiota using cpn60 sequence-based methods and investigation of animal-to-animal variation in microbial population structure. *Vet Microbiol.* (2008) 137:120–8. doi: 10.1016/j.vetmic.2008.12.019
43. Garcia-Mazxorro JF, Lanerie DJ, Dowd SE, Paddock CG, Grutzner N, Steiner JM, et al. Effect of a multi-species synbiotic formulation on faecal bacterial microbiota of healthy cats and dogs as evaluated by pyrosequencing. *FEMS Microbiol Ecol.* (2011) 78:542–54. doi: 10.1111/j.1574-6941.2011.01185.x
44. Suchodolski JS. Diagnosis and interpretation of intestinal dysbiosis in dogs and cats. *Vet J.* (2016) 215:30–7. doi: 10.1016/j.tvjl.2016.04.011

45. Butowski CF, Thomas DG, Young W, Cave NJ, McKenzie CM, Rosendale DI, et al. Addition of plant dietary fibre to a raw red meat high protein, high fat diet, alters the faecal bacteriome and organic acid profiles of the domestic cat (Felis catus). *PLoS ONE.* (2019) 14:e0216072. doi: 10.1371/journal.pone.0216072
46. Marsilo S, Pilla R, Sarawichitr B, Chow B, Hill SL, Ackermann MR, et al. Characterization of the fecal microbiome in cats with inflammatory bowel disease or alimentary small cell lymphoma. *Sci Rep.* (2019) 9:1–11. doi: 10.1038/s41598-019-55691-w
47. Hall JA, Jackson MI, Jewell DE. Chronic kidney disease in cats alters response of the plasma metabolome and fecal microbiome to dietary fiber. *PLoS ONE.* (2020) 5:e0235480. doi: 10.1371/journal.pone.0235480

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Effects of Vitamin D_2 and 25-Hydroxyvitamin D_2 Supplementation on Plasma Vitamin D Epimeric Metabolites in Adult Cats

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Feline vitamin D status is based on dietary consumption but metabolism of this essential nutrient and the efficacy of supplementation forms are poorly described in cats. The aim of this study was to further elucidate the metabolites of vitamin D_2 in cats and to compare the effectiveness of vitamin D_2 and 25(OH)D_2 for increasing feline vitamin D status. Eight adult male castrated domestic shorthair cats received vitamin D_2 or 25(OH)D_2 in a single crossover design. Vitamin D_2 was dosed daily in a molar equivalent dosage to vitamin D_3 ingested in the diet while 25(OH)D_2 was provided at a daily dose of 20% molar equivalent intake of dietary vitamin D_3 based on its expected higher potency. Plasma concentrations of 25-hydroxyvitamin D epimers were evaluated at baseline then every 2 weeks for a total of 10 weeks. Analysis of multiple vitamin D metabolite concentrations was completed at the end of each supplementation period, followed by a washout period preceding the second phase of the crossover trial. Results showed that supplementation with 25(OH)D_2 more effectively and rapidly raised circulating 25(OH)D_2 levels in cat plasma compared to vitamin D_2. Formation of C-3 epimers of 25(OH)D_3, 25(OH)D_2, and 24,25*R*$(OH)_2D_3$, but not 24,25$(OH)_2D_2$, were observed in feline plasma. The abundant concentrations of epimeric forms of vitamin D metabolites found in circulation suggest that these metabolites should be considered during vitamin D analyses in cats. Further studies using 25(OH)D and vitamin D_2 forms are needed to conclude safety and efficacy of these vitamers for supplementation in this species.

Keywords: feline, C-3 epimer, cholecalciferol, ergocalciferol, 24,25-dihydroxyvitamin D, 3-epi-24,25$(OH)_2D_2$, 1,25$(OH)D_2$, calcitriol

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INTRODUCTION

Unlike some mammals, cats cannot synthesize adequate vitamin D from UV light and therefore have a dietary requirement for this nutrient (1). Cholecalciferol (vitamin D_3) is found in animal tissues and is provided in most commercial feline diets through ingredients or supplementation (2). Ergocalciferol (vitamin D_2) is a fungal and plant source of vitamin D (3) that is currently found in feline supplements for homemade diets. This may offer an alternative for cats that must avoid specific animal protein sources due to adverse food reactions or food allergies.

Additionally, vitamin D_2 is less likely to induce toxicity in cats when provided at an equivalent dose to D_3 (4). However, knowledge regarding vitamin D metabolism, including utilization of vitamin D_2, in cats is extremely limited.

Ingested vitamin D is absorbed from the diet via enterocytes, incorporated into plasma lipoproteins, and transported to the liver by vitamin D binding protein, where it is stored and hydroxylated to 25(OH)D (5–7). This metabolite is then transported to the kidney where it is hydroxylated again at position 1 of the A-ring to form the hormone $1,25(OH)_2D$, or calcitriol (8). Together with other metabolites such as $24,25(OH)_2D$ and 1,24,25-dihydroxyvitamin D ($1,24,25(OH)_2D$), this hormone plays an important role in calcium homeostasis, skeletal health, and possibly other physiological functions (9, 10). Epimeric forms of many of these vitamers have been identified in multiple species, with an epimer at position 3 of the A-ring of 25-hydroxyvitamin D_3 (3-epi-$25(OH)D_3$) recently identified in cats (11). The function of C-3 epimers of vitamin D metabolites is unknown, but they appear to be endogenously produced and biologically active metabolites (12–17).

Since all vitamin D is essentially converted to 25(OH)D and this metabolite is found abundantly in circulation, it is the most commonly measured indicator of vitamin D status (18). Circulating $25(OH)D_3$ is an active metabolite and has been found to be more potent at influencing vitamin D status than vitamin D_3 when administered as a supplement to dogs (19, 20), rats (21, 22), chicks (21), and humans (23, 24). In fact, $25(OH)D_3$ is reported to be up to 10 times more potent than vitamin D_3 in humans (23) and may prove an effective form of supplementation for people and animals in diseases such as chronic kidney disease and cardiovascular disease (23, 25, 26).

Vitamin D supplementation is common in humans, and both cholecalciferol and ergocalciferol are used. With increasing recognition of vitamin D status in companion animals, supplementation may become more common in the future and supplement form will need to be considered (2). Although research in cats is limited, one study suggests a lower bioavailability of vitamin D_2 compared to D_3 in felines (27). Cats are not the only species that appear to discriminate against vitamin D_2; a similar pattern is observed in pigs (28), chicks (28, 29), horses (30), and monkeys (31, 32). Rats, on the other hand, exhibit a preference for vitamin D_2 (28, 33). In humans, vitamins D_2 and D_3 have generally been considered equipotent, though a recent meta-analysis suggests a superiority of D_3 in raising 25(OH)D concentrations compared to D_2 (34). Numerous explanations for the increased potency of D_3 have been suggested, including greater affinity for hydroxylase enzymes and vitamin D receptors (32, 35–37). Most of the vitamin D_3 metabolites have a D_2 counterpart, suggesting a similar metabolic pathway based on 25-hydroxylation (38). Additionally, a unique 24-hydroxylation pathway occurs in vitamin D_2 metabolism to form metabolites ($24(OH)D_2$, $1,24(OH)_2D_2$, $1,24,25(OH)_3D_2$), that appear less potent than those derived from vitamin D_3, which may lead to more rapid deactivation of vitamin D_2 (39–44).

Given the paucity of information surrounding vitamin D metabolism in cats in general, and the unknown effectiveness of vitamin D_2 supplementation in cats specifically, the purpose of this study was to investigate vitamin D metabolism when cats are supplemented vitamin D_2 or $25(OH)D_2$ in addition to vitamin D_3 found in the diet. As vitamin D_2 is derived from non-animal sources, its use in cats as a supplement in place of vitamin D_3 is hypothetically advantageous in cases of suspected allergic reaction to food of animal origin. In many such cases, a tolerated meat, poultry, or fish source is empirically identified and in turn used as a dietary ingredient, though it may contain vitamin D_3 and thereby provide a background amount of vitamin D_3 (45). Impetus for study of $25(OH)D_2$ relates to reputed superior bioavailability of 25(OH)D compared to vitamin D (46). Supplementation with 25(OH)D might correct vitamin D deficiency when dietary vitamin D is ineffective, as in cases of inflammatory bowel disease, which are associated with vitamin D deficiency, and may occur secondary to allergic intolerance of food (47). Specifically, the aims of this study were: (1) to identify vitamin D metabolites present in cats, including epimeric forms of vitamin D_2 and D_3 metabolites that have been previously identified in other species, and (2) to compare the effectiveness of vitamin D_2 and $25(OH)D_2$ for increasing vitamin D status in cats. A difference in effectiveness was hypothesized based on reports in other species of greater potency of $25(OH)D_3$ compared to vitamin D_3.

