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Nutrition and management of animals we keep as companions, volume II

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Editorial: Nutrition and management of animals we keep as companions, volume II

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Editorial on the Research Topic
Nutrition and management of animals we keep as companions, volume II

1 Summary and commentary

Addressing the conflict between global warming and consumption of food by agricultural, companion, and exotic animals and humans requires a holistic approach that considers sustainable agricultural practices, efficient ingredient and product supply chains, reduced food waste, consideration of upcycling of food waste, and changes in consumption patterns. Invention and innovation in technology, policy interventions, and knowledge translation to individual consumer choices all play a role in finding solutions to mitigate the environmental impact of food production and consumption. The profound challenges that we face oblige us to find solutions that optimize animal, human, and environmental health.

In the nutrition of dogs and cats, animal sources of ingredients have been postulated as more complete ingredients, considering the nutrient requirements of these animals. However, the number of pet owners willing to replace animal sources with plant-based ingredients is growing. Pet owners believe they can potentially reduce their pets' environmental impact when transitioning to plant-based pet foods. In fact, it makes sense, since animal sources come from another trophic level, and the environmental cost is higher than that of producing vegetable ingredients. However, many pet foods use animal by-products, which do not involve directly raising livestock. Further to this, plant-based ingredients do not deliver all the nutrients that dogs and cats require. Thus, in this edition, Yoosefzadeh-Najafabadi et al. discuss how plants could be selected in a breeding program to get high-quality cultivars to meet the future demands of ingredients in feed formulations intended for pets. The authors addressed that with the improvement of the nutritional quality of plant-based feedstuffs, it may be possible to reduce the number of ingredients used to formulate complete diets for pets.

In this context, Morris E. et al. included vegetable protein in diets for cats, replacing hydrolyzed chicken liver and heart with rice protein concentrate at 0, 7, 14, and 28% of the diet. A linear improvement in the digestibility of dry matter and energy was observed followed by a linear improvement in true protein and carbohydrate digestibility as well. Against all expectations, food consumption was greater and fecal consistency was improved in cats fed the rice concentrate protein, making it an excellent ingredient to include in cat diets.

On the other side of society, there are pet owners interested in going against processed food, looking to feed dogs and cats with raw food, which they call "natural" foods. However, the risk of feeding companion animals with raw food has been declared not just for the animals but also for humans living with them. According to Kiprotich and Aldrich, some strategies can be used to minimize the risk of pathogens in raw diets, such as using non-thermal processing, high-pressure pasteurization, or using Generally Recognized as Safe (GRAS) food additives approved such as organic acids, essential oils, and bacteriophages can be considered as effective methods. The association of different additives and their combination with methods holds promise for controlling pathogenic microbes in raw food and ensuring the safety of these foods for both animals and the humans they share their households with.

Hemida et al. suggested that raw and extruded diets can have effects on epigenetics related to otitis in dogs. After evaluating longitudinal data reported in mothers and puppies, they concluded that dogs fed an ultra-processed carbohydrate-based diet (UPCD, dry) - 75% or more during pregnancy or in the growing phase of puppies up to6 months - were associated with otitis in later life. Puppies fed a non-processed meat-based diet (NPMD, raw) - at least 25% of the total diet - were associated with an increase in the incidence of otitis. Also, exposure to sunlight for more than 1 h a day and dogs raised on a dirt floor had a lower risk of developing otitis suggesting that the environment we raise pets in also predicts the risk of otitis.

Homemade diets may be a risk to dogs' and cats' health when not well-balanced. However, well-adjusted homemade diets for specific animals can benefit health. Silva et al. reported a case of ALT likely increased by excessive hepatic copper of a dog fed a kibble diet. Despite the concentrations being under normal levels, it is recognizable that sensitive dogs can be affected by copper concentrations that are relatively low. Using a homemade diet with moderately low copper was enough to maintain the dog with a low concentration of serum ALT.

The association between grain-free diets and dilated cardiomyopathy (DCM) is still under investigation after 2018 when the US Food and Drug Administration reported a potential link between these two factors. Bokshowan et al. investigated if the replacement of rice by pea, or rice diet plus 1% raffinose, is related to changes in the bioavailability of taurine, sulfur amino acids, excretion of bile salts, and some changes in the dynamics of the heart that could be related to the development of DCM after 5 weeks of feeding. Taking all results together, they did not find a clear relationship between pea or oligosaccharide-containing diets. Instead, a control diet (commercial dental diet) with no oligosaccharides detectable, but with a higher level of insoluble fiber, produced changes in plasma N-terminal pro-brain natriuretic peptide (NT-proBNP), one of the indicators of the development of DCM. In fact, some nutritional traits common in grain-free diets, such as gluten-free and low-glycemic starch sources, may present beneficial physiological effects in specific cases. Baptista da Silva et al. reported a case of a German Spitz with epileptic seizures controlled by a gluten-free/hydrolyzed protein diet. Also, Vastolo et al. tested two grain-free diets (sweet potato vs. pea starch) against a control diet (spelt + oats) to look at the postprandial glycemia in dogs fed different starch sources. The diet containing pea starch had the smallest postprandial glucose and insulin area under the curve and the lowest serum concentration of fructosamine, followed by the diet containing sweet potato.

Different starch sources can release glucose differently in the intestine, presenting an impact on glycemia and the net disposal of glucose in peripheral tissues. The dynamics of glucose digestion and metabolism need further investigation. Dogs and cats with diabetes need special formulations, and functional ingredients are fundamental to producing therapeutic diets. Recently, Corbee et al., looking for another way to control glycemia in diabetic patients, reviewed the fibroblast growth factor-21 (FGF21) analogs as a possible option to help in the treatment of diabetes. FGF21 is produced normally in the body and is greater in obese and diabetic patients. In experimental models, FGF21 seems to improve hepatic glucose metabolism, enhance serum insulin concentrations, lower blood glucose, and stimulate β-oxidation of the fatty acids. Curiously, at increasing concentrations of FGF21, obese mice did not fully respond anymore, leading the authors to believe that FGF21 could be harmful to the body and metabolism, or obese patients could become resistant to FGF21. The analogs of FGF21 have not been tested in dogs, but the combination with diets and exercise would be an efficient proposal for weight loss and glycemia control.

Food processing has been the focus of research for a long time as a way to produce and store food safely. Dainton, Molnar et al. investigated the effects of the canned food container size and type (flexible and semi-rigid and rigid) on thermal processing and its effect on B vitamins. They observed that the more flexible the containers are, the faster they reach the target lethality (time vs. temperature) during retort processing. Thiamine and riboflavin were shown to be unstable during retort processing, independent of the container type evaluated. The other vitamins remained stable. However, the authors drew attention to the processing losses, which must be greater once the target lethality was reached in this study, and supplementation is needed to guarantee the required amount of vitamins. Also, Dainton, White et al. showed that supplementing vitamin premix and yeast could improve the levels of thiamine in canned food. After 6 months, diets containing yeast were associated with a greater thiamin concentration, suggesting that thiamine from yeast is more resistant to storage.

Vitamins and other nutrients are adjusted to the content of energy in the diet to secure adequate ingestion of essential nutrients. The lack of precision in estimating metabolizable energy could result in malnutrition or add excessive nutrients, which is environmentally wasteful. Jewell and Jackson reviewed the equations to predict energy in dry and wet food for dogs and cats using a large amount of data and compared them to the energy density prediction values of the modified Atwater factors, the NRC (crude fiber), and the Hall equations. New equations were proposed, tested, and compared to the others. Despite the errors associated with the equations, a new version reduced the difference between the measured and estimated metabolizable energy, and a better predictor of food consumption per metabolic body weight was reached for dogs and cats, helping to adjust nutrients to the content of energy and avoiding over formulation and waste of nutrients.

Lipids are an important energy source for dogs and cats. Also, fatty acids are functional molecules and play a role as structural compounds in the cells. Jackson and Jewell conducted a study on long-chain fatty acids using fish oil (FO) as a source of EPA (20:5n3) and DHA (22:6n3) and medium-chain fatty acids oil (MCT - 8% caproate, 6:0; 51.4% caprylate, C8:0; 39.1% caprate, C10:0; <0.1% laurate, C12:0; and <0.01% of other fatty acids). They fed dogs for 28 days, and serum metabolomics was performed. The inclusion of FO decreased levels of triglycerides and total cholesterol, whether isolated or in association with MCT. Overall, the metabolites found showed that MCT largely led to changes in serum lipids associated with energy metabolism, while FO consumption produced changes dominated by structural-type lipids, confirming the competition already known between n-3 and n-6 fatty acids on phospholipids according to their inclusion in the diet. Furthermore, a long list of lipids was found and explored in the study, most of them were linked to the immune system and signaling factors, and part of them responded to the FO diet. Richards et al. also investigated the effects of concentrated vegetable sources of n-3 in dogs. Camelina oil, the second richest ALA oil, was compared with flaxseed and canola oil, searching for changes in inflammatory and oxidative markers, and coat quality in adult dogs. Camelina oil improved the coat, as did all other sources of oil containing great concentrations of linolenic acid (18:2n-3). However, inflammatory and oxidative markers remained stable across the diets and for 16 weeks of supplementation; however, all dogs were healthy and without any disorders mediated by inflammation.

The lipidic and energetic metabolism of the organism depends on some nitrogenous compounds, such as creatine, choline, and carnitine to work properly. For example, phosphocreatine is readily used for ATP regeneration during initial high caloric demand, while choline and carnitine are important for phospholipid synthesis and hepatic export and β-oxidation of long-chain fatty acids, respectively. Banton et al. showed that feeding healthy dogs with a diet supplemented with a combination of creatine, carnitine, and choline (CCC diet) resulted in the elevation of plasma creatine concentration more than diets supplied with methionine or taurine. Plasma creatine remained elevated up to 6 h after the meal. As a consequence, concentrations of plasma creatinine were elevated from 1 to 3 h after the meal in dogs fed the CCC diet. The fact that plasma creatinine can be affected by dietary creatine in the diet warns of potentially misleading results in healthy patients when creatinine is used as a diagnostic. Choline and carnitine were also tested by Rankovic et al. in lean and obese cats. Choline supplied in the diet resulted in lower food intake in cats, but no changes in body weight, body condition score, and body composition were observed. Lipid metabolism was influenced by choline, and greater total cholesterol and its fractions were increased in plasma. It seems that choline improves the mobilization of lipids. Choline or carnitine seemed not to play any influence over obese and lean cats, although obese cats showed more serum triglycerides, alkaline phosphatase, VLDL, and HDL-C than lean cats.

Besides the relevance of studying the functional role of nutrients in dogs and cats, the evaluation of diet digestibility is crucial during pet food development. Research and development are one of the industrial sections that require special attention. Improving the digestibility of diets has an impact on nutrient availability and fecal consistency. Jadhav et al., adding a blend of enzymes to commercial diets *in vitro*, observed greater digestibility of nutrients and energy, protein molecular weight reduction, and an increase in antioxidant capacity, providing an effective strategy to enhance nutrient digestibility. Classically, digestibility is performed *in vivo* with trained animals following AAFCO and FEDIAF protocols. However, Bos et al. evaluated an in-home protocol of digestibility in cats using the marker method (TiO_2). They fed cats for 8 days, and feces were collected and analyzed on a daily basis. A steady state was reached in digestibility parameters within 2 days of adaptation to the diet suggesting digestibility studies do not require feeding animals for longer periods of time. Also, after adaptation, 1 day of fecal collection was accurate to define digestibility at home, and using 3 days improved accuracy based on the irregular defecation pattern of the cats. Depending on the nutrient class, the number of cats ranged between 6 and 12, assuming acceptable errors. However, the authors declare that a comparison between in-home and in-lab tests would be interesting to control the sources of variation with in-home digestibility tests. Adoption of in-home digestibility protocols would help to avoid the reduce the use of laboratory dogs and cats in these research protocols.

Complementary to diet digestibility evaluation, analysis of dietary salt content and effects on urine production and supersaturation are very important, especially to cats. The relative urine supersaturation (RSS) can be calculated by software (EQUIL2). Anthony et al. used EQUIL2 to calculate the RSS and compared it with the RSS calculated in a new version of the software (EQUIL-HL21), and both were considered satisfactory for data from dogs and cats. Also, Morris E. M. et al. demonstrated that the EQUIL-HL21 program can accurately detect expected differences between foods formulated for urinary and non-urinary indications for cats. Regression models revealed that pH, magnesium, ammonium, citrate, chloride, calcium, phosphorus, and sulfate are the main urinary analytes that contribute to the predicted RSS values for struvite and calcium oxalate crystal formation. Urinary foods produced lower urinary pH, ammonium, potassium, phosphorus, magnesium, oxalate, citrate, and sulfate concentrations. The greater amount of sodium and chloride in urinary diets improves their excretion and increased volume of urine both which helps with dilution and lowers the risk for struvite formation.

Finally, in this second edition of "*Nutrition and management of animals we keep as companions*," we had the opportunity to review a diverse array of studies employing various approaches, all aimed at addressing questions related to pet nutrition. Ingredients, nutrients, epigenetic effects, absorption, and metabolism were evaluated in both healthy and ill patients, providing a comprehensive perspective to integrate information and determine optimal feeding practices for the health and longevity of dogs and cats.

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High-throughput plant breeding approaches: Moving along with plant-based food demands for pet food industries

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Introduction

Moving toward consuming plant-based meat while shifting away from conventional meat may benefit the environment, animal welfare, and human health (1). For many decades, conventional plant-based proteins such as seitan, tempeh, and tofu, as well as other vegetarian meat-like foods, have been commercially accessible (2). However, plant-based food demands have significantly increased over the last decades, especially in Western countries (3). The high demands for plant-based foods are not only limited to humans, as many companion animal owners are continuously concerned about the possible correlations between the consumption of animal products, even for their pets, and animal welfare problems, herbicides and fertilizers, degenerative health conditions, and climate change (Figure 1) (4).

In the United States, cats and dogs outnumber children under the age of 18 by nearly two to one, with 83 and 95 million pet dogs and cats, respectively (5). In Europe, especially in the United Kingdom, more than 57 million pets have been spread across ~40% of households, and the pet population is increasing by one percent annually (6). Several studies have been done all around Europe to find the possible reasons for changing the current common pet diet to plant-based, and the results indicated that animal welfare, ethical, and moral concerns are the three most important concerns that make pet owners willingly select the plant-based diet for their pets (4, 7–9). Therefore, efficient and effective plant-based meat production is now a high trend in pet industries in order to keep pace with high plant-based meat demands in the near future.

Meat has long been seen as a vital part of a balanced diet since it contains many valuable nutrients, such as high biological value protein, iron, vitamin B12, other B complex vitamins, zinc, selenium, and phosphorus (10). In addition, meat is considered as an important dietary component due to its high protein content and complete amino acid profile. Twenty different amino acids, as building blocks of proteins, are used to construct proteins; eight are considered essential for humans (10 for dogs), meaning they must be ingested as food as the body does not produce them (10). The remaining amino acids can be created in the body and are hence considered non-essential. However, it

would be necessary to supply the required raw components for the body to produce the non-essential amino acids sufficiently.

Different plant protein sources, such as soybean, which contain all essential amino acids (11), can be used as textured vegetable protein (TVP), an alternative to meat consumption. Nonetheless, TVP made from soybeans lacks important nutrients such as low levels of tryptophan, methionine, vitamin B12, and vitamin D that meat provides sufficiently. In addition, there are some nutrients from TVP sources that may not be well absorbed in the body (12). Therefore, additional nutrients and/or various plant protein sources should be embedded/adjusted into TVP to be considered as nutritionally valuable/complete as meat for plant-based food diets (Figure 1).

While the plant-based food demands for pets are increasing continuously, it would be necessary to develop plant cultivars that require less adjustment for optimal pet foods. The typical plant breeding process, from crossing to releasing a cultivar, will take many years (e.g., at least 10 years in soybean) (13). In addition, breeding for complex traits that are under control by several genetic and environmental factors even makes the breeding process cumbersome, expensive, and time-consuming (14). However, the recent advances in high throughput approaches can facilitate the plant breeding process by measuring several traits in a less expensive and timely manner, making the breeding decision more accurate and shortening the breeding cycle (15).

Here in this opinion paper, we briefly explain the current status/progress of plant-based pet foods, describe the high throughput plant breeding approach, elaborate on the recent high throughput approaches in plant breeding programs, and finally provide a vision for possible financial investment in developing cultivars for plant-based food industries.

Consume meats to meet nutritional demands: Still valid for today's life?

Throughout history, humans have always consumed meats to meet nutritional demands. However, meat consumption has increased dramatically over the last century, despite the availability of non-meat-based foods (16). Therefore, meat production has expanded fourfold since the 1960s to fulfill these rising demands, outpacing population growth. As a result, by 2050, worldwide meat consumption is expected to treble from 2008 despite the negative impacts that can be made on environmental sustainability, human health, and ethical considerations, improving public health and minimizing animal suffering (17). The main question here is whether consuming meats is still required to meet nutritional demands?

Plant foods such as soybeans, peas, beans, chickpeas, peanuts, and lentils have a long history of being used as protein sources in a broad range of cultural and international cuisines. Many are excellent protein sources, but few contain all the essential amino acids at a level that is sufficient to offer a full nutritional profile required for dogs. In addition, plant-based diets are not offering taurine, which is essential for cats. Therefore, it should be synthetically added as a supplement to plant-based pet food. Consequently, various plant-based protein sources must be used to meet nutritional needs, while most animal protein, like meat or dairy, does the whole job by itself, being a complete protein (18). As an example, soy protein is one of the important plant-based protein sources containing all the essential amino acids (11). However, it has to be used in combination with other plant sources to offer a well-balanced dietary profile for dogs (Figure 1).

Plant-based pet foods are not perfect for your pets: Rumor or real?

Animal food is not all about getting the optimum amount of protein every day but considering the amino acids that those proteins are formed of Knight and Leitsberger (4). Most pet food industries usually consider the amino acid profile as the priority to ensure that all of the required amino acids are included in the pet food (8). The key point of producing plant-based pet foods is to obtain the right combinations of different ingredients, such as plants, vitamins, and minerals (4). However, some believe that the current plant-based pet foods serve as double-edged swords with their merits and demerits and plant-based pet foods are underpowered to meet the criteria for carnivorous pets, such as cats (19). Dogs are known for being opportunistic feeder and can feed well on balanced plant-based pet foods, while cats are conventionally known as obligated carnivores, and meat is supposed to be included in their diets (19). However, a significant and growing body of population studies and case reports have indicated that both dogs and cats maintained on vegetarian diets may be healthy and, indeed, may experience a range of health benefits (4, 20).

Plant-based pet food status in the post-COVID-19 pandemic era

As business shutdowns, travel restrictions, and lockdowns were the three main inevitable components of the COVID-19 pandemic, a significant number of businesses experienced a challenging time, especially the food and beverage industries (21). The closure of several facilities and companies is obstructing global supply chains and negatively affecting production processes, delivery schedules, and sales of various items (21). Although the value chain disruption during the early stages of the COVID-19 pandemic had negatively impacted the plant-based pet food market, most people added new pets to their homes due to the remote work situation (22). Therefore, the COVID-19 pandemic has not had a large detrimental

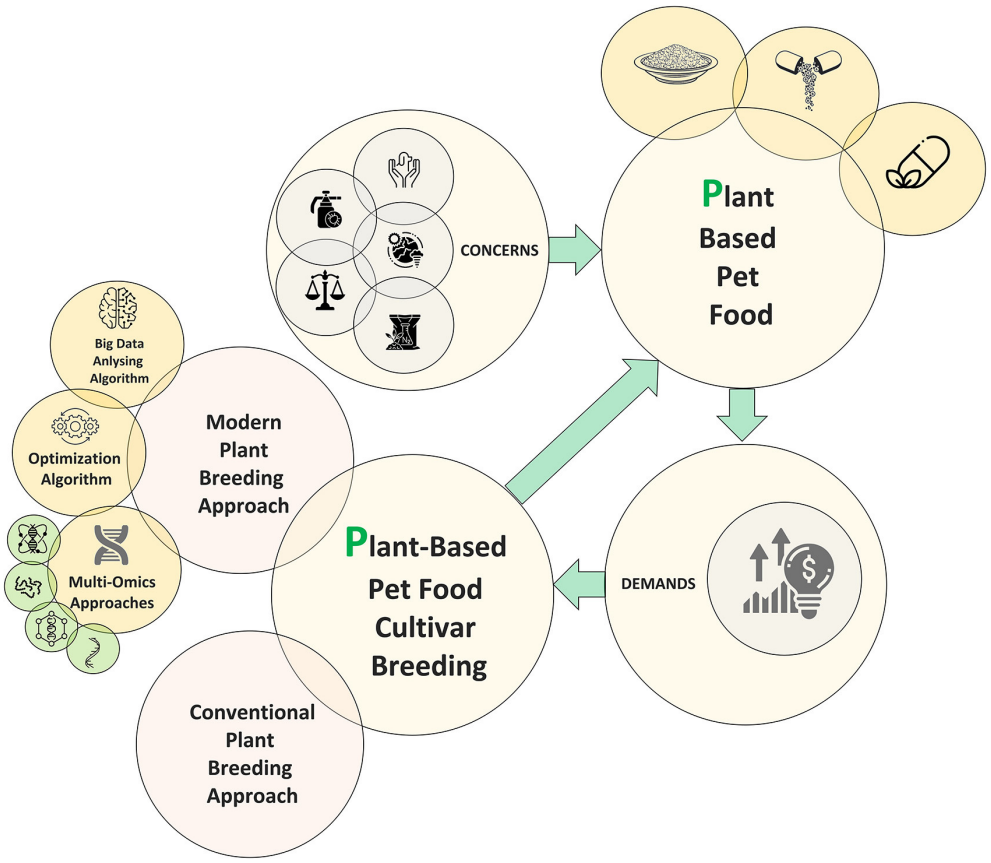

FIGURE 1
Schematic view of plant cultivar breeding pipeline for plant-based pet food production.

influence on the market for plant-based pet food, while the demand for plant-based pet food online shopping increased significantly (Figure 1) (22). Based on research conducted by Insight Partners, the global market for plant-based pet food production is expected to increase from nearly US$8.6 billion in 2021 to almost US$15.6 billion by 2028, which clearly indicates the huge investment in this area (23).

Cultivar development based on the pet food industries demands

One major problem that makes plant-based pet food production difficult is balancing the food based on several ingredients from different resources to ensure all nutritional needs are met (24). A single, complete protein source such as TVP is commonly used in plant-based pet food, which provides all the essential amino acids for dogs, but it has to be balanced for the right amount of essential amino acids to provide all the necessary nutrients at the required level that a meat-based diet offers (8). To face these challenges, several pet food companies provide dogs with all the nutrients they need by carefully formulating recipes based on a combination of yeast and plant proteins. The recipes are also supplemented with additional sources to ensure a complete and balanced vegan meal. However, adjusting plant-based pet foods is laborious and time-consuming, and pet food companies try to reduce their dependencies on several ingredients as much as possible. Here, the plant breeding approaches may be useful to breed for a well-balanced plant-based protein source. Instead of combining different plant protein sources, it would be convenient to develop a single resource that is equal to meat in terms of nutrition.

High throughput approaches to facilitate plant breeding programs

Nowadays, with significant advancements in high-throughput approaches in different plant omics such as phenomics, genomics, transcriptomics, proteomics, epigenomics, and metabolomics, plant breeders are now

able to speed up the breeding process and make decisions more accurate (Breeding era 4.0), especially for complex traits such as yield, protein, oil, and metabolite concentrations (25). As an example, increasing methionine concentration in soybean is a game changer parameter in plant-based pet food production which requires a comprehensive environment, genetic, and other omics information (Figure 1). The possible breeding pipeline to increase the methionine concentration in soybean is to (i) quantifying the amount of methionine using targeted approaches to select the superior genotypes that are high in methionine concentration, (ii) crossing the high methionine soybean genotype with high yield, high protein soybean cultivar, (iii) determining the genes and transcripts associate with the tested traits to detect the possible correlation between high yield, high protein and high methionine concentration genotypes using genome/transcriptome-wide association studies, (iii) detecting the possible linkage, epistasis, and/or pleiotropy effects existed with the loci governing methionine and other traits of interest, (iv) predicting the genotype performance using sophisticated bigdata analyzing methods to make the breeding decision more accurate, (v) speeding up the breeding progress by cultivating more than one generation in a year, (vi) making the breeding cycle short by using the associated genomic/transcriptomic regions with a trait of interest as markers, and (vii) using genome editing technologies in parallel with the conventional breeding methods to precisely modify several associated genomic regions with the tested traits per generation and add new value-added traits to the developed plant cultivar.

As several traits are required to be adjusted in developing cultivars for plant-based pet food production, the optimum values of the tested traits have to be determined based on their genetic relationship with other desirable traits (26). Nowadays, optimization algorithms (Figure 1), which optimize the input variables to maximize the value of the output variable, are implemented in plant breeding to improve multi-trait selection (26). In the methionine example, optimization algorithms can be employed considering the genotypic information of each genotype as input variables and methionine, protein, and yield as outputs. The outcome would be detecting the optimum condition of genomic regions in order to have a soybean cultivar with high yield, protein, and methionine concentration. Such results can be used in genome editing or/and marker-assisted selection pipelines to speed up the breeding process.

Conclusion and future perspective

Due to animal welfare, ethical, moral, and climate change concerns around producing meat-based food, pet owners are willing to change their pets' diets by shifting to plant-based pet foods. As a result, plant-based pet food production grows exponentially, and there is a dire need to facilitate the speed of food production to keep pace with future demands. Meanwhile, reaching the optimum amount of protein/amino acid profiles in plant-based pet foods requires a precise combination of different plant-sourced ingredients as there are no individual plant sources that are sufficient to meet all plant-based pet food criteria. However, by introducing plant breeding cultivar development pipelines specifically for pet food industries, we could be able to reduce the number of plant-sourced ingredients in plant-based pet foods. This endeavor requires multi-trait plant breeding programs as several plant components need to be adjusted simultaneously, for plant-based pet food production, which requires precise use of optimization algorithms in this area. Meanwhile, to speed up the breeding process and shorten the breeding cycle, breeders may want to use high-throughput methods and sophisticated bigdata analyzing methods in their breeding programs. In addition, this opinion paper suggested that financial agents invest in plant breeding programs to develop high-quality plant cultivars to meet the future demands of plant-based pet food production. As the future direction, several important areas are still less explored and need further investigations in plant-based pet food production, such as (1) ethical concerns to assure that pet owners are not imposing their personal views on their pets, and (2) longevity and palatability of plant-based pet foods.

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Author MV was employed by PawCo Foods.

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A review of food additives to control the proliferation and transmission of pathogenic microorganisms with emphasis on applications to raw meat-based diets for companion animals

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Raw meat-based diets (RMBDs) or sometimes described as biologically appropriate raw food (BARFs) are gaining in popularity amongst dog and cat owners. These pet guardians prefer their animals to eat minimally processed and more "natural" foods instead of highly heat-processed diets manufactured with synthetic preservatives. The market for RMBDs for dogs and cats is estimated at $33 million in the United States. This figure is likely underestimated because some pet owners feed their animals raw diets prepared at home. Despite their increasing demand, RMBDs have been plagued with numerous recalls because of contamination from foodborne pathogens like *Salmonella*, *E. coli*, or *Campylobacter*. Existing literature regarding mitigation strategies in RMBD's for dogs/cats are very limited. Thus, a comprehensive search for published research was conducted regarding technologies used in meat and poultry processing and raw materials tangential to this trade (e.g., meats and poultry). In this review paper, we explored multiple non-thermal processes and GRAS approved food additives that can be used as potential antimicrobials alone or in combinations to assert multiple stressors that impede microbial growth, ultimately leading to pathogen inactivation through hurdle technology. This review focuses on use of high-pressure pasteurization, organic acidulants, essential oils, and bacteriophages as possible approaches to commercially pasteurize RMBDs effectively at a relatively low cost. A summary of the different ways these technologies have been used in the past to control foodborne pathogens in meat and poultry related products and how they can be applied successfully to impede growth of enteric pathogens in commercially produced raw diets for companion animals is provided.

KEYWORDS

***Salmonella*, raw pet food, companion animals, raw meat-based diets (RMBDs), essential oils, organic acids**

Introduction

The domestication of the modern dog (*Canis lupus familiaris*) has resulted in a remarkable shift from the diet that its ancestor the wolf derived sustenance through scavenging and hunting small prey (1). The 2021–2022 American Pet Products Association (APPA) national pet owners survey reported that 70% of United States households owned a pet, which equates to 90.5 million homes (2). Because of anthropomorphism, there is an increasing number of pet owners who consider their animal a family member (3–6). Thus, the shift in human dietary choices due to increasing health consciousness are reflected in the ingredients pet owners prefer their animal consumed, thus there has been an increase in demand in foods that are considered "raw" and/or minimally processed (1, 7–9).

Raw meat-based diets (RMBDs) are a subset of minimally processed commercial diets (MPCD) or minimally processed home diets (MPHD) for companion animals and consist of raw or uncooked proteins sourced from animals such as lamb, pork, poultry, beef, venison, organ meats or offal, and supplemented with vegetables, tubers, dairy, or eggs (10, 11). However, the scope of this review will focus on RMBDs that are manufactured and marketed commercially in fresh and frozen forms. The market for MPCD diets is estimated at $120 million (11) of which RMBDs are a subcategory with estimates exceeding $33 million as of 2019. The feeding of companion animals with RMBDs is becoming increasingly popular because pet owners perceive these diets as "natural" and therefore presumed to provide additional health benefits to their animals, including but not limited to improved oral health, skin and coat compared to when these animals are fed conventionally heat sterilized foods manufactured through canning or extrusion (12–14). The shift toward RMBDs has resulted from concern that commercially available pet foods are over cooked when they are produced by extrusion (kibbles), baking (treats), or through canning (wet loaf and chunks and gravy styles). All undergo extremes in heat treatments during manufacturing to increase digestibility and assure microbial safety. However, these high temperatures are associated with increased degradation of some nutrients and formation of undesirable and potentially harmful compounds such as advanced glycation products (AGEs) (15–19). This tends to reinforce the argument for raw, and/or minimally processed pet food products (20–22). Presuming of course that safety can be assured by other means.