MATERIALS AND METHODS

Animals

Eight university-owned, purpose-bred male castrated domestic shorthair cats were included in the study. The median age of cats was 7.5 years (range 7–12 years) with median body weight of 4.7 kg (range 4.2–5.9 kg). Cats were considered healthy based on physical examination, activity, and results of complete blood count, plasma biochemistry, and total T4 concentrations evaluated immediately prior to enrollment. Cats were housed in an American Association for Laboratory Animal Science accredited, temperature, humidity, and light cycle-controlled facility. They were individually housed for ∼8 h during the day to monitor food intake, then group-housed in two separate groups for the remainder of the day. Food was provided once daily in the morning (typically 7:30–9:30); water was available at all times. Prior to beginning and every 2 weeks during the study, each cat's body weight was recorded using the same scale immediately before feeding. Body condition scoring using a 9-point scale (48) was also performed. Venous blood was then collected by jugular venipuncture and plasma was collected every 2 weeks for vitamin D metabolite determination. Biochemical analyses, including complete blood count and clinical biochemistry, were performed prior to and at the end of the experiment. Care of animals followed National Institutes of Health Guide for the Care and Use of Laboratory Animals (49) and was approved by the Animal Care and Use Committee of the University of Missouri (Protocol 9214).

Diet

The cats were maintained exclusively on the same commercial dry extruded feline diet (**Supplementary Table 1**) at an energy

intake known to maintain body weight for each individual (48–61 g/d; 877–1,110 kJ/d). This diet was fed for several months prior to initiation of the study and was readily accepted by the cats. Consumption of the offered amount was recorded daily in grams. As indicated by its label claim, the diet passed Association of American Feed Control Officials (AAFCO) feeding trials for maintenance of adult cats. A single batch of diet was fed throughout the duration of the experiment. Vitamin D_3 and D_2 concentrations in the study batch were measured by an external laboratory (Eurofins Nutrition Analysis Center, Des Moines, IA, USA). Vitamin D_3 content was 318 IU/100 g with no detectable (<4 IU/100 g) vitamin D_2.

Experimental Design

A single crossover design was used. Firstly, the cats were systematically assigned to one of two treatment groups. For this, the cats were ranked based on dietary intake of vitamin D_3 then assigned to the groups so that mean vitamin D_3 intakes between the groups were similar. Four cats initially received vitamin D_2 (Sigma-Aldrich, St. Louis, MO, USA) daily in a molar equivalent dosage to the vitamin D_3 they were ingesting from the diet based on commercial analysis of the diet and daily intake (0.80–0.86 μg/kg body weight per d). The other four cats initially received 25(OH)D_2 (Isosciences, Ambler, PA, USA) daily at a dosage that was equal to 20% of the daily molar equivalent intake of dietary vitamin D_3 (0.16–0.18 μg/kg body weight per d). Selected dosage of 25(OH)D_2 was based on data obtained by one of the investigators (RCB) in a prior canine study, indicating an ∼5-times greater oral potency of 25(OH)D_3 relative to vitamin D_3 for affecting change in plasma 25(OH)D_3 concentration (50). In this study, extraordinarily high plasma concentrations of 25(OH)D_3 resulted after only 2 weeks of dosing of 25(OH)D_3 at 2.3 μg/kg$^{0.75}$. Due to concern about potential hypervitaminosis D, potency of 25(OH)D_3 relative to vitamin D_3 in this work was not estimated from equilibration concentrations of 25(OH)D_3 in plasma. Rather, the relative potency was estimated from differences in rate of change in plasma 25(OH)D_3 concentration from baseline values. For dosing of 25(OH)D_2 in the present study, ethanol was used for dilution of stock solutions of vitamin D_2 and 25(OH)D_2 to 0.5 and 0.1 μg/μL, respectively. Each day, a small volume (7–10 μL) of ethanolic stock solution was applied to a single kibble of diet mixed in with the meal for voluntary consumption. Plasma concentrations of 25(OH)D_3 and its C-3 epimer, as well as 25(OH)D_2 and its C-3 epimer, were evaluated initially and then every 2 weeks. Following observation of an apparent plateau in plasma concentration of 25(OH)D_2 among the cats after 10 weeks, analyses of multiple vitamin D metabolite concentrations were completed. Thereafter, the D_2 vitamer supplementations were discontinued for a washout period of 12 weeks prior to beginning the second phase of the crossover. Length of the washout period was determined by monitoring plasma concentrations of 25(OH)D every 2 weeks until the concentrations of 25(OH)D_2 were below the level of quantification. Limits of quantification and detection were defined as follows: peaks of UV absorbance recorded during reverse-phase HPLC were quantified if they coincided with the retention time of D metabolite standard and their amplitude was 5-times greater than the amplitude of the standard deviation of background noise. Metabolites were considered detected when a peak coincided with retention time of standard and amplitude was 2-times greater than standard deviation of background noise. Limits of quantification for 25(OH)D_3, 3-epi-25(OH)D_3, 25(OH)D_2, 3-epi-25(OH)D_2, 24,25$(OH)_2D_3$, 3-epi-24,25$(OH)_2D_3$, and 24,25$(OH)_2D_2$ were determined to be 3.0, 2.0, 3.0, 3.8, 4.7, 10.1, and 9.1 ng/mL, respectively.

Laboratory Analyses

Clinical hematology (Sysmex xT-2000i; Sysmex, America, Inc.) and plasma chemistry analyses (Beckman AU480; Beckman Coulter, Inc.) were performed at the University of Missouri Veterinary Medical Diagnostic Laboratory, Columbia, MO prior to and at the end of each 10-week supplementation period. Clinical hematology and plasma chemistry were analyzed immediately after each supplementation period to confirm there were no adverse effects of supplementation. Plasma was also stored at −20°C for repeat chemistry analysis at the end of the trial to reduce inter-assay variation. Serum was reserved after each supplementation period and stored at −20°C, then analyzed for parathyroid hormone (PTH) and ionized calcium concentrations at an external laboratory (Michigan State University Veterinary Diagnostic Laboratory, East Lansing, MI, USA) upon conclusion of the study.

Plasma 25(OH)D Analyses

For monitoring of vitamin D status every 2 weeks, plasma concentrations of 25(OH)D_3 and 25(OH)D_2 and their respective C-3 epimers were determined using extraction and HPLC methods of Lensmeyer and colleagues (51) with modifications. The modifications were required because quantification of some metabolites was precluded by the overlap of HPLC peaks of 3-epi-25(OH)D_3 and 25(OH)D_2, as reported elsewhere (52). For the determinations, 0.5 mL of plasma was incubated overnight (14–16 h) with internal standard, tritiated 25(OH)D_3 (25-[26,27-^{3}H]-hydroxyvitamin D_3) (Perkin Elmer, Boston, MA, USA). The plasma was thereafter combined with 1.0 mL acetonitrile (CH_3CN), incubated for two 5 min periods, twice vortexed, and for 10 min centrifuged at 2,000 × g at ambient temperature (22–24°C). The supernatant was diluted with 0.5 mL water and loaded on a conditioned solid-phase extraction column (Strata^{TM}-X, 33 μm, 60 mg, Phenomenex, Torrance, CA, USA). The column was washed with 2 mL CH_3CN-water (35:65, v/v) and eluted with 2 mL CH_3CN. The eluent was dried by centrifugal evaporation for 75 min. Eluent residue was reconstituted with 250 μL hexanes-2-propanol (94:6, v/v). A portion of the reconstitute (200 μL) was injected on an equilibrated (2.0 mL/min, hexanes-2-propanol, 94:6, v/v) normal-phase column (4.6 x 250 cm, Zorbax Sil, Agilent Technologies, Santa Clara, CA, USA). Eluted fractions containing metabolites of 25(OH)D_2 and 25(OH)D_3 were dried and then reconstituted with 100 μL methanol and diluted with 50 μL water as needed for subsequent quantification by reverse-phase HPLC.

A portion (120 μL) of the reconstituted normal-phase fractions containing 25(OH)D_3 metabolites was injected on to a heated (50°C), reverse-phase HPLC column (Zorbax SB-CN,

Agilent Technologies, Santa Clara, CA, USA), equilibrated with methanol-water (67:33, v/v), flowing at 1.2 mL/min, as described by Lensmeyer et al. (51). Eluting 25(OH)D_3 and 3-epi-25(OH)D_3 were quantified from area-under-the-curve (AUC) of peak UV absorbance at 265 nm. Eluting 25-[26,27-^{3}H]-hydroxyvitamin D_3 was collected, dried, and quantified by liquid scintillation counting. Reconstitute of the normal-phase fraction containing 25(OH)D_2 metabolites was spiked with internal standard (25 ng of 25(OH)D_3) and a portion of it (120 μL) injected on the reverse-phase column for quantification of 25(OH)D_2, 3-epi-25(OH)D_2, and internal standard.

For calculation of the plasma metabolite concentrations, recovery of all the metabolites was assumed to be in equivalent proportion to recovery of tritiated internal standard, 25-[26,27-^{3}H]-hydroxyvitamin D_3. This assumption was verified for 25(OH)D_2 and 3-epi-25(OH)D_2 in pooled plasma collected from cats not consuming dietary vitamin D_2. For this, plasma aliquots (0.5 mL, $n = 5$) were spiked with 25 ng of 25(OH)D_2, 25 ng of 3-epi-25(OH)D_2, and 1,200 dpm of 25-[26,27-^{3}H]-hydroxyvitamin D_3. Mean (± standard deviation) recoveries of the metabolites were determined to be 43.1 ± 5.9, 44.0 ± 2.6, and 47.3 ± 2.6%, respectively.

Mono- and Di-Hydroxyvitamin D Metabolites

Plasma collected during the last week of each supplementation period was additionally analyzed for di-hydroxyvitamin D metabolites. For this, 1.0 mL of plasma was extracted by methods of Hollis (53) following overnight incubations with methanolic solutions (10 μL) of internal standards: 25-[26,27-^{3}H]-hydroxyvitamin D_3 and 1α,25-[26,27-^{3}H]-dihydroxyvitamin D_3 (Perkin Elmer Boston, MA, USA). Samples were vortex-mixed for 20 s in 1.0 mL CH_3CN and centrifuged (2,000 × g) for 20 min at ambient temperature. Resulting supernatant was vortex-mixed with 1.0 mL water and applied to a conditioned solid-phase column (Bond Elut C18; Agilent Technologies, Santa Clara, CA, USA). The column was washed with water (5 mL) and methanol-water (70:30, v/v; 5 mL), then air dried for 10 min. The column was eluted with hexanes-methylene chloride (90:10, v/v; 5 mL) and then hexanes-2-propanol (95:5, v/v; 5 mL). Residue of eluents was reconstituted and fractionated by the above described normal-phase HPLC method with two minor variations: The mobile-phase was hexanes-2-propanol (96:4,v/v) and six fractions were collected, each fraction coinciding with the retention time of standards of vitamin D_2 and D_3 forms of 25(OH)D, 24,25$(OH)_2$D, and 1,25$(OH)_2$D. The fractions were assumed to contain both C-3 epimers because little difference was observed between retention times of epimeric standards that were commercially available, i.e., 3-epi-25(OH)D_3, 3-epi-25(OH)D_2, and 3-epi-24*R*,25$(OH)_2D_3$ (Isosciences, Ambler, PA, USA).

The above described reverse-phase HPLC method was used to quantify both epimeric forms of 25(OH)D_3, 25(OH)D_2, 24,25*R*$(OH)_2D_3$ and 24,25$(OH)_2D_2$. The method was slightly modified for quantification of the 24,25$(OH)_2$D epimers: Internal standard, 25 ng of 25(OH)D_3, was added to the 24,25$(OH)_2$D reconstitutes prior to HPLC quantitation. The HPLC mobile-phase was changed to a gradient in which the methanol-water proportion was increased after 20 min from 60:40 (v/v) to 70:30 (v/v) for elution of internal standard, 25(OH)D_3. Plasma concentrations of the metabolites were calculated the same as described above for concentrations of the 25(OH)D_3 and 25(OH)D_2 metabolites.

The normal-phase fraction containing 1,25$(OH)_2D_2$ and that containing 1,25$(OH)_2D_3$ and 1α,25-[26,27-^{3}H]-dihydroxyvitamin D_3 were each aliquoted into thirds. One third of the fraction containing 1,25$(OH)_2D_3$ was dried and radioactivity in residue determined by liquid scintillation counting. The remaining aliquots of the fractions were added to borosilicate glass tubes (12 x 75 mm) to which 10 μL methanol was also added. The glass tube contents were dried and the amount of 1,25$(OH)_2D_3$ or 1,25$(OH)_2D_2$ in residue of the fractions was determined using a commercially available RIA kit (1,25-Dihydroxy Vitamin D RIA, Immunodiagnostic Systems, Ltd., Boldon, Tyne, & Wear, UK). A procedural deviation in use of the RIA kit was that standards were borosilicate glass tubes that contained dried residues of 10 μL aliquots of methanolic dilutions (0.4, 1.6, 6.3, and 25 pg/mL) of 1,25$(OH)_2D_3$ (Isosciences, Ambler, PA, USA) and organic mobile phase (hexanes-2-propanol, 96:4, v/v) of a volume equivalent to that aliquoted for RIA of samples (∼1.5 mL). Another variation in procedure in use of the RIA kit was that incubation of tubes with radiolabel was extended from 1 to 3 h before separation of bound from free radiolabel. The response in inhibition of binding was linear with natural log of mass of 1,25$(OH)_2D_3$ standard in tubes [bound % $= -14 \times \ln$ (1,25$(OH)_2D_3$ pg) + 92; $r = 0.998$]. The amount of 1,25$(OH)_2D_3$ calculated to affect a 50% binding was 19 pg.

The plasma concentrations of vitamin D metabolites were determined using the above methods in 1.0 mL replicates ($n = 6$) of pooled plasma from cats receiving no dietary vitamin D_2. The plasma replicates were spiked with 50 ng each of 25(OH)D_2, 3-epi-25(OH)D_2, and 24*RS*,25$(OH)_2D_2$ (Isosciences, Ambler, PA, USA) prior to analysis. Mean (± standard deviation) recoveries of the metabolites were found to be 41.4 ± 4.4, 44.3 ± 6.2, and 54.2 ± 18.7%, respectively. Recoveries of the tritiated labels of 25(OH)D_3 and 1,25$(OH)_2D_2$ were determined to be 44.8 ± 2.7 and 74.9 ± 13.2%, respectively. Coefficients of variation determined for 25(OH)D_2, 3-epi-25(OH)D_2, and 24*RS*,25$(OH)_2D_2$ were 10.7, 13.9, and 22.9%, respectively.

Metabolite Identity Confirmation

The LC-MS/MS methods of Kaufmann et al. (54) were used with modifications to confirm that UV absorbance of vitamin D metabolites were causal of reverse-phase HPLC peaks recorded during retention times of metabolite standards. Initially, HPLC eluent was collected during periods (∼1.5 min) when UV peaks of supposed metabolite were observed. The eluent fractions from each cat were combined across cats according to metabolite fraction. Metabolite in the pooled fractions was extracted into chloroform by methods of Bligh and Dyer (55). For this, water and chloroform were added to the fractions in amounts sufficient

to form a mixture of 0.9:1.0:1.0 (v/v/v) water-chloroform-methanol. The mixture was vigorously shaken for 1 min and thereafter allowed to stand until development of a chloroform layer, which was then removed and dried. Metabolite in residue of the chloroform extract was derivatized in a 1.0 mL glass vial by incubation for 30 min with freshly prepared labeling solution – 25 μL of briefly sonicated, dry, methylene chloride containing 0.1 mg/mL of 4-[2-(6,7-dimethoxy-4-methyl-3,4-dihydroquinoxalinyl-)ethyl]-1,2,4-triazoline-3,5-dione (Key Synthesis, Oaks, PA, USA). The incubation was extended another 60 min after addition of another 25 μL of labeling solution. The derivation was stopped by addition of 40 μL ethanol. After drying, derivatized metabolites were dissolved in 250 μL methanol-water (60:40, v/v) and quantified by the LC-MS methods of Kaufman et al. (54) by the University of Missouri, Charles W. Gehrke Proteomics Center.

Statistical Analyses

Statistical analysis was performed with statistical software [SAS 9.4, SAS Institute, Cary, NC, USA; Microsoft Excel (2016), Microsoft Corporation, Redmond, WA, USA]. Variable observations were accepted as normal if means and medians were similar and skew and excess kurtosis were both between −1.0 and 1.0. Plasma 25(OH)D_3 and 3-epi-25(OH)D_3 concentrations and anion gap, serum PTH concentration, and blood monocyte count were observations normalized by logarithmic transformation.

Repeated-measures, mixed-model ANOVA was used to determine the significance of effect of treatment (vitamin D_2 vs. 25(OH)D_2) on plasma concentrations of 25(OH)D_3 and 25(OH)D_2 and their C-3 epimers. Fixed effects were D_2 vitamer type, crossover phase, and their interaction while random effects were subjects (cats). Significance of differences of sampling week were identified using Tukey-adjusted, multiple comparisons. Significance of effect of oral D_2 vitamer form on plasma concentrations of dihydroxyvitamin D metabolites [24,25(OH)$_2$$D_2$, 24,25(OH)$_2$$D_3$, 1,25(OH)$_2$$D_2$, 1,25(OH)$_2$$D_3$] were determined with Wilcoxon two-sample tests comparing the group receiving vitamin D_2 first to the group receiving 25(OH)D_2 first. For single repeated measures, paired-*t* tests were used to test significance of oral D_2 vitamer type [D_2 vs. 25(OH)D_2] on normal observations. Sign-rank tests were used to test significance of single repeated, non-normal observations (i.e., serum ionized calcium concentration, blood basophil count, and plasma concentrations of phosphorus, urea nitrogen, creatinine, chloride, albumin, and total protein).

The number of cats selected for study was based on a power analysis using reported mean and variance observations on peak plasma 25(OH)D_2 concentrations in 10 adult cats given oral boluses of vitamin D_2 (27). The analysis indicated that paired observations of 25(OH)D_2 concentrations in seven cats should be sufficient for detecting a 50% mean difference caused by a treatment effect with $\alpha = 0.05$ with a power of 80%.

Central tendency and dispersion of non-normal observations are reported as median and range, respectively. Results for normal observations are reported as mean and SEM. Effects were considered significant if $P < 0.05$.