Typically, RMBDs are formulated with proteins from chicken, beef, lamb, duck, veal, and venison, organs like heart or liver, and are supplemented with bones, dairy products, fish, vegetables, fruits, and plant oils (10, 11). Vitamins and trace minerals may be added to these diets to adjust for any micronutrient shortcomings. Characteristically, these ingredients are ground and mixed into a batter and formed into patties, nuggets or placed into trays for commercial sale. Some pet owners prepare RMBDs from their homes because these diets are often expensive and not widely available in stores. Some pet owners opt to prepare these diets themselves because of the mistrust they have for "big" pet food companies due to numerous product recalls associated with aflatoxins and emerging research highlighting the ill health effects associated with animal consumption of AGEs, present in ultra-processed commercial diets (11). Regardless, the goal is that these diets meet the animal's nutrient requirements for amino acids, fatty acids, minerals, and vitamins.

Presuming nutrition can be met, the rest of the focus on these diets is safety and how to reduce microbial contamination by enteric foodborne pathogens such as non-typhoidal *Salmonella* spp. or *Campylobacter* that are inherently found in meat and poultry products. This is because raw diets cannot be heat processed, fermented, rendered, purified, extracted, or hydrolyzed by enzymolysis (23), thus leaving few avenues for efficient non-thermal antimicrobial interventions. Moreover, numerous studies have shown that RMBDs produced without adequate kill-steps are important vehicles for the transmission of pathogens to companion animals and to their human owners, during handling of food, or *via* cross-contamination with contact surfaces (24, 25). Foodborne enteric pathogens such as *Salmonella* spp., *Campylobacter jejuni*, *Listeria* spp., *Yersinia* spp., and *Escherichia coli* have been isolated from some commercial RMBDs globally (24, 25). Supplementary Table 1 provides a summary of pet food product recalls and withdrawals that were minimally processed due to contamination with foodborne pathogens reported by the Food and Drug Administration (FDA) from January 2017 to March 2021.

Raw meat-based diets, contaminated with foodborne pathogens have been linked to pathogenesis of certain diseases in pets for instance: Stiver et al. (25) prepared a case report of two cats that were diagnosed with *Salmonella* gastroenteritis and septicemia after necropsy, having been fed a home prepared RMBD. Morley et al. (24), observed cases of *Salmonella enterica* infections in a greyhound breeding facility that consumed raw diets and van Dijik et al. (26) reported that a dog fed with wild rabbit (hare) had tested positive for brucellosis. Although most healthy cats and dogs do not get ill from consuming contaminated RMBDs, some remain asymptomatic upon infection, and thus might shed the pathogen into the environment if animal excreta are not appropriately disposed (27, 28). Reports about the transmission of enteric foodborne pathogens from RMBDs to humans are still few with infections widely under-reported (24, 28). The CDC linked four outbreaks of multi-drug resistant *Salmonella* infections to raw turkey intended for feeding pets (29). Investigations by Public Health England (PHE) of the UK in (30) also linked an outbreak of Shiga toxin producing *Escherichia coli* (STEC) O157:H7 to contaminated raw pet food. Furthermore, antibiotic resistant strains of *Enterobacteriaceae* have been isolated from raw meats (beef, poultry, and fish) in retail shops by the World

Health Organization (WHO). To corroborate WHO findings, Baede et al. (31); Jans et al. (32) reported that *E. coli* isolated from RMBDs exhibited similar resistance mechanisms as antimicrobial isolates that had been isolated from food production animals such as cattle, and pigs. However, it is worth noting that the transmission of enteric foodborne pathogens from RMBDs and companion animals fed these diets is a complex phenomenon to describe. This is because companion animals like dogs have a unique relationship with their environment and thus may nibble at objects, wild animal excreta or dead animal matter contaminated with any pathogen during normal daily activities not associated with the meal, thus complicating the process of tracking and analyzing the risk factors associated with RMBDs. Therefore, the purpose of this review is to investigate the different non-thermal methods of microbial control that have been successfully applied to meat and poultry while exploring alternative ways that these technologies can be employed to control and impede the proliferation of foodborne pathogens in RMBDs for companion animals.

Contamination of raw meat-based diets

The choice of ingredients and the process of manufacturing RMBDs results into products that are highly perishable because they have a relatively high pH (5.5–6.5) and water activity of >0.98 (33). Animal and poultry carcasses are natural reservoirs of enteric foodborne pathogens such as *Salmonella* and *E. coli*, although muscle from healthy animals is sterile. These pathogens find their way into RMBDs because upstream harvesting techniques do not preclude fecal pathogens completely (34) and most processes do not involve an efficient pasteurization process and rely on the microbial quality of their ingredients and freezing/refrigeration of the product to control microbial growth during transportation or storage (10, 33). Contamination of RMBDs by foodborne pathogens such as *Salmonella, Campylobacter*, and enterohemorrhagic *E. coli* is not only a public health threat, but it leads to multiple product recalls annually which are also a significant financial loss to pet food manufacturers. The U.S. Department of Agriculture, Food Safety Inspection Service (USDA-FSIS) considers foodborne pathogens as adulterants in human foods whereas the FDA, the regulatory body for pet foods effectively have a zero-tolerance policy for enteric pathogens such as *Salmonella,* Shiga toxin-producing *E. coli* (STEC) and *Listeria monocytogenes* in commercial pet food, making the manufacturing and commercialization of RMBDs a herculean task. Discussed below are some of the most feasible non-thermal antimicrobial interventions that can be implemented in commercial pet food manufacturing plants to enhance the microbial safety of RMBDs.

High pressure pasteurization (HPP)

There are several non-thermal pasteurization technologies currently available such as irradiation and ultrasonication that could theoretically be used to pasteurize RMBDs. The pet food industry in the United States relies heavily on high-pressure pasteurization (HPP) as the main technology for microbial inactivation in RMBDs (35). High pressure pasteurization utilizes hydrostatic force derived from the compression of water (or any incompressible fluid) applied to a food product intended for pasteurization (36, 37). The pressure used during HPP ranges between 100 and 1,000 MPa and system temperatures ranges between 4 and 90°C for a short duration (a few seconds or minutes) depending on the microbiological quality of the product being pasteurized (38). Unlike thermal pasteurization, HPP has several benefits in that the pressure is transmitted uniformly across the product, has a low environmental impact (low energy consumption and gaseous emissions), preserves heat labile nutrients like vitamins, pigments, antioxidants, and flavor/volatile compounds (39–41). The demand for clean label, minimally processed human/animal food products is on the rise, and HPP offers an alternative to using extensive heat processing or synthetic food additives to ensure safety and prolong shelf life of a product (38, 41).

High pressure pasteurization technology is a promising antimicrobial intervention strategy currently being employed as a microbial inactivation step to address microbiological hazards and ensure compliance with federal food safety regulations (39). Raw meat-based diets for companion animals utilize raw meat as their main source of protein. This meat does not undergo any pasteurization or cooking step to kill pathogenic or spoilage bacteria which makes HPP a prime candidate for RMBDs. The biological composition of raw meat (high moisture, fat, and protein) makes it highly perishable and an important vehicle for pathogen transmission, thus safety and quality concerns are a high priority (38, 40). When spoilage bacteria contaminate meat, they metabolize low molecular weight compounds like glucose, amino acids, and lactate to produce off-odors, sliminess, and discolorations associated with putrefaction. This putrefaction affects the organoleptic, visual, and nutritional quality of raw pet food. Beyond food safety, it is imperative that the proliferation of these spoilage microbes be controlled.

Mechanism of microbial inactivation using HPP

High pressure pasteurization relies on the principles of Pascal's law which states that compression applied on one part of a liquid medium can be transmitted instantaneously through all parts of the mass being treated (37). The application of

high pressure might lead to a slight increase in temperature, and thus the net effect of HPP might be a combination of heat, pH change, or other microbial stressors that could achieve cellular disruption and inactivation. Either way, it is an example of hurdle technology that involves increasing the number of barriers for microorganism growth and survival. In various applications, HPP has been used successfully to inactivate enzymes, and pathogenic and spoilage microorganisms (36, 38, 39). The effects observed on meats treated with high pressure are dependent on the amount of pressure applied, temperature, and the duration (time) of the process. Thus, components of meat sensitive to high pressures such as myosin and myoglobin may limit the application of HPP to fresh meats in favor of fermented, precooked, or restructured meats (38, 39) due to weeping and syneresis.

The mechanism in which high pressure processing kills or mitigates the growth of pathogenic and spoilage bacteria is *via* cellular injury. This leads to death or impedes the ability of the microbes to repair, resuscitate or grow. The events that lead to cell death by high pressure processing are not well-understood even though several bacterial species have been studied (39). High pressure processing carried out at ambient conditions and hydrostatic pressure held between 300 and 800 MPa showed significant inactivation of vegetative cells. The inactivation of vegetative cells was because of denaturation and unfolding of critical metabolic and physiological enzymes in the cytoplasm, happening simultaneously with cell membrane rapture resulting from phase transitions of the cytoplasmic fluids (36, 38, 39, 41). The method of inactivation is reliant on hydrostatic pressure and intrinsic and extrinsic factors associated with a given microorganism. For instance, synergism has been observed with increased pressure and increased adiabatic temperatures that potentiate the lethality process (39, 41, 42).

Application of HPP in processing of RMBDs

There is limited published research investigating the use of HPP to inactivate enteric foodborne pathogens in RMBDs. Thus, to understand applicable research, we conducted a systematic search of the literature. The search was conducted by selecting key words, which were input into selected databases, and then the inclusion/exclusion criterion was established. The key words included "pet food," "dog," "RMBD," "raw meat-based diet," "raw pet food," "BARF," "meat," "poultry," "high pressure pasteurization," "high pressure processing," "HPP," "ground meat," "ground poultry," "minced," and "filets." These key words were applied to Google Scholar and Scopus with no limit to years or language. Original research and review articles investigating the use of HPP in microbial inactivation of RMBDs and comminuted meats were considered in this section. Comminuted meats have an increased surface area for pathogen attachment and proliferation, which decreases the antimicrobial efficacy of HPP treatments. Articles in book chapters, patents, trade publications, extension bulletins, and conference abstracts were excluded from this section.

Pasteurization of meat and poultry using HPP has been demonstrated as an effective process to control spoilage and pathogenic bacteria in meat and poultry products (38, 43–48). These studies demonstrated that whole chunks/cuts of meat were easier to pasteurize as the interior was sterile compared to when comminuted meats were used. Serra-Castelló et al. (49) reported that the antimicrobial efficacy of HPP (450–750 Mpa) against *Salmonella* inoculated in RMBDs formulated with lactic acid (0–7.2 g/kg) was enhanced as they observed log reductions ranging from 0.76 to 9.0 Log CFU/g depending on different combination of factors (time, pressure, and lactic acid concentrations). However, Simonin et al. (50) conceded that high-pressure treatments above 400 MPa resulted in significant reduction in microbial counts but induced adverse changes in the quality attributes of meat such as color, texture, and accelerated lipid oxidation.

The process of comminuting meat and poultry products increases surface area for microbial attachment and facilitates the redistribution of spoilage and pathogenic bacteria making pasteurization by HPP less effective. For instance, Sheen et al. (51) was able to achieve a 5 Log CFU/g reduction after treating 90 g of ground chicken using HPP at 500 Mpa for 10 min. The log reduction achieved by Sheen et al. (51) was notable but could not be feasibly applied industrially to pasteurize ground chicken. This is because high levels of pressure are required to inactivate pathogens, increasing energy costs which are exacerbated by the low throughput (90 g/10 min) that was reported in this study.

New studies indicate that the antimicrobial efficacy of HPP can be potentiated through combinations with food additives such as organic acids and essential oils to achieve higher log reductions while keeping the required pressure relatively low whilst increasing the shelf-life and safety of the meat (52, 53). Combination of HPP and organic acidulants or essential oils allows for the destruction of sub-lethally injured bacterial cells that often resuscitate and multiply, leading to product recalls. However, HPP operations may require that products be transported in chubs into a "clean room" for reformation, which might result in recontamination of the product during handling, packaging, or transit. Thus, the costs and contamination risks associated with HPP can be avoided through the utilization of generally recognized as safe (GRAS) food additives since they are relatively inexpensive, can be uniformly distributed in a product and have a residual antimicrobial effect which enhances safety of the RMBD products over prolonged periods of time compared to HPP.

Use of generally recognized as safe (GRAS) food additives to control foodborne pathogens in raw meat-based diets

There is limited research regarding organic acidulants to control foodborne pathogens in RMBDs. To understand the published work that might be applicable, we conducted a two-part systematic search of the literature. The search was organized by selecting key words, identifying the appropriate databases, and determining inclusion and/or exclusion criterion. Search one key words included "pet food," "dog," "RMBD," "raw meat-based diet," "raw pet food," and "BARF," applied to Google Scholar and Scopus with no limit to years or language. Original research, and review papers with synthesis of new findings were included, and book chapters, patents, trade publications, extension bulletins, and conference abstracts were excluded. Search two key words included "essential oils," "organic acids," "bacteriophages," "ground," "minced," "cubed," "trimmings," "skin," "filets," "beef," "chicken," "lamb," "pork," and "turkey" was also applied to Google Scholar and Scopus with no limitations to years and language. Only research and review articles evaluating the antimicrobial efficacy of food additives in comminuted meats were considered for this section of the review paper. Cases where whole chunks and comminuted meats were analyzed concurrently were also considered and included in the summary tables. This is because comminuted meats have increased surface area for pathogen attachment, proliferation, and difficulty of decontamination as these mimicked the way RMBDs are manufactured and retailed. Articles in book chapters, patents, trade publications, extension bulletins, and conference abstracts were also excluded from the summary tables.

Use of organic acidulants to control enteric pathogens in RMBDs

Organic acidulants are considered by the Code of Federal Regulations (CFR) as generally recognized as safe (GRAS) additives. They have been commonly applied to animal and poultry meats because these acids are relatively inexpensive and have been demonstrated to be efficient antimicrobials (54, 55). Examples of these acids are lactic, citric, succinic, propionic, malic, and acetic and their salts. Most GRAS organic acids do not have a daily (maximum) acceptable intake for humans or animals which increases their applicability. However, their dosage is limited by their negative impact on organoleptic and color attributes of meat and poultry products. Most organic acids are described as weak acids because they do not fully dissociate in water but rather dissociate in a pH-dependent manner (56). When organic acids are added to meats, the pH of the meat is lowered to that which is equal to or lower than the acid dissociation constant (pKa) of the acid, resulting in an increased concentration of protonated acid which is responsible for the antimicrobial activity of the organic acid (56).

There are two primary mechanisms by which organic acids elicit antimicrobial activity: first, by cytoplasmic acidification which impedes ATP production and regulation, and secondly through accumulation of dissociated anions from the organic acid to toxic levels affecting cell physiology and metabolism (56). A transmembrane gradient may be created if the cytoplasmic pH is higher than that of the surrounding membrane leading to diffusion of undissociated acid through the cell membrane. The more alkaline pH of the cytoplasm then encourages the dissociation of the acid yielding anions and protons (57, 58). Accumulation of undissociated acid in the cytoplasm was associated with shifting the cytoplasmic pH which affected enzymatic activity, protein, and nucleic acid synthesis (59). Lactic acid was reported to make the cell membrane more permeable in Gram-negative bacteria, causing a leakage of lipopolysaccharides (60). Alakomi et al. (61), further reported that the chelating properties of citric and malic acids caused an intercalation of the outer membrane of *Salmonella*. Additionally, mold inhibitors such as sorbic acids contain more hydrophobic compounds and have been reported to increase the permeability of the membranes while interfering with membrane proteins; thus, helping to inhibit mold (62). However, recent research shows that the mechanisms of cellular inhibition or death by organic acidulants are not unilateral as these acids interact with different bacterial membranes and structures creating crippling hurdles that lead to either growth inhibition or inactivation. This would suggest that one mechanism is inadequate to accurately describe the mode of action for a singular organic acid as a food additive for control of spoilage and (or) enteric pathogens in human and animal foods (63).