RESULTS

Animals

A total of eight cats were initially included in the study. All diet presented each day was typically consumed. The median (range) energy intake for cats at baseline was 79.8 (73.2–82.4) kcal/kg$^{0.67}$. Cats received dietary vitamin D_3 in their commercial diet (0.65–0.95 μg/kg body weight per d). Prior to starting the D_2 vitamer supplementations, body condition score (BCS) of the cats was ideal (BCS 5/9, 20% body fat, $n = 3$) or slightly above ideal condition (BCS 6/9, ∼ 25 to 30% body fat, $n = 5$). Food presentation was adjusted by 5–10% to maintain/achieve ideal BCS throughout the study. While vitamin D_3 intake varied slightly with adjustments to food intake, this did not appear to have a substantive effect on D status as there was no significant crossover phase effect on plasma 25(OH)D_3 concentrations.

One cat was euthanized after the first phase of the crossover and therefore excluded from statistical analysis. During the washout period following 10 weeks of supplementation with 25(OH)D_2, this cat was found to have an increase in plasma urea nitrogen (from 34 to 40 mg/dL, reference interval 19–35 mg/dL), while plasma creatinine was unchanged from the pre-trial value (1.4 mg/dL) and within the reference interval (0.9–2.0 mg/dL). The concentrations of the total of 25(OH)D_3 and 25(OH)D_2 at this time point were 44.8 ng/mL for the beta epimers and 13.1 ng/mL for alpha C-3 epimers, respectively. During the 10 weeks of supplementation, the mean (range) plasma concentrations of the of total 25(OH)D for this cat were 47.3 (44.8–70.1) ng/mL for beta epimers and 15.0 (9.1–21.8) ng/mL for alpha epimers. These values were similar to plasma concentrations observed in other cats in the study who received 25(OH)D_2 supplementation (**Table 1**). This cat was later euthanized during the washout period, 11 weeks after discontinuing 25(OH)D_2 supplementation, following 1 week of clinical signs (anorexia, vomiting, lethargy) and the development of azotemia (BUN 163 mg/dL, creatinine 6.0 mg/dL) and other plasma biochemistry changes consistent with renal failure. Abnormal necropsy findings included asymmetric and overall diminished renal mass with left nephrolithiasis. Histopathological findings were lymphocytic interstitial nephritis, with the smaller, left kidney having diffuse interstitial, cortical fibrosis between dilated medullary and cortical tubules, and the larger, right kidney having similar pathological changes but with adjacent and greater regions of normal cortex with tubular vacuolation and minimal interstitial lymphocytic inflammation.

After conclusion of the second phase of the crossover, one cat experienced syncopal episodes attributed to paroxysmal third-degree AV block and persistent left bundle branch block. The cat had received supplementation with 25(OH)D_2 during the second phase of the trial. An echocardiogram revealed mild dilation of the right atrium and ventricle. Blood glucose, cardiac troponin, ionized calcium, and other clinical hematological and biochemistry analyses repeated at the time were within laboratory reference intervals. Echocardiograms were subsequently performed on all other cats which had received 25(OH)D_2 supplementation during the second phase

TABLE 1 | Mean and SEM values of plasma concentrations (ng/mL) of 25(OH)D_3 and 25(OH)D_2 and their epimers in seven cats during 10 weeks of oral supplementation with either vitamin D_2 or 25(OH)D_2 while consuming a commercial feline diet containing vitamin D_3.

	Supplement (S)			Week (W)						*P*-value		
Metabolite	**D_2**	**25(OH)D_2**	**SEM**	**0**	**2**	**4**	**6**	**8**	**10**	**SEM**	**S**	**W**	**S x W‡**
25(OH)D_3	26.8	32.1	1.1	36.2	31.8	28.9	27.7	27.3	25.5	1.1	<0.01	<0.01	<0.01
25(OH)D_2	13.0	25.5	2.3	0.0*	16.4*	21.0*	23.4	28.4	26.4	2.6	<0.01	<0.01	<0.01
Total 25(OH)D	39.5	56.0	1.1	36.2	47.7	47.6	50.0	53.3	49.4	1.1	<0.01	<0.01	<0.01
3-epi-25(OH)D_3	14.0	15.1	1.1	12.4^{a}	13.2^{a,b}	13.9^{b}	15.1^{b,c}	16.8^{c}	16.5^{c}	1.1	0.08	<0.01	0.23
3-epi-25(OH)D_2	6.7	5.3	1.0	0.0^{a,†}	7.0^{b,†}	6.4^{b,†}	6.8^{b,†}	8.2^{b,†}	7.6^{b,†}	1.2	0.06	<0.01	0.79
Total 3-epi-25(OH)D	20.3	20.1	1.1	12.4^{a}	20.0^{b}	20.3^{b}	21.8^{b,c}	25.4^{c}	24.3^{b,c}	1.1	0.78	<0.01	0.56

The listed P-values indicate significance of effect of supplement form on metabolite concentration for the period of oral supplementation. Mean metabolite values with superscript letters indicate significance of differences of values between experimental weeks during the supplementations; values that do not have symbols in common differ ($P < 0.05$).

**Less than 25(OH)D_3 value for the same week ($P < 0.05$).*

†Less than 3-epi-25(OH)D_3 value for the same week ($P < 0.05$).

*‡Significance of Supplement-Week interaction; see **Supplementary Figures 1**, **2** for plots of metabolite concentrations where the interaction was significant.*

($n = 3$) and the findings were unremarkable. Serial 24-h evaluations with a Holter monitor over the next 12 weeks indicated significant improvement in conduction abnormalities in the affected cat. Rapid and complete resolution of clinical signs was observed with no further abnormalities appreciated over the next several months. Results from this cat were included in the analysis for a total of seven cats in the study.

Effects of D_2 Supplementation on 25(OH)D Concentrations

Plasma concentrations of 25(OH)D_2 increased over time with both D_2 vitamer treatments during the 10 weeks of supplementation. There was no detectable 25(OH)D_2 in any plasma sample initially (week 0). Following supplementation with D_2 vitamers, 25(OH)D_2 concentrations gradually increased to plateaus by week 6. Supplementation with 25(OH)D_2 was associated with a more rapid and greater rise in plasma 25(OH)D_2 concentration compared to treatment with vitamin D_2 ($P < 0.01$; **Table 1**).

Following supplementation with vitamin D_2, plasma concentrations of 25(OH)D_3 declined over time and were significantly reduced from baseline by week 8 ($P < 0.02$). Plasma concentrations of 25(OH)D_3 were greater than those of 25(OH)D_2 until week 6 ($P < 0.01$), after which the concentrations of 25(OH)D_3 and 25(OH)D_2 were not statistically different. The combined concentrations of 25(OH)D_2 and 25(OH)D_3 [total 25(OH)D] were unchanged by vitamin D_2 supplementation (**Table 1**; **Supplementary Figure 1**). On the contrary, supplementation with 25(OH)D_2 did not affect 25(OH)D_3 concentrations but did result in an increase in total 25(OH)D by week 2 of supplementation ($P < 0.01$). The concentrations of 25(OH)D_2 and 25(OH)D_3 were not different at any time point other than week 0, when 25(OH)D_2 concentrations were below 25(OH)D_3 ($P < 0.01$; **Supplementary Figure 2**).

Effects of D_2 Supplementation on Alpha C-3 Epimeric Forms of 25(OH)D

Peaks of UV absorbance were observed coinciding with the retention time of 3-epi-25(OH)D_2 standard in reverse-phase HPLC of extracted and fractionated plasma from all cats. Elution of the epimer at the time of the UV peaks was confirmed by the LC-MS/MS analysis described in the methods.

Similar to initial 25(OH)D_2 concentrations, there was no detectable 3-epi-25(OH)D_2 in any plasma sample initially (week 0). With both D_2 vitamer supplementations, 3-epi-25(OH)D_2 concentrations increased by week 2 ($P < 0.03$), after which no significant changes were observed (**Table 1**). There was no effect of D_2 supplementation form [vitamin D_2 vs. 25(OH)D_2] on plasma concentrations of 3-epi-25(OH)D_2.

Plasma concentrations of the alpha C-3 epimers remained below concentrations of their respective beta epimers [i.e., 25(OH)D_2 and 25(OH)D_3] at all time points. By week 10 of the D_2 vitamer supplementations, mean concentration of 3-epi-25(OH)D_2 was less ($P < 0.01$) and 29% of the concentration of 25(OH)D_2 and 3-epi-25(OH)D_3 was less

TABLE 2 | Median and range values of plasma vitamin D metabolites in cats after 10 weeks of oral supplementation with vitamin D_2 ($n = 4$) or 25(OH)D_2 ($n = 3$) while consuming a commercial feline diet containing vitamin D_3.

	Supplementation form				*P*-value
	Vitamin D_2		25(OH)D_2		
Metabolite	Median	Range	Median	Range	
1,25(OH)$_2$D$_3$ (pg/mL)	37.2	17.9–55.8	28.4	23.6–29.9	0.86
1,25(OH)$_2$D$_2$ (pg/mL)	28.4	23.6–29.9	13.5	10.7–16.0	0.71
24,25(OH)$_2$D$_3$ (ng/mL)	15.3	9.3–25.8	17.3	16.5–17.8	1.00
24,25(OH)$_2$D$_2$ (ng/mL)	nd*	nd	nd	nd	–
3-epi-24,25*R*(OH)$_2$D$_3$ (ng/mL)	2.9	0.0–7.5	2.7	2.3–5.6	1.00
3-epi-24,25(OH)$_2$D$_2$ (ng/mL)	nd	nd	nd	nd	–

**nd, not detected.*

($P < 0.01$) and 65% of the concentration of 25(OH)D_3 (**Table 1**). The C-3 epimer of 25(OH)D_3 was present in higher concentrations than 3-epi-25(OH)D_2 at all weeks during both vitamer supplementations ($P < 0.02$). After week 0, there were no significant changes in and differences between concentrations of 3-epi-25(OH)D_2 and 3-epi-25(OH)D_3. There was, however, a difference between the alpha epimer concentrations of 25(OH)D_2 and 25(OH)D_3 at weeks 8 and 10 when cats received 25(OH)D_2 supplementation ($P < 0.01$). The total 3-epi-25(OH)D concentrations remained unchanged after week 0 during both D_2 vitamer supplementations (**Table 1**).

Effects of D_2 Supplementation on 24,25(OH)$_2$D and 1,25(OH)$_2$D Metabolites

At the end of supplementations, analysis of week 10 plasma from both crossover phases was conducted to determine concentrations of dihydroxyvitamin D metabolites, including the D_3 and D_2 forms of 1,25(OH)$_2$D, as well as 24,25(OH)$_2$D and its putative C-3 epimers. Due to an analysis error that caused sample loss, concentrations of these metabolites could not be reliably determined for week 10 of the second crossover phase. Results of analyses of samples of the first phase of the crossover trial are reported here when cats received oral supplementation with vitamin D_2 ($n = 4$) and 25(OH)D_2 ($n = 3$) (**Table 2**).

There was no effect of D_2 vitamer form on the concentrations of 1,25(OH)$_2$D and 24,25(OH)$_2$D$_3$ metabolites (**Table 2**). Vitamin D_3 and D_2 forms of 1,25-dihydroxyvitamin D were identified and quantified. Median plasma concentrations of 1,25(OH)$_2$D$_2$ were 49% less than 1,25(OH)$_2$D$_3$ ($P = 0.02$).

Peaks of UV absorbance were observed coinciding with the retention times of 24,25*R*(OH)$_2$D$_3$ and the C-3 epimer, 3-epi-24,25*R*(OH)$_2$D$_3$, standards in reverse phase HPLC of extracted and fractionated plasma. Identity of the metabolites were confirmed by LC-MS/MS analysis described in the methods. No UV detectable peaks were observed during or closely following the retention time for 24*RS*,25(OH)$_2$D$_2$ standard. Only the vitamin D_3 form of 24,25(OH)$_2$D was detected in plasma samples of all cats. This included the alpha C-3 epimer of 24,25(OH)$_2$D$_3$ which was detected in six of the seven cats. Among all cats, median plasma concentrations of 24,25*R*(OH)$_2$D$_3$ were greater than 3-epi-24,25*R*(OH)$_2$D$_3$ ($P < 0.01$; **Table 2**).

Effects of D_2 Supplementation on Plasma Biochemistry and Blood Count Variables

Oral 25(OH)D_2 compared to vitamin D_2 supplementation was not associated with greater plasma calcium or phosphorus concentrations after 10 weeks. Additionally, there were no differences in indicators of kidney function including blood urea nitrogen, creatinine, and PTH concentrations between supplement forms [25(OH)D_2 and vitamin D_2] (**Table 3**). Clinical hematology and biochemistry values following 10 weeks of oral supplementation with 25(OH)D_2 were not significantly different from those following vitamin D_2 supplementation (**Supplementary Tables 2**, **3**).

DISCUSSION

We have described feline vitamin D metabolite formation when dietary sources of vitamin D_3 plus vitamin D_2 or 25(OH)D_2 are provided. Our hypothesis that supplementation with 25(OH)D_2 would more effectively and rapidly raise circulating 25(OH)D_2 levels in cat plasma compared to vitamin D_2 was supported. We report here observations of C-3 epimer formation for 25(OH)D_3, 25(OH)D_2, and 24,25*R*(OH)$_2$D$_3$. To the authors' knowledge, this is the first report confirming circulating concentrations of 1,25(OH)$_2$D$_2$, 24,25*R*(OH)$_2$D$_3$, and 3-epi-24,25*R*(OH)$_2$D$_3$ in the cat. We were unable to identify any circulating 24,25(OH)$_2$D$_2$ or 3-epi-24,25(OH)$_2$D$_2$ in these samples. This information may provide insight into the similarities and differences between feline vitamin D_2 and D_3 metabolism.

The results of this study illustrate that supplementation with vitamin D_2 and 25(OH)D_2 result in increased plasma concentrations of 25(OH)D_2 and 3-epi-25(OH)D_2 when they are provided in addition to vitamin D_3 in the diet. During supplementation with vitamin D_2, total 25(OH)D concentrations remained stable over time despite an increase in 25(OH)D_2 (**Table 1**, **Supplementary Figure 1**). This could be explained by the concurrent reduction in 25(OH)D_3 concentration. A similar effect of vitamin D_2 supplementation on 25(OH)D_3 levels in humans has been reported, but is not a consistent finding (56, 57). This suggests that vitamin D_2 and vitamin D_3 are competing substrates for 25-hydroxylase in the liver and that available 25-hydroylase activity may limit elevation of plasma 25(OH)D concentration when dietary vitamin D content is increased. An alternative explanation is that vitamin D_2 may induce catabolism of 25(OH)D_3, which has been theorized based on an increased ratio of 24,25(OH)D_3 to 25(OH)D_3 following vitamin D_2 supplementation in humans (58). This is unlikely to be the case in cats since this pattern was not observed in our study during supplementation with 25(OH)D_2, which resulted in an initial increase in total 25(OH)D with no change in 25(OH)D_3 concentration (**Supplementary Figure 2**). The stable concentrations of plasma 25(OH)D_3 during 25(OH)D_2

TABLE 3 | Median and range of select biochemistry and endocrinology variables in seven cats after 10 weeks of oral supplementation with vitamin D_2 or 25(OH)D_2 while consuming a commercial feline diet containing vitamin D_3.

		Supplementation form				
		Vitamin D_2		25(OH)D_2		
	Reference interval	Median	Range	Median	Range	*P*-value
Urea nitrogen (mg/dL)	19–35	24	18–29	22	19–30	1.00
Creatinine (mg/dL)	0.9–2.0	1.4	1.0–1.7	1.5	0.9–1.8	0.69
Calcium (mg/dL)	8.6–10.4	8.9	8.6–9.8	9.2	8.9–9.5	0.44
Phosphorus (mg/dL)	2.3–5.0	4.3	3.1–4.8	4.2	3.3–4.7	0.80
Parathyroid hormone (pmol/L)	0.40–2.50	0.80	0.30–0.90	0.60	0.30–1.70	0.67
Ionized calcium (mmol/L)	1.00–1.40	1.19	1.17–1.26	1.22	1.14–1.25	0.56

supplementation are consistent with our knowledge of vitamin D hydroxylation in the liver, described as a relatively uncontrolled process with little negative feedback from circulating 25(OH)D concentration (59).

The ability of 25(OH)D_2 to raise total 25(OH)D levels compared to vitamin D_2, even when dosed at a 20% molar equivalent, indicates a greater than five times increased potency of 25(OH)D_2 compared to vitamin D_2. This is consistent with multiple studies and meta-analyses showing increased effectiveness of 25(OH)D_3 compared to vitamin D_3 in humans (24, 60, 61).

Similar to previous studies, we found that the C-3 epimer of 25(OH)D_3 is present in cats in quantities greater than observed in other species, such as rats, dogs, and humans (11, 17, 62). There are reports of C-3 epimers of numerous vitamin D metabolites, including 1,25(OH)$_2D_3$, 1,25(OH)$_2D_2$, and 24,25(OH)$_2D_2$ which have been detected in other species and cell lines (12, 13, 15, 16, 62–67). With the methods employed here, we could not investigate occurrence of C-3 epimers of 1,25(OH)$_2D_3$ and 1,25(OH)$_2D_2$, but we did find quantifiable concentrations of 3-epi-25(OH)D_2 and 3-epi-24,25*R*(OH)$_2D_3$ in feline plasma samples, the presence of which have not been previously reported in cats. The C-3 epimer of 25(OH)D_2 was first detected after 2 weeks of supplementation with both D_2 forms (**Table 1**). At all time points during supplementation and for both supplement forms, concentrations of 3-epi-25(OH)D_2 were below 25(OH)D_2. The equilibration of 3-epi-25(OH)D_2 was much more rapid (2 weeks) than that of 25(OH)D_2 (8 weeks). Although the role of C-3 epimerization is unknown, it has been suggested that the epimers are products of a clearance pathway for vitamin D metabolites (11). Our results are consistent with this theory, as demonstrated by increased concentrations of 3-epi-25(OH)D_2 after initiating D_2 vitamer supplementations. A previous report of 3-epi-25(OH)D_3 supports C-3 epimerization as a means of protection against hypervitaminosis D by demonstrating this metabolite's ability to affect calcium homeostasis and bone mineralization (12). The presence of epimeric forms of vitamin D metabolites in high abundance may be part of the reason cats seem relatively resistant to vitamin D toxicosis even when ingesting very large amounts of vitamin D (68).