Potential application of organic acids in RMBDs

Generally recognized as safe (GRAS) organic acids such as acetic (21CFR184.1005), citric (21CFR184.1033), and lactic (21CFR184.1061) acids have been approved by the FDA for direct addition to manufactured foods as antimicrobial interventions on meat carcasses and derived cuts pre- and post-chilling at concentrations of <5% (54, 64). Studies regarding the antimicrobial efficacies of these organic acids in the meat industry have been widely conducted and reported. Lactic acid at 150 mM was vacuum infused into boneless/skinless chicken breast cubes that had been inoculated with 10^8 Log CFU/g of *S.* Typhimurium and stored at 4°C and a 2.5 log reduction was observed by the 6th day while there were no significant reductions on day 9 and 12 (65). Over et al. (65) further tested different organic acids, citric, malic, and tartaric

TABLE 1 Summary of antimicrobial efficacy for organic acids at various doses used to control spoilage and pathogenic bacteria in meat and poultry products.

Food/meat	Organic acid	Dose	Microorganism	Log reduction	References
Ground Beef	Acetic acid	2.0%	S. Typhimurium	1.5 Log CFU/g	(66)
	Lactic acid	4.0%	*E. coli* O157:H7	2.5 Log CFU/g	
Beef cubes	Acetic acid	1.0%	Aerobic Plate counts	1.8 Log CFU/cm^2	(67)
	Lactic acid	1.0%		4.3 Log CFU/ cm^2	
Broiler chicken skins	Acetic acid	4.0%	*Salmonella*	2.0 Log CFU/ cm^2	(68)
Raw Chicken	Sodium acetate	6 g/chicken	Enterobacteriaceae	3.0 Log CFU/chicken	(69)
Fresh pork sausage	Sodium citrate	1.5%	S. Kentucky	0.3 Log CFU/g	(70)
Beef tissue	Lactic acid	2.0%	S. Typhimurium	1.2 Log CFU/g	(71)
Skinless chicken breast	Lactic acid	150 mM	S. Typhimurium	2.5 Log CFU/g	(65)
Fresh pork	Lactic acid	3.0%	S. Typhimurium	2.33 Log CFU/cm^2	(72)
Chicken breast skin	Malic acid	1.0%	S. Typhimurium	2.16 Log CFU/cm^2	(73)
Chicken breast skin	Tartaric acid	1.0%	S. Typhimurium	2.16 Log CFU/cm^2	(73)

acids at the same concentration of 150 mM using the same procedure described above. By day 6, the initial inoculum of *S.* Typhimurium had dropped from its initial concentration of 10^8 Log CFU/g to just 10^2 Log CFU/g and were undetectable by day 9. Citric acid was just as effective as acetic acid in the control of *S.* Typhimurium compared to lactic acid, but its application was limited by the negative impact on the quality attributes of the chicken.

Most studies have evaluated the antimicrobial efficacy of organic acids in ridding surfaces of pathogenic contaminants in both animal and poultry meats because the inside tissues of the meat are considered sterile. However, when these tissues/cuts/trimmings are ground, a new challenge arises when utilizing organic acids as antimicrobial interventions due to an increase in surface area available for microbial attachment and proliferation. For example, Harris et al. (66) inoculated beef trimmings with strains of *Salmonella* or *E. coli* O157:H7 at a concentration of 4.0 Log CFU/g and then ground the beef trimmings with two different levels of lactic acid and citric acid at 2.0 and 4.0%, respectively. Microbial analysis of the ground inoculated meats revealed a 2.5 log reduction in *E. coli* O157:H7 and a 1.5 log reduction of *Salmonella* after the ground meats were held frozen for a month.

Published studies (Table 1) that utilized organic acids at various doses to control enteric foodborne pathogens in meat/poultry products and the log reductions that occurred in the microbial challenge studies. Overall, the log reductions reported in Table 1 ranged from 0.3 to 4.3 Log CFU/g. When whole chunks/cuts of meat were treated with organic acids, and challenged against a foodborne pathogen, relatively higher log reductions were observed than when ground meats and sausages were used in a study. Also, the types of acidulant used to treat the meat or poultry may affect the log reductions observed, for instance, when Hamby et al. (67) treated beef cubes with both acetic and lactic acid at 1.0%, the latter resulted in a significant reduction of the aerobic plate counts (APC). However, Tamblyn and Conner (73) reported no differences in log reductions when they treated chicken breast skins with malic or tartaric acids at 1.0%. The log reductions were also dependent on the dose of acidulants used as higher concentrations of acid resulted in higher log reductions.

The broad potential applicability of organic acids in food products to enhance safety and quality is complicated because the high acid and low pH usually alters the sensory properties of meats and poultry. Application of acids directly at higher concentrations alters the quality of meat products resulting in changes in meat color and syneresis perceived as negative by pet owners (82). Consequently, there needs to be a means to slowly deliver the acidulants into the meat product to ensure minimal changes in product quality. For instance, encapsulating organic acidulants with soluble and edible vegetable oil films allows for a "slow release" mechanism, melting and releasing the acid into the meat at a slow and controlled rate, avoiding the acid shock effect observed when direct/raw acids are applied to meat (82). Ultimately, one way to increase the utilization of organic acids in RMBDs as antimicrobial interventions would be through encapsulation.

Use of essential oils as antimicrobials in RMBDs

Essential oils (EOs) are types of phytochemicals produced by aromatic plants primarily for defense against microbial invasion (83–85). These EOs consist of many components, such as terpenes, alcohols, acids, esters, aldehydes, and ketones (83, 86). Of these components, the volatile bioactive components are responsible for the antimicrobial activity of EOs (87).

TABLE 2 Summary of antimicrobial efficacy for essential oils at various doses used to control spoilage and pathogenic bacteria in meat and poultry products.

Food/meat	Essential oil	Dose of EO	Microorganism	Log reduction	References
Beef filets	Oregano oil	0.8%	*L. monocytogenes*	2 Log CFU/g	(74)
			Lactic acid bacteria	3 Log CFU/g	
Minced meat	Oregano oil	1.0%	Total viable counts	1 Log CFU/g	(75)
Chicken breast cubes	Thyme oil + lemon juice	0.5%	*S. enterica*	3.48 Log CFU/cube	(76)
Broiler breast meat	Thyme oil + orange essential oil	0.5% each EO	*S.* Enteritidis	2.6 Log CFU/mL	(77)
			Campylobacter coli	2.6 Log CFU/mL	
Minced sheep meat	Oregano oil	0.9%	*S.* Enteritidis	2.53 Log CFU/g	(78)
Minced pork meat	Thyme oil + vacuum conditions	0.3%	*S. enterica* (Infantis, Typhimurium, Montevideo, Enteritidis)	1.69 Log CFU/g	(79)
		0.6%		2.00 Log CFU/g	
		0.9%	Cocktail	3.85 Log CFU/g	
Ground chicken	Carvacrol	0.1%	*S. enterica* (Heidelberg, Typhimurium, Montevideo, Kentucky)	0.12 Log CFU/g	(80)
	Mustard oil	0.75%	Cocktail	0.31 Log CFU/g	
Minced meat	Thyme oil	0.1%	Mesophilic bacteria (*Salmonella, E. coli, L. monocytogenes*)	Log CFU/g	(81)
	Ginger oil	0.1%		1.3 Log CFU/g	

Examples of EOs are thyme, rosemary, cinnamon, eucalyptus oils, etc. Furthermore, certain components of these oils have been extracted and used as antimicrobials such as thymol, eugenol or cinnamaldehyde. As an example, the adaptation of the list of EOs by Bajpai et al. (88) (Figure 1) shows the different chemical structures of components that make up EOs.

To achieve microbial decontamination by these EOs, a few theories on their mechanism of action have been proposed. Many studies have demonstrated that components of EOs work synergistically to control the proliferation of microorganisms. Burt (89) reported that the hydrophobicity of the components of EOs increased cell permeability which allowed antimicrobial compounds to enter the cell cytoplasm. Essential oils contain different forms of phenols that disrupt cell membranes increasing permeability, leakage of cell contents, inhibition of ATPases which affects ATP production, and ultimately leading to cell death (88, 90, 91).

Application of essential oils in raw meat-based diets

Biochemical reactions such as lipid oxidation, autolytic enzymatic spoilage, and microbial spoilage result in significant losses of meat and poultry products along the production chain and have substantial economic and environmental impacts (92, 93). Essential oils (EOs) and their components can be used as a natural alternative to synthetic preservatives and there are several studies that have explored their use in meat and poultry products (76, 84, 88). Spoilage microorganisms that lead to deterioration of meat quality include, *Pseudomonas, Acinetobacter, Lactobacillus spp., Enterobacter* etc., yeast, and mold (83). These microorganisms' metabolic activity results in the formation of off-flavors, odors, and changes in color which are associated with deterioration in meat products. In addition to spoilage organisms, meat potentially harbor pathogenic enteric microbes such as *Salmonella spp., Staphylococcus aureus, Listeria monocytogenes, Clostridium perfringens, Clostridium botulinum,* Enterohemorrhagic *E. coli*, and *Campylobacter spp*. that are inherent in meat and can be controlled using EOs (91).

Table 2 shows a summary of published studies in which different meat and poultry products were treated with EOs and challenged against enteric foodborne pathogens. The data in Table 2 show the type and dose of EO, the kind of pathogen or serovar challenged against the EO, and the log reduction observed after microbial analysis. The log reductions observed varied from 1 to 3 Log CFU/g when the EOs were challenged against different pathogens or their serovars. However, the difference in antimicrobial efficacies of the EOs observed in the various studies could be attributed to interaction of various factors such as type of meat, dose of EO, the strain/serovar of

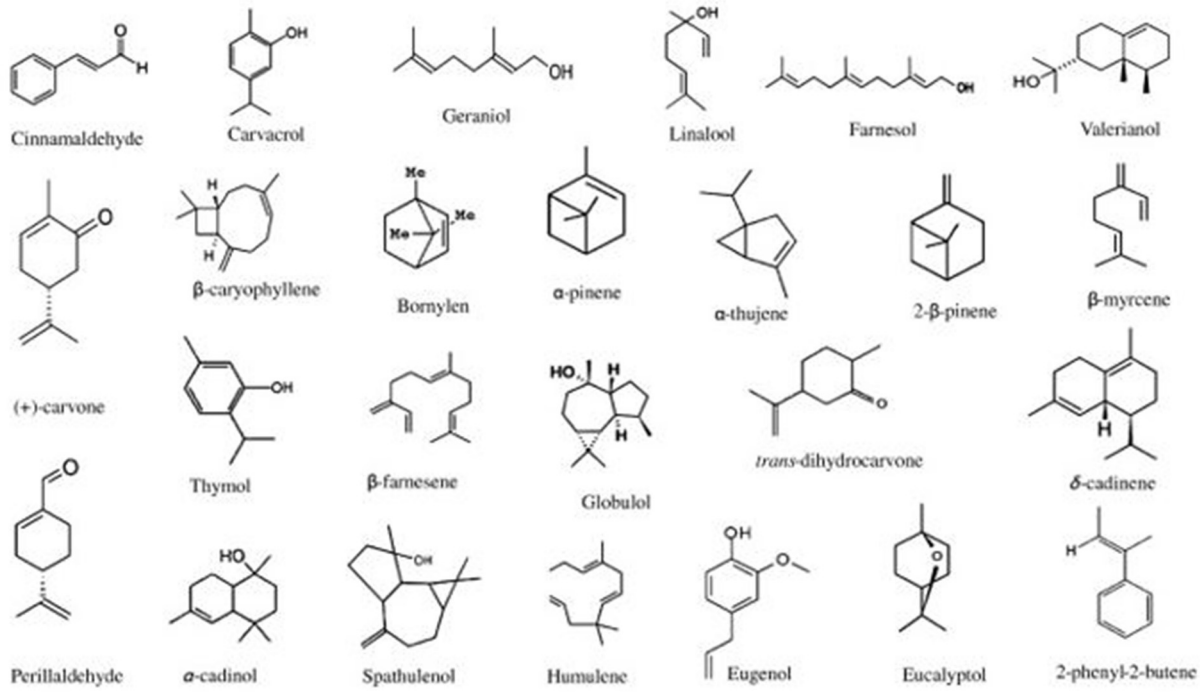

FIGURE 1
Chemical structures of components of essential oils [adopted from Bajpai et al. (88)].

a pathogen or the duration of pathogen exposure to the EOs. The interaction of some of these factors resulted in varying log reductions across studies and among similar pathogens. For instance, Kiprotich et al. (76) reported a 3.48 Log CFU/mL reduction when *Salmonella enterica* was challenged against 0.5% (v/v) thyme oil. However, Boskovic et al. (79) applied 0.3% thyme oil and only observed a 1.69 Log CFU/g reduction. The difference between these two studies was that Kiprotich et al. (76) added thyme oil into lemon juice and supplemented the mixture with *Yucca schidigera*, a natural emulsifier, and allowed the mixture to stand at 23°C for 8 h when microbial analysis was performed; whereas Boskovic et al. (79) pulled a vacuum on the packaging of the minced meat and stored their samples at 3 ± 1°C for 15 days. The difference in the results obtained in these two studies can be attributed to the synergistic effects of the different conditions offered to the essential oils. The other avenue would be to combine different types of EOs because each may contain different concentrations and modes of action. For instance, Thannisery and Smith (77) combined thyme oil and orange essential oil at 0.5% (v/v) each and achieved a 2.6 Log CFU/mL of *Salmonella* Enteritidis and a 3.6 Log CFU/mL reduction of *Campylobacter coli* in chicken breast meat. Combinations of EOs and other antimicrobial strategies such as emulsifiers, modified atmospheric packaging or refrigeration might increase the applicability of EOs by fostering a synergistic, complimentary antimicrobial effect, which in turn circumvents the strong flavors and damage to sensory properties of food usually associated with application of higher concentrations of EOs (76, 94, 95).

To apply EOs in RMBDs, they might have to be added to the product during the grinding and mixing process. A formula for most commercially available pet foods consists of ground meats with bones, tubers, vegetables, and fruits. In this form, they present a challenge to decontamination since surface treatment alone is not sufficient. Unlike whole chunks of meat or poultry which have been successfully decontaminated with EOs, grinding reduces particle size while increasing surface area for pathogen attachment and distribution throughout the product. Supplementary measures such as modified atmospheric packaging (MAP), freezing or vacuumizing might synergize the antimicrobial processes discussed above.