The detection of similar D_2 and D_3 plasma metabolites following supplementation with D_2 forms indicates similar pathways of metabolism in cats with some possible exceptions. Firstly, we did not detect plasma concentrations of 24,25(OH)$_2D_2$ in any of the cats. It is conventionally believed that 24-hydroxylation of 25(OH)D to form 24,25(OH)$_2$D is the first step in degradation of the vitamer (8). Here, the cat may be different. If the role of degradation is adopted by C-3 epimerization in cats, then we might expect plasma concentrations of 24,25(OH)$_2$D to be lower in cats than plasma concentrations of 3-epi-25(OH)D. In fact, we found that 24,25*R*(OH)$_2D_3$ concentrations were similar to those of 3-epi-25(OH)D_3 after 10 weeks. This finding seems consistent with a possible physiologic role for 24,25*R*(OH)$_2D_3$ in cats. Some investigators have found evidence that 24,25(OH)$_2$D may have independent or synergistic physiological actions on bone (69). The reason we were unable to detect 24,25(OH)$_2D_2$ in plasma is unknown, though we postulate that cats may have 24-hydroxylases with lower affinity for 25(OH)D_2 compared to 25(OH)D_3.

Our observations of plasma 1,25(OH)$_2$D concentrations may also indicate a difference between metabolism of D_2 and D_3 vitamers in cats. The plasma concentrations of 1,25(OH)$_2D_2$ were significantly lower than those of 1,25(OH)$_2D_3$ following 10 weeks of supplementation with both forms of vitamin D. We do not believe that the relative concentrations of the two vitamer forms of 1,25(OH)$_2$D reflect a difference in substrate availability, since plasma concentrations of 25(OH)D_2 were not significantly different from those of 25(OH)D_3 after 10 weeks for either D_2 supplement form (**Table 1**). A possible explanation is that 1α-hydroxylase in the feline kidney has a lower affinity for 25(OH)D_2 compared to 25(OH)D_3. Even if such an affinity difference occurs, its physiological importance is uncertain.

Of important note is that the total amount of vitamin D intake (vitamin D_3 + D_2 form) by the cats was well below the safe upper limit (19 $\mu g/kg^{0.67}$) recommended by the National Research Council, which is based on observations for vitamin D_3 (4). The upper limit is based in part on observations that chronic intake (18 months) of diet containing 33,840 IU/kg vitamin D by kittens and adult cats was not detrimental to reproduction, growth, or renal health (68). Nevertheless, one cat in our study

was diagnosed with renal failure 11 weeks after discontinuing supplementation with 25(OH)D_2 (0.80 μg/kg/d). After reviewing blood chemistry values for the months and years prior to the study, and based on the chronic changes detected on necropsy, we suspect this cat had an insidious progression of chronic renal disease that was not detectable on routine blood chemistry prior to study enrollment. Chronic renal failure of unknown etiology is a common cause of morbidity and mortality in domestic cats as they age (70). Although there is no evidence that renal disease was caused by supplementation with 25(OH)D_2, this cannot be completely ruled out. However, total 25(OH)D concentrations after 10 weeks of supplementation were 44.8 ng/mL for the beta C-3 epimer and 13.1 ng/mL for the alpha C-3 epimer, which was within the range observed for the other cats supplemented with 25(OH)D_2 (**Table 1**), which makes hypervitaminosis D an unlikely cause for this cat's renal disease. In addition, a second cat experienced adverse effects following supplementation with 25(OH)D_2 for 10 weeks during the second phase of the study. This cat was diagnosed with paroxysmal third-degree AV block and persistent left bundle branch block which rapidly resolved once the supplement was discontinued. No other cats receiving 25(OH)D_2 had cardiac abnormalities based on echocardiograms, and the relationship between these findings and supplementation are unclear.

Since use of 25(OH)D is being investigated for supplementation in multiple species, further studies to determine dose and safety of this metabolite in cats are warranted to ensure that the potent effects do not result in toxicity when supplemented at apparent physiologic doses. Ingestion of 25(OH)D in place of vitamin D has potential clinical applications for when a rapid increase in vitamin D status is sought or when absorption or metabolism of vitamin D is impaired. Although there was no difference between 25(OH)D_2 and vitamin D_2 on plasma hematology and biochemistry parameters, including indicators of calcium homeostasis (total calcium, phosphorus, PTH, and ionized calcium), long-term safety studies are indicated based on the adverse events observed in two of eight cats in this study. This is especially important given reports in other species that 25-hydroxyvitamin D is the more active form of vitamin D and ∼2–6 times more potent than vitamin D, a feature currently being exploited in studies of human and canine vitamin D supplementation (19, 22, 23, 25, 60).

One limitation of this study was the small sample size which increased the likelihood of type-2 statistical error, especially in comparisons of outcomes between supplementation of 25(OH)D_2 and vitamin D_2. For the dihydroxy metabolites in particular, sample loss resulted in comparisons of only 3–4 observations. Given the low number of observations, it is possible that there is a statistical difference between the supplementation groups, but because of low power we could not detect any differences.

Additionally, differentiation between effects of the D_2 vitamers was complicated by consumption of vitamin D_3 in the diet. Nevertheless, our findings are relevant in practice. If cats receive diets supplemented with vitamin D_2, some vitamin D_3 likely would be ingested because many animal-source ingredients contain vitamin D_3 (46). Appropriately designed studies are needed to evaluate outcome differences between D vitamers (D_2 and D_3) when background vitamin D status is low as in vitamin D deficiency.

In conclusion, this study reports on oral administration of D_2 vitamers and identification of novel vitamin D metabolites that may help us to better understand vitamin D metabolism in cats. The high concentrations of C-3 epimers detected in cat plasma consistent with previous studies emphasizes the importance of considering epimeric forms of vitamin D metabolites when reporting on vitamin D status in cats, as these are present in abundant quantities and may cause under- or over-estimation of circulating vitamin D depending on methods of analysis. Further studies using 25(OH)D and vitamin D_2 forms in cats are needed before conclusions can be made about safety and efficacy of these vitamers for supplementation, or their efficacy compared to vitamin D_3. Nonetheless, the present research indicates that oral supplementation with 25(OH)D_2 compared to vitamin D_2 is more potent and more rapid in raising vitamin D status in adult cats.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by Animal Care and use Committee of the University of Missouri.

AUTHOR CONTRIBUTIONS

CR was the principal investigator of the study and contributed to designing and implementing the study, data analyses, and writing the manuscript. RB was the co-investigator of the study and contributed to study design, implementing the study, processing and analyzing the data, and writing the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2021.654629/full#supplementary-material

REFERENCES

1. Morris JG. Ineffective vitamin D synthesis in cats is reversed by an inhibitor of 7-dehydrocholestrol-delta7-reductase. *J Nutr.* (1999) 129:903–8. doi: 10.1093/jn/129.4.903
2. Parker VJ, Rudinsky AJ, Chew DJ. Vitamin D metabolism in canine and feline medicine. *J Am Vet Med Assoc.* (2017) 250:1259–69. doi: 10.2460/javma.250.11.1259
3. Association of American Feed Control Officials, Inc. 2017 Official Publication. In: *[Table], AAFCO Dog Food Nutrient Profiles Based on Calorie Content*. Manhattan, KS: Association of American Feed Control Officials, Inc. (2017).
4. National Research Council. *Nutrient Requirements of Dogs and Cats*. Washington, DC: National Academies Press (2006).
5. Blomstrand R, Forsgren L. Intestinal absorption and esterification of vitamin D3-1,2-3H in man. *Acta Chem Scand.* (1967) 21:1662–3. doi: 10.3891/acta.chem.scand.21-1662
6. Silva MC, Furlanetto TW. Intestinal absorption of vitamin D: a systematic review. *Nutr Rev.* (2018) 76:60–76. doi: 10.1093/nutrit/nux034
7. Haddad JG Jr. Transport of vitamin D metabolites. *Clin Orthop Relat Res.* (1979) 142:249–61. doi: 10.1097/00003086-197907000-00040
8. DeLuca HF. Vitamin D: historical overview. *Vitam Horm.* (2016) 100:1–20. doi: 10.1016/bs.vh.2015.11.001
9. Mellanby RJ. Beyond the skeleton: the role of vitamin D in companion animal health. *J Small Anim Pract.* (2016) 57:175–80. doi: 10.1111/jsap.12458
10. Zafalon RVA, Risolia LW, Pedrinelli V, Vendramini THA, Rodrigues RBA, Amaral AR, et al. Vitamin D metabolism in dogs and cats and its relation to diseases not associated with bone metabolism. *J Anim Physiol Anim Nutr.* (2020) 104:322–42. doi: 10.1111/jpn.13259
11. Sprinkle MC, Hooper SE, Backus RC. Previously undescribed vitamin D C-3 epimer occurs in substantial amounts in the blood of cats. *J Feline Med Surg.* (2018) 20:83–90. doi: 10.1177/1098612X17693523
12. Djekic-Ivankovic M, Lavery P, Agellon S, Weiler HA. The C-3alpha epimer of 25-hydroxycholecalciferol from endogenous and exogenous sources supports normal growth and bone mineral density in weanling rats. *J Nutr.* (2017) 147:141–51. doi: 10.3945/jn.116.231753
13. Brown AJ, Ritter CS, Weiskopf AS, Vouros P, Sasso GJ, Uskokovic MR, et al. Isolation and identification of 1alpha-hydroxy-3-epi-vitamin D3, a potent suppressor of parathyroid hormone secretion. *J Cell Biochem.* (2005) 96:569–78. doi: 10.1002/jcb.20553
14. Bianchini C, Lavery P, Agellon S, Weiler HA. The generation of C-3alpha epimer of 25-hydroxyvitamin D and its biological effects on bone mineral density in adult rodents. *Calcif Tissue Int.* (2015) 96:453–64. doi: 10.1007/s00223-015-9973-9
15. Brown A, Ritter C, Slatopolsky E, Muralidharan K, Okamura W, Reddy G. 1α, 25-Dihydroxy-3-Epi-vitamin D3, a natural metabolite of 1α, 25-dihydroxyvitamin D3, is a potent suppressor of parathyroid hormone secretion. *J Cell Biol.* (1999) 73:106–13. doi: 10.1002/(SICI)1097-4644(19990401)73:1<106::AID-JCB12>3.0.CO;2-Q
16. Rehan VK, Torday JS, Peleg S, Gennaro L, Vouros P, Padbury J, et al. 1Alpha, 25-dihydroxy-3-epi-vitamin D3, a natural metabolite of 1alpha, 25-dihydroxy vitamin D3: production and biological activity studies in pulmonary alveolar type II cells. *Mol Genet Metab.* (2002) 76:46–56. doi: 10.1016/S1096-7192(02)00022-7
17. Wiebe D, Binkley N. Case report: three patients with substantial serum levels of 3-epi-25(OH)D including one with 3-epi-25(OH)D2 while on high-dose ergocalciferol. *J Clin Endocrinol Metab.* (2014) 99:1117–21. doi: 10.1210/jc.2013-3953
18. Heaney RP, Armas LA, Shary JR, Bell NH, Binkley N, Hollis BW. 25-Hydroxylation of vitamin D3: relation to circulating vitamin D3 under various input conditions. *Am J Clin Nutr.* (2008) 87:1738–42. doi: 10.1093/ajcn/87.6.1738
19. Young LR, Backus RC. Serum 25-hydroxyvitamin D3 and 24R,25-dihydroxyvitamin D3 concentrations in adult dogs are more substantially increased by oral supplementation of 25-hydroxyvitamin D3 than by vitamin D3. *J Nutr Sci.* (2017) 6:e30. doi: 10.1017/jns.2017.8
20. Parker VJ, Rudinsky AJ, Benedict JA, Beizaei A, Chew DJ. Effects of calcifediol supplementation on markers of chronic kidney disease-mineral and bone disorder in dogs with chronic kidney disease. *J Vet Intern Med.* (2020) 34:2497–506. doi: 10.1111/jvim.15949
21. Blunt J, Tanaka Y, DeLuca H. Biological activity of 25-hydroxycholecalciferol, a metabolite of vitamin D3. *Proc Natl Acad Sci USA.* (1968) 61:1503. doi: 10.1073/pnas.61.4.1503
22. Sitrin MD, Pollack KL, Bolt MJ, Rosenberg IH. Comparison of vitamin D and 25-hydroxyvitamin D absorption in the rat. *Am J Physiol.* (1982) 242:G326–32. doi: 10.1152/ajpgi.1982.242.4.G326
23. Stamp T, Haddad J, Twigg C. Comparison of oral 25-hydroxycholecalciferol, vitamin D and ultraviolet light as determinants of circulating 25-hydroxyvitamin D. *Lancet.* (1977) 309:1341–3. doi: 10.1016/S0140-6736(77)92553-3
24. Graeff-Armas LA, Bendik I, Kunz I, Schoop R, Hull S, Beck M. Supplemental 25-hydroxycholecalciferol is more effective than cholecalciferol in raising serum 25-hydroxyvitamin D concentrations in older adults. *J Nutr.* (2020) 150:73–81. doi: 10.1093/jn/nxz209
25. Sprague SM, Silva AL, Al-Saghir F, Damle R, Tabash SP, Petkovich M, et al. Modified-release calcifediol effectively controls secondary hyperparathyroidism associated with vitamin D insufficiency in chronic kidney disease. *Am J Nephrol.* (2014) 40:535–45. doi: 10.1159/000369939
26. Mann MC, Hollenberg MD, Hanley DA, Ahmed SB. Vitamin D the autonomic nervous system, cardiovascular risk. *Physiol Rep.* (2015) 3:e12349. doi: 10.14814/phy2.12349
27. Morris JG. Cats discriminate between cholecalciferol and ergocalciferol. *J Anim Physiol Anim Nutr.* (2002) 86:229–38. doi: 10.1046/j.1439-0396.2002.00379.x
28. Horst RL, Napoli JL, Littledike ET. Discrimination in the metabolism of orally dosed ergocalciferol and cholecalciferol by the pig, rat and chick. *Biochem J.* (1982) 204:185–9. doi: 10.1042/bj2040185
29. DeLuca HF, Nakada M, Tanaka Y, Sicinski R, Phelps M. The plasma binding protein for vitamin D is a site of discrimination against vitamin D-2 compounds by the chick. *Biochim Biophys Acta.* (1988) 965:16–21. doi: 10.1016/0304-4165(88)90145-6
30. Harrington DD, Page EH. Acute vitamin D3 toxicosis in horses: case reports and experimental studies of the comparative toxicity of vitamins D2 and D3. *J Am Vet Med Assoc.* (1983) 182:1358–69.
31. Hunt RD, Garcia FG, Walsh RJ. A comparison of the toxicity of ergocalciferol and cholecalciferol in rhesus monkeys (Macaca mulatta). *J Nutr.* (1972) 102:975–86. doi: 10.1093/jn/102.8.975
32. Marx SJ, Jones G, Weinstein RS, Chrousos GP, Renquist DM. Differences in mineral metabolism among nonhuman primates receiving diets with only vitamin D3 or only vitamin D2. *J Clin Endocrinol Metab.* (1989) 69:1282–90. doi: 10.1210/jcem-69-6-1282
33. Holmberg I. Differences in the metabolism of vitamin D2 and vitamin D3 by subcellular fractions from rat liver. *Biochim Biophys Acta.* (1984) 800:106–9. doi: 10.1016/0304-4165(84)90100-4
34. Tripkovic L, Lambert H, Hart K, Smith CP, Bucca G, Penson S, et al. Comparison of vitamin D2 and vitamin D3 supplementation in raising serum 25-hydroxyvitamin D status: a systematic review and meta-analysis. *Am J Clin Nutr.* (2012) 95:1357–64. doi: 10.3945/ajcn.111.031070
35. Houghton LA, Vieth R. The case against ergocalciferol (vitamin D2) as a vitamin supplement. *Am J Clin Nutr.* (2006) 84:694–7. doi: 10.1093/ajcn/84.4.694
36. Hoy DA, Ramberg CF, Horst RL. Evidence that discrimination against ergocalciferol by the chick is the result of enhanced metabolic clearance rates for its mono- and dihydroxylated metabolites. *J Nutr.* (1988) 118:633–8. doi: 10.1093/jn/118.5.633
37. Jones KS, Assar S, Harnpanich D, Bouillon R, Lambrechts D, Prentice A, et al. 25(OH)D2 half-life is shorter than 25(OH)D3 half-life and is influenced by DBP concentration and genotype. *J Clin Endocrinol Metab.* (2014) 99:3373–81. doi: 10.1210/jc.2014-1714
38. Jones G. Extrarenal vitamin D activation and interactions between vitamin D, vitamin D, and vitamin D analogs. *Annu Rev Nutr.* (2013) 33:23–44. doi: 10.1146/annurev-nutr-071812-161203
39. Jones G, Schnoes HK, Levan L, Deluca HF. Isolation and identification of 24-hydroxyvitamin D2 and 24,25-dihydroxyvitamin D2. *Arch Biochem Biophys.* (1980) 202:450–7. doi: 10.1016/0003-9861(80)90449-X