Bacteriophages

Bacteriophages refer to host-specific viruses that parasitize bacteria by lysing, breaking, and penetrating through the cell membrane and multiplying inside the cell, causing its death (96, 97). Bacteriophages are ubiquitous in the environment, and highly specific making them ideal for the biocontrol of

bacteria as they attack a wide range of spoilage and pathogenic microorganisms while maintaining their specificity (96, 98). These phages may belong to the Order Caudovirales, with their respective families including *Myoviridae, Siphoviridae*, and *Podoviridae* (99–101). Bacteriophages are increasingly being applied to liquid foods as an alternative to chlorine-based decontaminants which are associated with rising incidences of antimicrobial resistance (102).

The mechanism by which bacteriophages parasitize bacteria is based upon the specificity of the phage virus to a singular bacterial species or one very similar (98, 99, 103). Despite their ubiquity in the environment, a relatively small proportion of phage viruses possess the specificity required to bind with a target pathogen, thus their overall impact on the microbial ecosystem remains insignificant regarding negative effects (104, 105). As an example of bacteriophage specificity, Ricci and Piddock (106) demonstrated that ST27, ST29, and ST35 phages only bound to TolC receptors present on outer membranes of *Salmonella* serovars but were totally inactive against receptors found in the *Enterobacteriaceae* family. Whereas, some phages express a phenomenon described as "local adaptation," that allows them to infect bacteria across several genera (105, 107, 108).

The phage attaches to specific receptors on the outer cell membrane and then injects itself by adsorption. Once in the cell, the phage will either follow a lytic or lysogenic lifecycle. The lytic or virulent cycle causes rapid cell death as the phage uses the cell to replicate (96). Daughter phages are released upon cell lysis to infect the next line of bacterium. For lysogenic phages they transfer their genome to bacterial cells and use the host replication which results in the transmission of phage genome through host daughter cells but does not result in cell death (101). Lytic phages minimize transduction of their genome into their host leading to cells resisting phage viruses (phage resistance) whereas lysogenic phages contribute to phage resistance as they transfer their genome through the host cells (99, 101). From the mechanisms of action discussed above, lytic phages would be appropriate for use in therapeutic and antimicrobial interventions in both animal and human food.

Application of bacteriophages to control pathogens in raw diets

The relationship between bacteria and phages is expressed as ratio, described as "multiplicity of infection (MOI)," and multiplicity of adsorption (MOA) which is a ratio of the phage forming units to colony forming units (PFU/CFU) (96, 98). This ratio allows for phages to be applied as an antimicrobial intervention with the efficacies of different phage concentrations determined by the number (CFU) of bacterial cells inactivated by a specific concentration of phage viruses (PFU) (105, 109–111). However, the concentration of bacterial cells has been shown to have no effect on the antimicrobial potency of the phages as demonstrated by Bigwood et al. (112) who increased the concentrations of *Salmonella* while keeping constant *Salmonella* phages (P7) and observed no difference in inactivation efficiency. Likewise, Bigwood et al. (112) increased the phage concentration from 1.8×10^4 to over 5×10^8 PFU/mL and observed increased inactivation of *Salmonella,* and vice versa when the phage concentration was lowered. Bacteriophages have been mainly applied to liquid foodstuffs, but progress has been made for application to solid foods. The current challenges of phage application are the development of resistance to phages by bacteria which necessitates the use of phage cocktails to control mutating (adapting) cells. Secondarily not all phages are recognized by the FDA as GRAS.

Phages are ubiquitous which allows for flexibility when they come into contact against a serotype of a spoilage or pathogenic bacterium. They offer an alternative non-thermal method to treat minimally processed or raw foods or ingredients. Studies that employed bacteriophages in meat and poultry to control enteric pathogens is summarized in Table 3. There was a higher log reduction of the pathogens challenged against the phages in whole chunks of meat compared to ground meat. The phages' antimicrobial activity also appeared to depend on the type of serovar of pathogen they were exposed to. For instance, Spricigo et al. (113) challenged *Salmonella enterica* serovar Typhimurium and Enteritidis inoculated in poultry meat against a phage solution at 10^9 PFU/mL and observed a significant difference in log reduction (2.2 and 0.9 Log CFU/g, respectively). Also, when different types of phages were challenged against the same *Salmonella* serovar different log reductions were observed after treatment. Furthermore, Hungaro et al. (102) isolated bacteriophages from poultry feces and used them against *Salmonella* Enteritidis on chicken skin and reported a 1.0 Log CFU/cm^2 reduction as an alternative to chlorine, a chemical disinfectant. Higgins et al. (114) sprayed carcasses of broilers and turkey inoculated with *Salmonella* Enteritidis with rinse water containing 10^9 PFU/mL of PHL4 bacteriophages and reported a 93.0% (on broilers) and 58% (on turkeys) reduction of the initial concentration of pathogens compared to the control carcasses that were sprayed with only water.

The application of bacteriophages is still limited by factors such as pH and temperature which affect their antimicrobial potency. For instance, Leverentz et al. (115) applied a specific phage cocktail to honeydew melon (pH 5.8) and apple slices (pH 4.2), stored at 5, 10, and 20°C. A 2.5–3.5 log reduction of *Salmonella* Enteritidis was observed on the honeydew slices that were stored at 5 and 10°C, whereas no significant log reduction was observed at 20°C. There was no significant reduction of *Salmonella* Enteritidis on the apple slices at any temperature level leaving the authors to hypothesize that the phages had been deactivated by low pH of the apple

TABLE 3 Summary of antimicrobial efficacy for bacteriophages used to control enteric foodborne pathogens in meat and poultry products.

Food/meat	Bacteriophage	Phage dose	Target pathogen	Log reduction	References
Ground beef	S16 and FO1a	10^9 PFU/mL	S. Typhimurium	1.0 Log CFU/mL	(55)
			S. Infantis		
			S. Heidelberg		
Pig skin	UAB_Phi20	10^{10} PFU/mL	S. Typhimurium	2.9 Log CFU/cm^2	(113)
Poultry	UAB_Phi78	10^9 PFU/mL	S. Enteritidis	2.2 Log CFU/cm^2	
Fresh eggs	UAB_Phi87	10^{10} PFU/mL	S. Typhimurium	2.2 Log CFU/g	
			S. Enteritidis	0.9 Log CFU/g	
			S. Typhimurium	0.9 Log CFU/g	
			S. Enteritidis	0.9 Log CFU/g	
Poultry skin	*Podoviridae* (phiSE)	10^9PFU/mL	S. Enteritidis	2.2 Log CFU/cm^2	(102)
Broiler carcass	PHL4	10^9PFU/mL	S. Enteritidis	93.0% reduction	(114)
Turkey	PHL4	10^9 PFU/mL	S. Enteritidis	58.0% reduction	

slices. The implication of their observation is that more acid-resistant phages need to be developed for application in low pH food systems or matrices if they are to be deployed as antimicrobial interventions.

Summary

The high-pressure pasteurization and food additives discussed as interventions in this review have exhibited antimicrobial efficacies of varying successes against spoilage and pathogenic bacteria in poultry and meat products. However, commercialization and adoption of these novel interventions by the animal and pet food industry has been slow because of the varying antimicrobial efficacies obtained from using these technologies when applied to control enteric foodborne pathogens in meat and poultry products. Variation in experimental design, microbial strains, equipment, and outcomes have made the adoption and scale-up of these interventions difficult due to inadequate reproducibility of the results from these studies. For instance, different studies that utilized the same intervention (i.e., essential oil, organic acidulant, or bacteriophage) against a similar pathogen resulted in different results under comparable conditions (Tables 1–3). The lack of consistency makes standardization of these antimicrobial interventions difficult given that they are mainly applicable to minimally processed foods which are at a higher risk of being contaminated. Furthermore, effective pasteurization requires that higher doses of these non-thermal interventions be applied which can have undesirable effects on the sensory and nutritional attributes of a given pet food, warranting additional research to address palatability concerns.

A path forward is rooted in hurdle technology on the premise that combining technologies will act synergistically. Harnessing this synergism could allow for lower doses to be applied to products, may lower the negative impact on quality and sensory attributes of the treated foods and has the potential to increase consistency in effective pathogen control. Combinations of essential oils, high-pressure processing, and low pH tolerant phages should be developed which would allow the combination of organic acids and bacteriophages to become a reality. Improving the safety of RMBDs for companion animals, given the biological hazards discussed in this review will require a holistic approach. First, utilization of food additives like organic acids or essential oils considered as GRAS and "natural" should be a first step. Secondly, these interventions should be evaluated in combination by taking advantage of their different mechanisms of antimicrobial action.

Also, strategies like modified or controlled atmospheric packaging should be researched in addition to these new emerging technologies because air composition affects microbial life and, thus, it might introduce a stressor, impeding the growth of pathogenic microbes. Kinetic mechanistic maps of bacteria from different genera can help scale up these proposed antimicrobial interventions by highlighting the more robust and resistant microbes in the matrices of a RMBD. In conclusion, as the demand for RMBDs increases, so will safety challenges associated with them. Innovative and holistic approaches will need to be developed and utilized to address microbial safety and hazards associated with commercial RMBDs. Therefore, the antimicrobial interventions discussed in this review may be a framework for future research aimed at controlling foodborne pathogens in commercially manufactured RMBDs for companion animals.

Author contributions

SK and CA came up with the idea for this review. SK wrote the manuscript. CA edited the final drafts of this review

paper. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2022.1049731/full#supplementary-material

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Addition of a combination of creatine, carnitine, and choline to a commercial diet increases postprandial plasma creatine and creatinine concentrations in adult dogs

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Creatine is a nitrogenous compound essential for cellular energy homeostasis found in animal protein; however, when heat-processed for pet food, creatine is degraded to creatinine, which is not metabolically active and excreted in urine. The objective of the present investigation was to define the postprandial plasma creatine and creatinine response in dogs fed a commercial diet (CON) formulated for adult dogs, top-dressed with a combination of creatine (9.6 g/kg dry matter, DM), carnitine (2.13 g/kg DM) and choline (0.24 g/kg DM; CCC), methionine (2.6 g/kg DM; MET), or taurine (0.7 g/kg DM; TAU). Eight adult Beagles were fed one of the four diets for 7 days in a Latin Square design with no washout period. On day 7, cephalic catheters were placed and blood samples were collected before being fed (fasted) and up to 6 h post-meal. Creatine and creatinine were analyzed using HPLC and data analyzed using PROC GLIMMIX in SAS. Plasma creatine concentrations were higher in dogs fed CCC (103 ± 10 μmol/L) compared to MET (72 ± 7 μmol/L) at fasted ($P < 0.05$) and higher compared to all other treatments from 15 to 360 min post-meal ($P < 0.05$). Plasma creatinine concentrations were higher in dogs fed CCC from 60 to 180 min compared to all other treatments. These data suggest that when creatine, carnitine and choline are top-dressed for 7 days, plasma creatine is rapidly absorbed and remains elevated up to 6 h post-meal. This may have implications for energy metabolism and should be considered when using creatinine as a diagnostic tool in dogs.

KEYWORDS

creatine, creatinine, dogs, meal response, amino acid metabolism

Introduction

Creatine, an essential nitrogenous compound, is synthesized from the amino acids glycine and arginine to produce ornithine and guanidinoacetate (GAA). Once synthesized, GAA can be methylated by S-adenosylmethionine (SAM) to creatine and S-adenosylhomocysteine, which is quickly metabolized to homocysteine. This reaction

uses more methyl groups from SAM than any other methylation reaction (1). Creatine is stored in high concentrations in both skeletal and cardiac muscle as both creatine and phosphocreatine, which is available for immediate regeneration of ATP during short bouts of intense exercise [reviewed in (2)]. More specifically, during initial intense exercise, phosphocreatine is readily and quickly used for ATP regeneration whereas aerobic metabolism provides a significant amount of ATP during subsequent bouts of intense exercise (3). Creatine may also play a role in modulating neurological, neuromuscular and atherosclerotic disease [reviewed in (4)].

Creatine synthesis has the potential to present a burden for amino acid metabolism as it involves three amino acid precursors, glycine, arginine and methionine, the precursor to SAM. In fact, some studies in rats supplemented with creatine report greater plasma glycine concentrations (5) and lower plasma homocysteine concentrations (5, 6). Similar to creatine, carnitine is a major methyl acceptor and endogenous metabolite of methionine that plays a role in generating energy *via* fatty acid transport into the mitochondria (7). On the other hand, choline can act as a methyl donor in the remethylation pathway to generate methionine from homocysteine.

In vertebrates, creatine is irreversibly and non-enzymatically dehydrated to produce creatinine at a rate of about 1.5–2% of the total creatine pool per day (8) and, as such, serum and urine creatinine is used as a measure of glomerular filtration rate in dogs to clinically assess kidney disease. Although no reference range exists for plasma or serum creatine in dogs, several healthy reference ranges have been defined for serum creatinine in dogs. Extremes in serum creatinine have been reported from 35 to 250 μmol/L but reference ranges can depend on the laboratory method used and on the breed of dog (9). In fact, a separate reference range has been defined for greyhound dogs from 106 to 168 μmol/L given that creatinine concentrations are directly related to muscle mass (10). Accordingly, this reference range is higher than the reference range determined in the same study for healthy mixed breed dogs [70–150 μmol/L; (10)]. The serum concentrations of creatinine need to be interpreted carefully with an understanding of dietary composition and total feed intake, the body composition of the individual, and the intensity and duration of exercise that the individual participates in.

Sources of creatine in pet food are animal tissues and by-products; however, after heat-processing, creatine in kibble and meat and bone meal is rapidly degraded to creatinine resulting in lower concentrations of creatine and higher concentrations of creatinine in cooked pet food compared to raw meats (11). Although not currently supplemented, or even commonly measured in pet food, creatine has important implications for amino acid and energy metabolism in the dog. As such, the objective of the current study was to define the postprandial plasma creatine and creatinine response in dogs fed a commercial meat-based diet top-dressed with methyl acceptors, creatine and carnitine, and methyl donor, choline, or methionine, or a downstream metabolite of methionine, taurine. We hypothesize that dogs supplemented with creatine, carnitine and choline will have greater postprandial plasma creatine and creatinine concentrations compared to dogs fed methionine, taurine and control.

Materials and methods

Animals, dietary treatments, and meal response

All data that will be presented herein were collected but not published in Banton et al. (12) and as such, a detailed explanation of the methods can be found there. In short, eight pair-housed healthy Beagle dogs (1.6 ± 0.04 yrs, 7.8 ± 1.5 kg) were fed a commercial control diet formulated for all life stages and fed to maintain body weight (BW; Nutrience Grain-Free Pork, Lamb and Duck Formula, single batch, Rolf C. Hagen Inc., QC, Canada, Supplementary Table 1). This diet was fed either on its own (CON) or supplemented with 2.6 g/kg DM 99% DL-methionine (MET) to achieve 2.2 times the National Research Council (13) recommended allowance for adult dogs (7.2 g/kg dry matter; DM); 0.7 g/kg DM 98.5% taurine (TAU) to achieve a similar amount to a taurine supplemented diet for dogs with early cardiac disease (Royal Canin Early Cardiac Dry Dog Food, Mars Pet Care, St. Charles, MO; 1.6 g/kg DM); or a mixture of 9.6 g/kg DM 99.5% creatine monohydrate, 2.13 g/kg DM 60% choline chloride and 0.24 g/kg DM 50% L-carnitine (CCC). The control diet contained 0.195 g/kg of creatine and 0.563 g/kg creatinine on an as-fed basis. After supplementation, dogs fed CCC received a total of 9.123 g/kg creatine on an as-fed basis. The creatine dose was selected based on McBreairty et al. (14) study done in pigs, which is 200 mg/kg BW/day and is similar to a typical creatine dosing level in humans (15). Choline was supplemented to achieve 2.3 times the NRC recommended allowance for adult dogs (3.8 g/kg DM) and carnitine was supplemented to exceed the commonly supplemented dose of 0.1–0.2 g/kg DM in dog food [0.33 g/kg DM; (16)]. Dogs were fed one of the treatments or CON once daily for 7 d in a complete, randomized 4 × 4 Latin Square design. On d 7, a cephalic catheter was placed, a fasted blood sample was taken, the meal was fed and blood samples were collected at 15, 30, 60, 90, 120, 180, 240, 300, and 360 min after the meal. Blood was placed in a sodium-heparin tube and centrifuged at 4°C at 1,200 × *g* for 10 min. Plasma was separated and stored at −80°C until analysis.

Creatine and creatinine analysis

The creatine and creatinine content of the control diet was analyzed at AlzChem Trostberg GmbH (Trostberg, Germany)

using an ion chromatography system (Dionex ICS3000 or 5000) as reported in van der Poel et al. (17). The creatine and creatinine concentrations in the plasma was analyzed using High Performance Liquid Chromatography (HPLC) [Adapted from (18); Aligent Technologies, Santa Clara, CA]. Briefly, 60 uL of trifluoroacetic acid (TFA) was added to 300 uL of plasma and 300 uL of tris buffer in order to deproteinate the plasma. The sample was set on ice for 10 min before centrifuging at 14,500 rpm for 10 min. The supernatant was then filtered through a 0.2 uM nylon filter. Creatine and Creatinine (100 uL injection volume) were separated in a Hypercarb column (4.6 × 100 mm, 5 um; Fisher Scientific, Ottawa, ON) that was maintained at room temperature using HPLC with UV detection (210 nm) with a mobile phase of 3% acetonitrile and 0.1% TFA and a flow rate of 0.8 mL/min. Creatine and creatinine peaks were compared with known standards (Creatine Monohydrate, 99% and Anhydrous Creatinine, 98% from Sigma-Aldrich, St. Louis, MO).

Statistical analysis

Concentrations of plasma creatine and creatinine were analyzed as repeated measures using the PROC GLIMMIX procedure in SAS (SAS version 9.4, SAS Inst., Inc., Cary, NC). Dietary treatment and time were treated as fixed effects and period and dog were treated as random effects. In the statistical model, the effect of dietary treatment, time, and their interaction was evaluated. For each variable, model assumptions were assessed through residual analysis. Residuals for creatine were not normally distributed and as such, data were log-transformed for analysis. Means were separated using the Tukey–Kramer adjustment, and results were deemed significant at $P \leq 0.05$ and trends at $0.05 < P \leq 0.10$.

Results

Residuals for creatine were not homogenous after log-transformation of the data but they were normally distributed. Although the homogeneity assumption of the model was not met, this is likely due to the much larger variation in plasma creatine concentrations in dogs fed CCC compared to the other treatment groups, which was expected since these dogs were supplemented with creatine.

There was a significant time by treatment interaction effect for both plasma creatine ($P < 0.0001$) and creatinine ($P < 0.0001$; Figures 1, 2, respectively). Fasted plasma creatine concentrations were higher in dogs fed CCC (103 ± 10 μmol/L) compared to dogs fed MET (72 ± 7 μmol/L; $P = 0.0347$), but similar to dogs fed CON and TAU. In addition, plasma creatine concentrations were higher in dogs fed CCC compared to all other treatments from 15 to 360 min post-meal ($P < 0.05$). Plasma creatinine concentrations were higher in dogs fed CCC compared to dogs fed CON ($P = 0.0117$) and MET ($P = 0.0285$) at 30 min post-meal and higher in dogs fed CCC from 60 to 180 min post-meal compared to all other treatments ($P < 0.05$). In addition, plasma creatinine was higher in dogs fed CCC compared to CON ($P = 0.0458$) and tended to be higher compared to dogs fed MET ($P = 0.0861$) at 240 min post-meal. Plasma creatinine concentrations tended to be higher in dogs fed CCC compared to dogs fed CON ($P = 0.0779$) and MET ($P = 0.0994$) at 300 min post-meal.

As dogs on MET, TAU and CON did not differ in their plasma creatine or creatinine concentrations at time 0 (fasted), a reference range for this population of dogs was calculated using pooled data across these three treatments ($n = 24$). Fasted plasma creatine ranged from 45.8 to 137.0 μmol/L with an average concentration of 83.0 ± 24.7 μmol/L, therefore the reference range was determined to be 33.6–132.4 μmol/L (mean ± 2 SDs). Fasted plasma creatinine ranged from 62.7 to 182.3 μmol/L with an average concentration of 139.4 ± 28.1 μmol/L, therefore the reference range was determined to be 83.3–195.5 μmol/L.

Discussion

As hypothesized, creatine concentrations increased following the meal proving effective dosing in the CCC treatment and agreeing with previous reports. Harris et al. (19) reported peak creatine concentrations that were 4 times higher than fasted at 3 h post-meal in Beagles supplemented

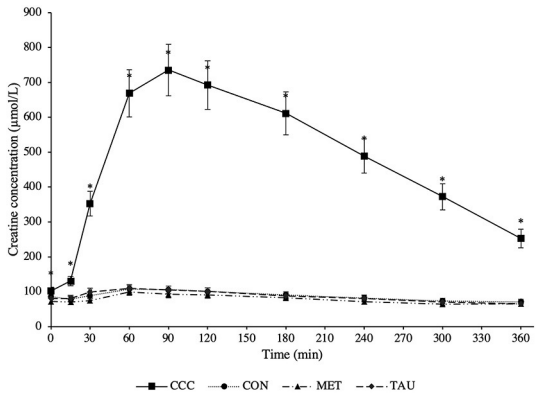

FIGURE 1
Mean plasma creatine concentrations (μmol/L) in dogs from fasted (0 min) to 360 min post-meal on either creatine, carnitine and choline (CCC), control (CON), methionine (MET), or taurine (TAU) supplementation. Values are presented as least squares means (lsmeans) ± SEM. The asterisk indicates a significant time by treatment interaction effect ($P < 0.05$) at each time point.

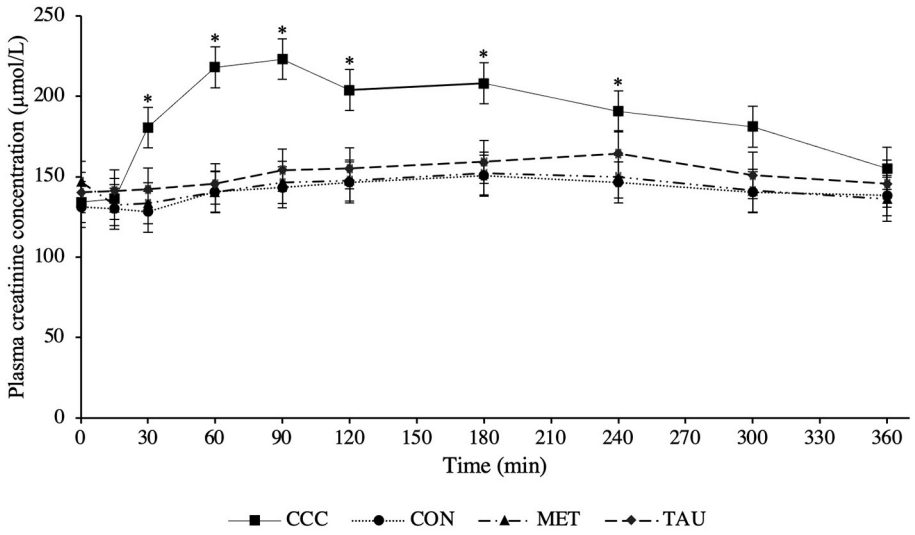

FIGURE 2
Mean plasma creatinine concentrations (µmol/L) in dogs from fasted (0 min) to 360 min post-meal on either creatine, carnitine and choline (CCC), control (CON), methionine (MET), or taurine (TAU) supplementation. Values are presented as least squares means (lsmeans) ± SEM. The asterisk indicates a significant time by treatment interaction effect ($P < 0.05$) at each time point.

with creatine monohydrate at ∼167 mg/kg after only 1 day. Lowe et al. (20) reported peak creatine concentrations that were 4.5 or 5.7 times higher than fasted at 1 and 2 h post-meal in Beagles supplemented with creatine monohydrate at either 200 or 400 mg/kg BW/day, respectively for 28 days. In the present study where we supplied creatine at 200 mg/kg BW/d, creatine reached peak concentrations that were seven times higher than the control diet after 90 min and remained elevated in plasma until at least 6 h post-meal. Therefore, when creatine monohydrate is top-dressed, it is rapidly absorbed and remains elevated in circulating plasma for up to 6 h.

Although an established healthy reference range for plasma creatine does not exist in dogs, we attempted to define one, 33.6–132.4 µmol/L, noting that this is limited to only eight dogs of the same age and breed. Different serum creatinine reference ranges have been defined for different breeds of dogs; 70–150 µmol/L in healthy mixed breed dogs and 106–168 µmol/L in greyhounds (10). Dogs supplemented with creatine in this trial had plasma creatinine concentrations above both reference ranges from 30 to 300 min following the meal. Furthermore, at certain time points following the meal, dogs fed CON, TAU, and MET also had concentrations that exceeded the mixed breed reference range. Although this reference range is in serum and we measured plasma, this should be considered when using creatinine as a diagnostic tool for kidney disease in dogs. In fact, in several case reports in human medicine, people consuming large amounts of protein and creatine supplements have been misdiagnosed with kidney disease due to elevated levels of serum creatinine (21). Based on the data from the present study, it is recommended that fasted blood samples be collected when assessing creatinine concentrations in dogs in a clinical setting, especially if the dog is consuming supplementary creatine, as these values could be elevated and additional diagnostics are required.

In the only study to investigate a source of creatine (guanidinoacetic acid, GAA) supplementation to Foxhound mixed breed dogs undergoing a light exercise regimen, Dobenecker and Braun (22) reported greater plasma creatine concentrations up to 7 h post-meal, as well as a decrease in body fat and an increase in muscle. This avenue of research remains largely unexplored in dogs, but supplementing creatine to human athletes has been well-studied and suggests that creatine supplementation can improve both physical performance (23, 24) and recovery after short-term, high intensity exercise (25, 26). The meal response of creatine appearance in plasma suggests that after only 7 days of supplementation, peak concentrations of creatine can reach up to seven times that of fasted concentrations. Although more research is needed, this data provides insight into the application of creatine supplementation in dogs and suggests that working breeds who perform shorter bouts of high intensity exercise may benefit from creatine supplementation, as has been shown to be the case in exercising humans (23–26).

Several studies in both humans (27) and rats (5, 6) suggest that creatine supplementation decreases homocysteine concentrations. Homocysteine has long been suggested as a risk factor for the development of coronary heart disease in humans [reviewed in (28)] and more recently, in dogs

(29, 30). In previously published findings from this study, we reported similar plasma homocysteine and methionine concentrations in the CCC supplemented group compared to CON and TAU, despite high levels of supplemental creatine, whereas dogs supplemented with methionine had elevated plasma homocysteine and methionine concentrations from 60 to 360 min following the meal (12). There are several explanations for the lack of change in methionine and homocysteine concentrations in the CCC group. First, the control diet was formulated for dogs of all life stages and far exceeded amino acid requirements at maintenance. Brosnan et al. (31) highlight that the demand for *de novo* creatine synthesis in growing piglets is far greater than at maintenance and presents a considerable burden on methionine metabolism and this demand may not be present in the dogs on the current study. Second, the dogs used in the current study were healthy adult dogs not undergoing any exercise or immune challenge and therefore there was no additional demand for amino acids or downstream metabolites. Deminice and Jordao (32) also highlight the ability of creatine to reduce oxidative stress markers induced by exercise, suggesting a demand for creatine during exercise. We also did not see any treatment differences in plasma arginine or glycine in the previous report (12), which may be explained by this reasoning as well. Likely the oversupply of amino acids in the control diet circumvented the need for any sparing effect of creatine supplementation. Future studies may attempt to investigate the sparing effect of creatine with either growing or exercise-challenged dogs or perhaps a diet limited in methionine.

Recent work in human medicine has begun exploring creatine's role in disease. For example, patients with rheumatoid arthritis supplemented with creatine for 12 weeks had increased appendicular and total lean mass, improving muscle wasting caused by the disease (33). A study done in healthy elderly men and women reported that those supplemented with creatine for 2 weeks had improved upper body grip strength and delayed neuromuscular fatigue (34). Some work in rats suggests an anti-inflammatory effect of supplemental creatine in models of both acute and chronic induced inflammation (35, 36). However, studies done in humans remain contradictory. Although creatine supplementation in healthy athletes has led to decreases in several pro-inflammatory cytokines after exercise (37, 38), supplemental creatine in patients with osteoarthritis had no effect on pro-inflammatory compounds compared to unsupplemented patients (39). Together, this work suggests a possible application for creatine supplementation in aging dogs, but research is yet to be done in this area.

In conclusion, 7 days of creatine monohydrate supplementation at 9.6 g/kg DM (200 mg/kg BW/day) fed to healthy adult beagles led to elevated plasma creatine concentrations up to at least 6 h post-meal and elevated plasma creatinine concentrations up to 4 h post-meal. Although our previous findings suggest no differences in plasma methionine, arginine or glycine in the CCC group, rat and human studies indicate that this may be a possibility and may be relevant for future investigations. For instance, exercising dogs or puppies may be favorable populations of dogs to benefit from elevated energy stores or spare precursor amino acids necessary for growth. These populations should be considered when thinking about supplementing creatine in dogs and when conducting future research.

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 Committee, University of Guelph.

Author contributions

SB and AS were responsible for designing the study. SB was responsible for statistical analysis and writing of the manuscript. UB and ES were responsible for laboratory analysis. All authors contributed to editing of the manuscript.

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Conflict of interest

Author AS is the Champion Petfoods Chair in Canine and Feline Nutrition, Physiology and Metabolism, a Champion Petfoods consultant, receives research funding from private industry, and was a former employee of P&G Petcare and Mars Petcare. Author UB is a former employee of AlzChem Group AG.

The remaining authors declare 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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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2022.1063169/full#supplementary-material

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Impacts of vitamin premix and/or yeast ingredient inclusion in a canned cat food on thiamin retention during 6 months of storage

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Introduction: Low thiamin levels in thermally processed canned cat foods are concerning for the pet food industry. However, there is little information on storage stability of thiamin in this food format or if inclusion of select ingredients, such as dried yeasts, has an effect. Therefore, the objective was to evaluate the storage stability of thiamin when a vitamin premix and/or yeasts ingredients were included in a canned cat food.

Materials and methods: The factorial treatment arrangement consisted of 2 levels of vitamin premix (with or without) and 4 inclusions of yeast (NY = none, LBV = Lalmin B Complex Vitamins, BY = product #1064B, or EA = BGYADVANTAGE). Diets were stored for 6 months and analyzed every month for thiamin. Data were analyzed as a mixed model (SAS v. 9.4; SAS Institute, Cary, NC) with fixed effects (vitamin premix, yeast, time, and their two-way and three-way interactions) and random effects (production day and the interaction of production day, vitamin premix, and yeast). Significance was set at $P < 0.05$ and Fisher's LSD was used to separate means.