40. Horst R, Prapong S, Reinhardt T, Koszewski N, Knutson J, Bishop C. Comparison of the relative effects of 1,24-dihydroxyvitamin D(2) [1, 24-(OH)(2)D(2)], 1,24-dihydroxyvitamin D(3) [1,24-(OH)(2)D(3)], and 1,25-dihydroxyvitamin D(3) [1,25-(OH)(2)D(3)] on selected vitamin D-regulated events in the rat. *Biochem Pharmacol.* (2000) 60:701–8. doi: 10.1016/S0006-2952(00)00378-6
41. Mawer EB, Jones G, Davies M, Still PE, Byford V, Schroeder NJ, et al. Unique 24-hydroxylated metabolites represent a significant pathway of metabolism of vitamin D2 in humans: 24-hydroxyvitamin D2 and 1,24-dihydroxyvitamin D2 detectable in human serum. *J Clin Endocrinol Metab.* (1998) 83:2156–66. doi: 10.1210/jc.83.6.2156
42. Horst RL, Reinhardt TA, Ramberg CF, Koszewski NJ, Napoli JL. 24-Hydroxylation of 1,25-dihydroxyergocalciferol. An unambiguous deactivation process. *J Biol Chem.* (1986) 261:9250–6. doi: 10.1016/S0021-9258(18)67647-1
43. Horst RL, Koszewski NJ, Reinhardt TA. 1 alpha-hydroxylation of 24-hydroxyvitamin D2 represents a minor physiological pathway for the activation of vitamin D2 in mammals. *Biochemistry.* (1990) 29:578–82. doi: 10.1021/bi00454a035
44. Reinhardt TA, Ramberg CF, Horst RL. Comparison of receptor binding, biological activity, and in vivo tracer kinetics for 1,25-dihydroxyvitamin D3, 1,25-dihydroxyvitamin D2, and its 24 epimer. *Arch Biochem Biophys.* (1989) 273:64–71. doi: 10.1016/0003-9861(89)90162-8
45. Mattila PH, Piironen VI, Uusi-Rauva EJ, Koivistoinen PE. Contents of cholecalciferol, ergocalciferol, and their 25-hydroxylated metabolites in milk products and raw meat and liver as determined by HPLC. *J Agricult Food Chem.* (1995) 43:2394–9. doi: 10.1021/jf00057a015
46. Ovesen L, Brot C, Jakobsen J. Food contents and biological activity of 25-hydroxyvitamin D: a vitamin D metabolite to be reckoned with? *Ann Nutr Metab.* (2003) 47:107–13. doi: 10.1159/000070031
47. Lalor S, Schwartz AM, Titmarsh H, Reed N, Tasker S, Boland L, et al. Cats with inflammatory bowel disease and intestinal small cell lymphoma have low serum concentrations of 25-hydroxyvitamin D. *J Vet Intern Med.* (2014) 28:351–5. doi: 10.1111/jvim.12294
48. Laflamme D. Development and validation of a body condition score system for cats: a clinical tool. *Feline Practice.* (1997) 25:13–8.
49. Committee for the Update of the Guide for the Care and Use of Laboratory Animals. *Guide for the Care and Use of Laboratory Animals*. 8th ed. Washington, DC: National Academies Press (2011). 246 p.
50. Backus RC, Foster LR, Dietary 25-hydroxyvitamin D3 and its potency relative to vitamin D3 for affecting vitamin D status of dogs. *Am J Vet Res*. (In Press).
51. Lensmeyer GL, Wiebe DA, Binkley N, Drezner MK. HPLC method for 25-hydroxyvitamin D measurement: comparison with contemporary assays. *Clin Chem.* (2006) 52:1120–6. doi: 10.1373/clinchem.2005.064956
52. Lensmeyer G, Poquette M, Wiebe D, Binkley N. The C-3 epimer of 25-hydroxyvitamin D(3) is present in adult serum. *J Clin Endocrinol Metab.* (2012) 97:163–8. doi: 10.1210/jc.2011-0584
53. Hollis BW. Assay of circulating 1,25-dihydroxyvitamin D involving a novel single-cartridge extraction and purification procedure. *Clin Chem.* (1986) 32:2060–3. doi: 10.1093/clinchem/32.11.2060
54. Kaufmann M, Gallagher JC, Peacock M, Schlingmann KP, Konrad M, DeLuca HF, et al. Clinical utility of simultaneous quantitation of 25-hydroxyvitamin D and 24,25-dihydroxyvitamin D by LC-MS/MS involving derivatization with DMEQ-TAD. *J Clin Endocrinol Metab.* (2014) 99:2567–74. doi: 10.1210/jc.2013-4388
55. Bligh EG, Dyer WJ. A rapid method of total lipid extraction and purification. *Can J Biochem Physiol.* (1959) 37:911–7. doi: 10.1139/y59-099
56. Shieh A, Chun RF, Ma C, Witzel S, Meyer B, Rafison B, et al. Effects of high-dose vitamin D2 versus D3 on total and free 25-hydroxyvitamin D and markers of calcium balance. *J Clin Endocrinol Metab.* (2016) 101:3070–8. doi: 10.1210/jc.2016-1871
57. Trang HM, Cole DEC, Rubin LA, Pierratos A, Siu S, Vieth R. Evidence that vitamin D-3 increases serum 25-hydroxyvitamin D more efficiently than does vitamin D-2. *Am J Clin Nutr.* (1998) 68:854–8. doi: 10.1093/ajcn/68.4.854
58. Batacchi Z, Robinson-Cohen C, Hoofnagle AN, Isakova T, Kestenbaum B, Martin KJ, et al. Effects of vitamin D2 supplementation on vitamin D3 metabolism in health and CKD. *Clin J Am Soc Nephrol.* (2017) 12:1498–506. doi: 10.2215/CJN.00530117
59. Zhu J, DeLuca HF. Vitamin D 25-hydroxylase - four decades of searching, are we there yet? *Arch Biochem Biophys.* (2012) 523:30–6. doi: 10.1016/j.abb.2012.01.013
60. Cesareo R, Falchetti A, Attanasio R, Tabacco G, Naciu AM, Palermo A. Hypovitaminosis D: is it time to consider the use of calcifediol? *Nutrients.* (2019) 11:1016. doi: 10.3390/nu11051016
61. Quesada-Gomez JM, Bouillon R. Is calcifediol better than cholecalciferol for vitamin D supplementation? *Osteoporos Int.* (2018) 29:1697–711. doi: 10.1007/s00198-018-4520-y
62. Bailey D, Veljkovic K, Yazdanpanah M, Adeli K. Analytical measurement and clinical relevance of vitamin D(3) C3-epimer. *Clin Biochem.* (2013) 46:190–6. doi: 10.1016/j.clinbiochem.2012.10.037
63. Higashi T, Sakajiri K, Shimada K. Analysis of C-3 epimerization in (24R)-24,25-dihydroxyvitamin D3 catalyzed by hydroxysteroid dehydrogenase. *J Pharm Biomed Anal.* (2004) 36:429–36. doi: 10.1016/j.jpba.2004.07.012
64. Kamao M, Tatematsu S, Hatakeyama S, Sakaki T, Sawada N, Inouye K, et al. C-3 epimerization of vitamin D3 metabolites and further metabolism of C-3 epimers: 25-hydroxyvitamin D3 is metabolized to 3-epi-25-hydroxyvitamin D3 and subsequently metabolized through C-1alpha or C-24 hydroxylation. *J Biol Chem.* (2004) 279:15897–907. doi: 10.1074/jbc.M311473200
65. Masuda S, Kamao M, Schroeder NJ, Makin HL, Jones G, Kremer R, et al. Characterization of 3-epi-1alpha,25-dihydroxyvitamin D3 involved in 1alpha,25-dihydroxyvitamin D3 metabolic pathway in cultured cell lines. *Biol Pharm Bull.* (2000) 23:133–9. doi: 10.1248/bpb.23.133
66. Siu-Caldera ML, Sekimoto H, Weiskopf A, Vouros P, Muralidharan KR, Okamura WH, et al. Production of 1alpha,25-dihydroxy-3-epi-vitamin D3 in two rat osteosarcoma cell lines (UMR 106 and ROS 17/2.8): existence of the C-3 epimerization pathway in ROS 17/2.8 cells in which the C-24 oxidation pathway is not expressed. *Bone.* (1999) 24:457–63. doi: 10.1016/S8756-3282(99)00019-8
67. Kamao M, Tatematsu S, Sawada N, Sakaki T, Hatakeyama S, Kubodera N, et al. Cell specificity and properties of the C-3 epimerization of Vitamin D3 metabolites. *J Steroid Biochem Mol Biol.* (2004) 89–90:39–42. doi: 10.1016/j.jsbmb.2004.03.048
68. Sih TR, Morris JG, Hickman MA. Chronic ingestion of high concentrations of cholecalciferol in cats. *Am J Vet Res.* (2001) 62:1500–6. doi: 10.2460/ajvr.2001.62.1500
69. van Leeuwen JP, van den Bemd GJ, van Driel M, Buurman CJ, Pols HA. 24,25-Dihydroxyvitamin D(3) and bone metabolism. *Steroids.* (2001) 66:375–80. doi: 10.1016/S0039-128X(00)00155-0
70. Marino CL, Lascelles BD, Vaden SL, Gruen ME, Marks SL. Prevalence and classification of chronic kidney disease in cats randomly selected from four age groups and in cats recruited for degenerative joint disease studies. *J Feline Med Surg.* (2014) 16:465–72. doi: 10.1177/1098612X13511446

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The remaining author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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