Results and discussion: Diets including the vitamin premix [average 55.1 mg/kg dry matter basis (DMB)] contained more ($P < 0.05$) thiamin than diets that did not (average 7.5 mg/kg DMB). Inclusion of LBV (average 40.3 mg/kg DMB) resulted in the highest ($P < 0.05$) levels of thiamin, followed by BY ($P < 0.05$; average 26.9 mg/kg DMB). Diets with NY and EA contained the lowest ($P < 0.05$) levels of thiamin and were not different from each other ($P > 0.05$; average 19.3 mg/kg DMB). The diet containing vitamin premix without yeast lost ($P < 0.05$) 17.8% thiamin while diets containing a yeast ingredient maintained thiamin levels better during storage. This suggested that thiamin from yeast ingredients was more resistant to degradation during storage and should be considered when designing new canned cat foods.

KEYWORDS

canned cat food, degradation, retention, storage, thiamin, vitamin B1, wet cat food, yeast

Introduction

Thiamin is a water-soluble B-vitamin. It is found in plants in its free form (molecular formula: $C_{12}H_{17}N_4OS^+$) and in animals as a phosphorylated form, typically thiamin diphosphate [also called thiamin pyrophosphate; molecular formula: $C_{12}H_{19}N4O_7P_2S+$; (1)]. Thiamin can be produced synthetically as thiamin mononitrate (molecular formula: $C_{12}H_{17}N_4OS{\cdot}NO_3$) or thiamin hydrochloride (molecular formula: $C_{12}H_{17}N_4OS^+Cl^-{\cdot}HCl$). Once thiamin is consumed, it is digested to the free form, absorbed in the small intestine, and the majority is phosphorylated in the liver (1). Thiamin is a critical coenzyme for the conversion glucose to pyruvate, pyruvate to acetyl-CoA, and α-ketoglutarate to succinyl-CoA as well as for the oxidation of branched α-keto acids. Additionally, thiamin is involved in normal functions of the nervous system (1).

Due to the water solubility of thiamin, excesses of the vitamin cannot be stored in the body and are excreted in urine (1). This poses a risk for cats, who have a high thiamin requirement of 5.6 mg of thiamin per kg of diet on a dry matter basis [DMB; (2)]. Cats who do not consume diets with sufficient levels of thiamin may exhibit instability, decreased appetite, ventroflexion, and seizures among other clinical symptoms (3–6). Prolonged deficiency can lead to permanent brain damage that affects learning (7) and death within a month. As such, achieving sufficient levels of thiamin in foods for cats after processing and throughout the product's shelf life is critical.

Many cats in the United States are fed canned foods that undergo thermal processing with a retort. This is a regulated process to commercially sterilize food under high pressure and high temperatures (8). Canned foods contain high levels of moisture and experience prolonged heat exposure, both of which often result in decreased thiamin levels post-processing. Many researchers have addressed factors that can influence thiamin degradation due to thermal processing (9–11). A previous research paper evaluated two dried brewer's yeasts (spray dried brewer's yeast #1064B, abbr. BY; BGYADVANTAGE, abbr. EA) and a fortified inactive yeast (Lalmin B-Complex Vitamins, abbr. LBV) for their thiamin stability in a canned cat food through thermal processing. This research identified a spray-dried brewer's yeast (BY) with similar thermal degradation levels compared to thiamin mononitrate, the standard thiamin source for canned cat foods, and a fortified inactive yeast ingredient (LBV) with high levels of thiamin (12). While these were meaningful findings, previous research in extruded pet foods found that thiamin levels decreased during storage of the diet (13). As it stands, there are no published values for thiamin degradation during storage in a canned cat food, let alone in canned cat foods containing either of the previously mentioned yeast ingredients.

The objective of this experiment was to determine the effect of 6 months of storage on thiamin content of canned cat foods containing a yeast ingredient and/or a thiamin mononitrate from a vitamin premix. The hypothesis was that inclusion of a yeast ingredient would improve the storage stability of dietary thiamin compared to thiamin mononitrate.

Materials and methods

Experimental treatment production

The experimental treatments and their production have been described previously (12). Briefly, 8 experimental treatments were arranged as a factorial with 2 categorical levels of vitamin premix (no vitamin premix or 0.08% vitamin premix containing thiamin mononitrate) and 4 categorical levels of dried yeast [no yeast (NY), 0.65% LBV, 5.00% BY, or 5.00% EA; Table 1]. The 0.08% vitamin premix inclusion was chosen to simulate a commercial canned cat food with around 23 times the AAFCO minimum level of thiamin for adult cats supplied by the vitamin premix prior to diet processing. The level of LBV (Lallemand Bio-Ingredients, a division of Lallemand Inc., Montréal, QC, CA) was chosen to match the thiamin supplied by the vitamin premix. However, formulation levels were based on a yeast ingredient screening analysis and the LBV used in experimental treatment production contained less thiamin than expected (12). The levels for BY (The Peterson Company, Kalamazoo, MI) and EA (The F.L. Emmert Company, Cincinnati, OH) were capped at 5.00% for practicality as levels >5.00% could impose processing challenges.

All 8 diets were produced each day for 3 consecutive days in a commercial pet food cannery. All ingredients except the brewer's rice, vitamin premix, and yeast ingredients were prepared, mixed into a basal batter, and heated daily to 50°C daily. Then, the respective brewer's rice, vitamin premix, and/or yeast ingredient were added to 7.6 kg of the basal batter and blended for 1 min while maintaining temperature at 50°C to create each experimental treatment. Aliquots of 156 g of the final batter were transferred to cans (size 307 × 109; Crown Holdings, Philadelphia, PA), which were subsequently closed (Pneumatic Scale Angelus, Stow, OH) with an easy-open lid. All experimental treatments produced on the same day were processed in a horizontal steam batch retort (Versatort Multimode 1520; Allpax, Covington, LA) at the same time. The process schedule, or the thermal conditions utilized to effectively eliminate microorganisms that reproduce in food stored under ambient conditions and microorganisms and spores that pose a public health risk (8), consisted of a 10 min come-up cycle, a 63 min cooking cycle with a target temperature of 123°C, and a 27 min cooling cycle. After the process schedule was complete, cans were removed from the retort and cooled to room temperature. This process created 8 canned diets to serve as experimental treatments for the storage study with ranging thiamin contents. Ranking the experimental treatments by their

TABLE 1 Ingredient composition of canned cat foods containing different levels of a vitamin premix and/or a dried yeast ingredient[a].

	No vitamin premix				Contains vitamin premix			
Ingredient, % as-is	**NY**	**LBV**	**BY**	**EA**	**NY**	**LBV**	**BY**	**EA**
Basal batter[b]	94.92	94.92	94.92	94.92	94.92	94.92	94.92	94.92
Ground brewer's rice	5.08	4.43	0.08	0.08	5.00	4.35	-	-
Vitamin premix	-	-	-	-	0.08	0.08	0.08	0.08
Yeast ingredient	-	0.65	5.00	5.00	-	0.65	5.00	5.00

[a]NY, no yeast; LBV, Lalmin B-Complex Vitamins; BY, spray-dried brewer's yeast #1064B; EA, BGYADVANTAGE.
[b]1 kg of basal batter contains 283.48 g mechanically deboned low ash chicken, 230.33 g frozen pork liver, 223.59 g water, 148.83 g ground chicken, 106.30 g steam, 3.19 g guar gum, 1.35 g potassium chloride, 1.06 g mineral premix for cats, 0.74 g kappa carrageenan, 0.57 g taurine, 0.53 g salt, and 0.02 g 50% vitamin E.

average thiamin contents immediately after processing appears as follows: NY without the vitamin premix (0.7 mg/kg DMB), EA without the vitamin premix (2.2 mg/kg DMB), BY without the vitamin premix (5.8 mg/kg DMB), LBV without the vitamin premix (19.9 mg/kg DMB), EA with the vitamin premix (46.5 mg/kg DMB), NY with the vitamin premix (54.7 mg/kg DMB), LBV with the vitamin premix (59.6 mg/kg DMB), and BY with the vitamin premix [60.7 mg/kg DMB; (12)].

Storage of processed diets

Three cans from each combination of experimental treatment and production day were submitted to a commercial lab (Midwest Laboratories; Omaha, NE) for nutritional analysis. This timepoint was considered as "Month 0." All other cans were stored in a commercial pet food warehouse (Emporia, KS) for 6 months. This length was chosen because canned pet foods may take 2–6 months from diet production to be purchased by a pet owner. Therefore, it is important that thiamin degradation is minimal during this time. Storage took place from October 2020 to April 2021. Temperature was not controlled but never fell below 4.4°C. Diets were stored in their original sealed cans.

Chemical analyses

A composite of three cans of each treatment replicate was analyzed monthly for the duration of diet storage by a commercial laboratory (Midwest Laboratories; Omaha, NE). Treatment replicate composites were analyzed in duplicate for moisture (AOAC 930.15), thiamin (AOAC 942.23), crude protein (AOAC 99003), crude fat (AOAC 954.02), crude fiber (AOCS Ba 6a-05), ash (AOAC 942.05), and minerals (sulfur, phosphorus, potassium, magnesium, calcium, sodium, iron, manganese, copper, and zinc; AOAC 985.01). Results below the minimum detection level were treated as zeros and duplicate values were averaged for one value per sample. Nitrogen free extract (NFE) was calculated by subtracting the DMB contents of crude protein, crude fat, crude fiber, and ash from 100%.

Statistical analysis

Macronutrient (moisture, crude protein, crude fat, crude fiber, ash, and NFE) and mineral contents were averaged across all 7 time points for each of the 8 diets produced. Values for these nutrients were presented as mean values ± one standard deviation. This was chosen rather than conducting an analysis of variance (ANOVA) because previous research did not find meaningful differences in moisture, nitrogen, crude fat, and ash contents of a pet food consisting of chunks in gravy, jelly, or water stored for 28 days (14).

Thiamin contents of diets were transformed using the square root function to meet the model assumptions of normality and equal variances. Data were analyzed as a split-plot in time with the fixed effects of vitamin premix, yeast, and time and the random effects of production day and production day by vitamin premix by yeast. Significance of the main effects (vitamin premix, yeast, and time) and all 2-way and 3-way interactions were determined with an ANOVA. Denominator degrees of freedom were adjusted using the Kenward-Roger adjustment. Means were separated using the Fisher's least significant difference (LSD). Single degree of freedom contrasts were analyzed on the main effect of time to determine if the change in thiamin content followed a linear, quadratic, or cubic relationship. Significance for the ANOVA, means separation, and single degree of freedom contrasts was set at $\alpha = 0.05$. The GLIMMIX procedure was employed in this analysis (SAS v. 9.4; SAS Institute, Cary, NC). Data were presented as back-transformed mean values with 95% confidence intervals (CI).

Results

Average macronutrient and mineral contents

Macronutrients were relatively unaffected by ingredient composition and storage time (Supplementary Table 1). Average moisture content ranged from 82.1% (EA with and without vitamin premix) to 83.1% (BY without vitamin premix). Crude fat (average minimum 26.9% DMB for BY without vitamin

premix; average maximum 30.9% DMB for NY with vitamin premix), crude fiber (average minimum 0.67% DMB for LBV without vitamin premix; average maximum 1.83% DMB for EA with vitamin premix), and ash (average minimum 5.51% DMB for NY without vitamin premix; average maximum 7.70% DMB for EA with vitamin premix) were more spread out with greater variation. Crude protein exhibited the widest range with an average minimum of 38.2% DMB (NY without vitamin premix) and an average maximum of 51.0% DMB (BY with vitamin premix). Contents of NFE exhibited a similar range width; on average the minimum NFE was observed in EA with vitamin premix (10.5% DMB) and the maximum NFE observed in NY without vitamin premix (25.5% DMB).

Similarly, average mineral contents of the 8 diets during 6 months of storage were close when variation was considered (Supplementary Table 1). Notable ranges were observed for calcium (average minimum 0.96% DMB for BY without vitamin premix; average maximum 1.46% DMB for EA with vitamin premix), magnesium (average minimum 0.072% DMB for NY without vitamin premix; average maximum 0.142% DMB for EA with vitamin premix), iron (average minimum 241 mg/kg DMB for BY without vitamin premix; average maximum 293 mg/kg DMB for EA with vitamin premix), copper (average minimum 32.59 mg/kg DMB for LBV with vitamin premix; average maximum 39.23 mg/kg DMB for BY without vitamin premix), and manganese (12.69 mg/kg DMB for BY without vitamin premix; average maximum 26.38 mg/kg DMB for EA with vitamin premix).

Thiamin content during storage

The main effects of vitamin premix ($P < 0.05$; Figure 1), yeast ($P < 0.05$; Figure 2), and time ($P < 0.05$; Figure 3) were all significant. Thiamin content when the vitamin premix was included in the formula (55.1 mg/kg DMB; 95% CI = 42.9 mg/kg DMB, 68.9 mg/kg DMB) was 6.4 times higher ($P < 0.05$) than when the vitamin premix was left out of the formula (7.5 mg/kg DMB; 95% CI = 3.5 mg/kg DMB, 13.1 mg/kg DMB). Thiamin content was 49.8% higher ($P < 0.05$) when formulas contained LBV (40.3 mg/kg DMB; 95% CI = 31.0 mg/kg DMB, 50.9 mg/kg DMB) than formulas that contained BY (26.9 mg/kg DMB; 95% CI = 19.4 mg/kg DMB, 35.7 mg/kg DMB). Formulas that contained EA (19.3 mg/kg DMB; 95% CI = 13.0 mg/kg DMB, 26.8 mg/kg DMB) or NY (19.3 mg/kg DMB; 95% CI = 13.1 mg/kg DMB, 26.8 mg/kg DMB) were not different ($P > 0.05$) from each other. However, formulas that included either NY or EA contained 52.1% and 28.2% less ($P < 0.05$) thiamin than when LBV or BY, respectively, were included in the formula.

Thiamin decline during 6 months of storage was not linear ($P > 0.05$; Figure 3). Instead, the quadratic and cubic contrasts ($P < 0.05$) were both significant. However, thiamin content was not different ($P > 0.05$) at the beginning (month 0 average = 23.4 mg/kg DMB; 95% CI = 15.6 mg/kg DMB, 32.7 mg/kg DMB), middle (month 3 average = 23.3 mg/kg DMB; 95% CI = 15.5 mg/kg DMB, 32.6 mg/kg DMB), or the end of storage (month 6 average = 24.6 mg/kg DMB; 95% CI = 16.6 mg/kg DMB, 34.1 mg/kg DMB). However, the greatest ($P < 0.05$) thiamin content was observed in month 1 (average = 30.1 mg/kg DMB; 95% CI = 21.1 mg/kg DMB, 40.6 mg/kg DMB) and a 12.4% increase ($P < 0.05$) was observed in months 4 (average

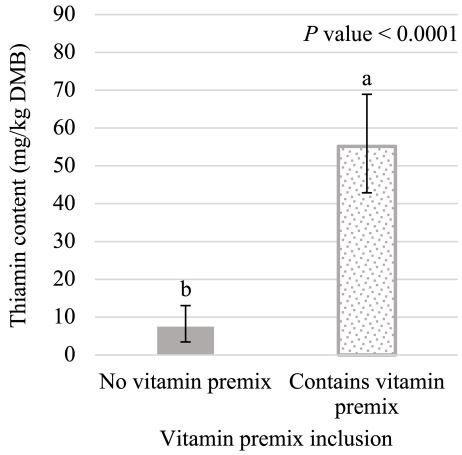

FIGURE 1
Main effect of vitamin premix inclusion on dry matter basis (DMB) thiamin content (average with 95% confidence interval) of canned cat foods stored in a commercial warehouse for 6 months. [a,b]Means without a common superscript are different ($P < 0.05$).

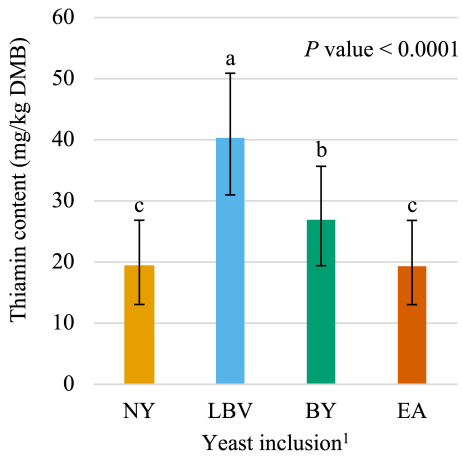

FIGURE 2
Main effect of yeast inclusion[1] on dry matter basis (DMB) thiamin content (average with 95% confidence interval) of canned cat food stored in a commercial warehouse for 6 months. [abc] Means without a common superscript are different ($P < 0.05$). [1]NY, no yeast; LBV, Lalmin B-complex vitamins; BY, spray dried brewer's yeast #1064B; EA, BGYADVANTAGE.

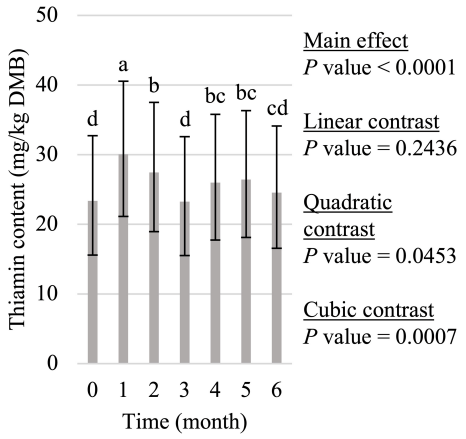

FIGURE 3
Main effect of time on dry matter basis (DMB) thiamin content (average with 95% confidence interval) of canned cat food stored in a commercial warehouse for 6 months. [a–d]Means without a common superscript are different ($P < 0.05$).

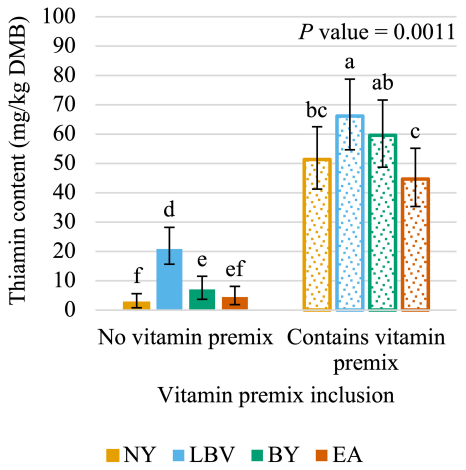

FIGURE 4
Interaction of vitamin premix inclusion and yeast inclusion[1] on dry matter basis (DMB) thiamin content (average with 95% confidence interval) of canned cat food stored in a commercial warehouse for 6 months. [abcdef] Means without a common superscript are different ($P < 0.05$). [1]NY, no yeast; LBV, Lalmin B-complex vitamins; BY, spray dried brewer's yeast #1064B; EA, BGYADVANTAGE.

= 26.0 mg/kg DMB; 95% CI = 17.7 mg/kg DMB, 35.8 mg/kg DMB)) and 5 (average = 26.4 mg/kg DMB; 95% CI = 18.1 mg/kg DMB, 36.3 mg/kg DMB) compared to month 3.

The interaction of vitamin premix inclusion and yeast inclusion was significant ($P < 0.05$; Figure 4). The diet containing LBV and the vitamin premix (66.1 mg/kg DMB; 95% CI = 54.6 mg/kg DMB, 78.7 mg/kg DMB) and the diet containing BY and the vitamin premix (59.6 mg/kg DMB; 95%

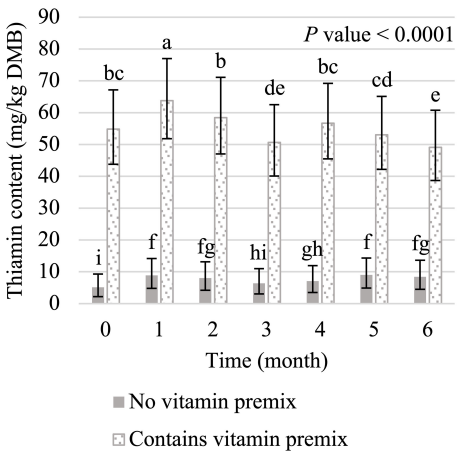

FIGURE 5
Interaction of vitamin premix inclusion and time on dry matter basis (DMB) thiamin content (average with 95% confidence interval) of canned cat food stored in a commercial warehouse for 6 months. [a–i]Means without a common superscript are different ($P < 0.05$).

CI = 48.7 mg/kg DMB, 71.6 mg/kg DMB) contained 47.2% and 33.4%, respectively, more ($P < 0.05$) thiamin than the formula with EA and the vitamin premix (44.9 mg/kg DMB; 95% CI = 34.3 mg/kg DMB, 55.1 mg/kg DMB). The DMB thiamin content of the diet containing NY and the vitamin premix (51.3 mg/kg DMB, 95% CI = 41.3 mg/kg DMB, 62.5 mg/kg DMB) was not different ($P > 0.05$) than of diets containing the vitamin premix and either BY or EA. However, the NY and vitamin premix diet did contain 22.4% less ($P < 0.05$) thiamin than the LBV and vitamin premix diet. All diets including the vitamin premix contained more ($P < 0.05$) thiamin than all diets that did not include the vitamin premix, regardless of yeast inclusion. The diet with LBV and no vitamin premix (20.9 mg/kg DMB; 95% CI = 14.6 mg/kg DMB, 28.2 mg/kg DMB) contained more ($P < 0.05$) thiamin than the diets containing no vitamin premix and either BY (7.0 mg/kg DMB, 95% CI = 3.7 mg/kg DMB, 11.5 mg/kg DMB), EA (4.4 mg/kg DMB; 95% CI = 1.9 mg/kg DMB, 8.1 mg/kg DMB), or NY (2.7 mg/kg DMB; 95% CI = 0.8 mg/kg DMB, 5.6 mg/kg DMB). Including BY without the vitamin premix resulted in 2.66 times as much ($P < 0.05$) thiamin as NY without the vitamin premix. The diet containing EA without the vitamin premix had similar ($P > 0.05$) levels of thiamin compared to diets without the vitamin premix and containing either NY or BY.

The interaction of vitamin premix and time was significant ($P < 0.05$; Figure 5). Thiamin content increased ($P < 0.05$) during storage when vitamin premix was not included in the diet; a 64.7% increase in thiamin content was observed between month 0 and month 6. However, thiamin levels for all diets without vitamin premix were low and averages ranged from 5.1

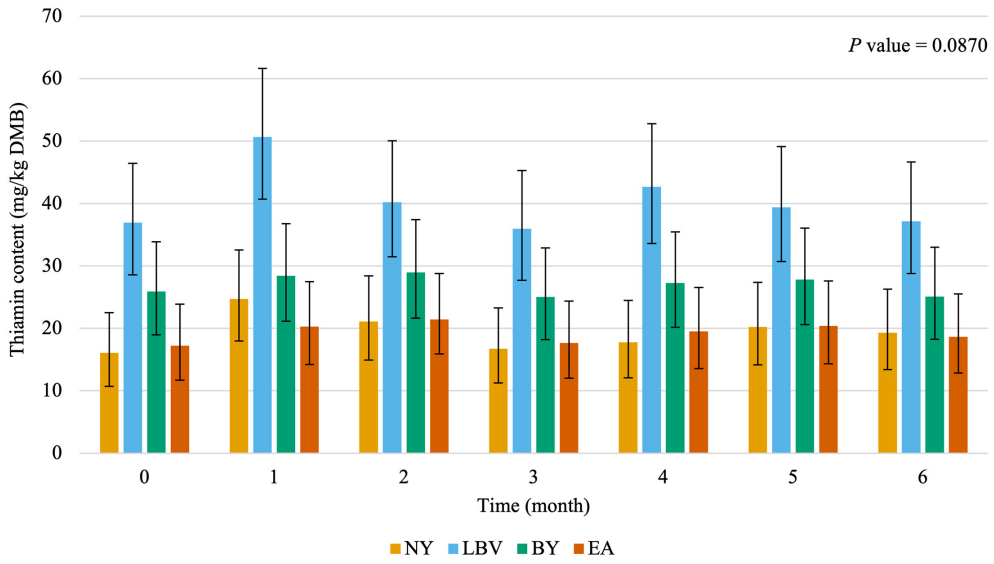

FIGURE 6
Interaction of yeast inclusion[1] and time on dry matter basis (DMB) thiamin content (average with 95% confidence interval) of canned cat food stored in a commercial warehouse for 6 months. [1]NY, no yeast; LBV, Lalmin B-complex Vitamins; BY, spray dried brewer's yeast #1064B; EA, BGYADVANTAGE.

mg/kg DMB to 9.0 mg/kg DMB. Thiamin content also fluctuated slightly when vitamin premix was included in the diet. The highest content was observed in month 1 (average = 63.8 mg/kg DMB; 95% CI = 51.8 mg/kg DMB, 77.0 mg/kg DMB) and the lowest content in month 6 (average = 49.1 mg/kg DMB; 95% CI = 38.7 mg/kg DMB, 60.8 mg/kg DMB). Overall, thiamin content decreased ($P < 0.05$) by 10.6% when month 0 (average = 54.9 mg/kg DMB; 95% CI = 43.8 mg/kg DMB, 67.2 mg/kg DMB) and month 6 were compared.

The interaction of yeast and time was not significant ($P > 0.05$; Figure 6). Average thiamin content across yeast inclusions during the 6 months of storage was 26.5 mg/kg DMB.

The interaction of vitamin premix, yeast, and time was significant ($P < 0.05$; Supplementary Figure 1). The diets containing LBV or BY without vitamin premix and diets containing BY or EA with vitamin premix did not ($P > 0.05$) exhibit thiamin degradation during 6 months of storage. The highest ($P < 0.05$) level of thiamin in the diet with no vitamin premix and no yeast was observed in months 1, 5, and 6 (average = 4.6 mg/kg DMB) and the lowest ($P < 0.05$) level in month 0 (average = 0.5 mg/kg DMB; 95% CI = 0.0 mg/kg DMB, 2.2 mg/kg DMB). This represented a >8-fold increase in thiamin content on a very small starting base. Thiamin content of the diet containing EA without vitamin premix was lower ($P < 0.05$) at month 0 (average = 5.8 mg/kg DMB; 95% CI = 2.7 mg/kg DMB, 10.1 mg/kg DMB) than at months 1, 2, 5, and 6 (average = 7.8 mg/kg DMB), representing a 34.5% increase. The diet containing vitamin premix with no yeast had less ($P < 0.05$) thiamin after 6 months of storage with a 17.2% decrease. Thiamin content in the diet containing vitamin premix and LBV was higher ($P < 0.05$) in month 1 (average = 89.6 mg/kg DMB; 95% CI = 75.7 mg/kg DMB, 104.7 mg/kg DMB) than in any other month of storage (average = 62.7 mg/kg DMB). Replicates of this diet had a wider range of thiamin contents (54.2 mg/kg DMB to 126.9 mg/kg DMB) compared to replicates at other sample collection times.

Discussion

The aim of this work was to identify yeast ingredients as potential thiamin sources and to evaluate the stability of their intrinsic thiamin during storage of 6 months in a commercial warehouse. Preliminary studies identified that retort processing conditions (10), package size and material (9), protein source (11), and the presence of sulfites (11) influence thiamin content in canned cat food. However, none of those experiments identified a solution to minimize thiamin degradation that could be applied to all thermally processed cat foods. If a yeast ingredient had similar or better shelf life compared to thiamin mononitrate, then it could be a suitable alternative thiamin source.

Macronutrient and mineral contents

Commercial cat foods contain moisture ranging from 78 to 82% maximum (15, 16). The diets in the present experiment fall just outside the upper end of that range. Data from a pilot study suggested that pre-retort batter moisture content could

influence the concentration of thiamin after retort processing (10). However, all 8 diets contained similar levels of moisture, which likely did not influence thiamin content during storage differently. Future experiments could achieve lower moisture contents by decreasing water and/or steam addition in a production setting.

All 8 diets met the AAFCO crude protein (26% DMB) and crude fat (9% DMB) requirements for adult cats (2). In general, crude protein content was higher for diets containing BY or EA vs. NY. This is likely due to the nutritional composition of the yeast ingredients and the brewer's rice used as a space-holding ingredient. The brewer's rice contained 9.2% DMB crude protein whereas the three yeast ingredients contained an average of 53.5% DMB crude protein (12). Diets containing LBV were intermediate in terms of crude protein content; this is likely due to the ingredient's lower inclusion level compared to BY and EA. This influenced the differences observed in NFE content, a calculation which typically accounts for starches, sugars, and some non-starch polysaccharides (17). Diets containing BY and EA contained less NFE compared to diets containing NY with diets containing LBV in the middle. Interestingly, diets containing EA contained slightly less NFE than diets containing BY. This was likely influenced by the slightly higher crude fat and crude fiber contents of the two ingredients that were not as detectable in the complete diets.

Similarly, all 8 diets met the AAFCO minimum requirements for the minerals analyzed in the experiment. While this is not unexpected, it is meaningful to note that inclusion of yeast ingredients as thiamine sources did not affect the formulas' abilities to meet minimum levels. Differences in the minerals contents of finished diets could be attributed to the mineral contents of the yeast ingredients themselves, as ground brewer's rice made relatively little impact (12).

Limited data are available on typical macronutrient and mineral contents of commercial canned cat foods. Reports of the macronutrient content of three commercial products suggested the typical diet may range from 41.3 TO 51.5% DMB crude protein and 33.9–40.7% DMB crude fat by acid hydrolysis (18–20). Two studies reported ash contents between 7.2 and 11.05% DMB (19, 20). Only one study reported crude fiber and NFE contents (1.2–1.5% DMB and 2.0–5.2% DMB, respectively) for a diet across a longitudinal feeding study (19). Regardless, the diets in the present experiment contained similar nutrient levels, which suggested that they were fair representations of commercially available diets.

Thiamin content during storage

It was expected that the main effects of vitamin premix and yeast and their interaction would be significant due to the experimental design. However, it was not expected that inclusion of EA would result in similar thiamin levels compared to inclusion of NY when the effects of vitamin premix and time were not considered. Thiamin degradation during thermal processing of these canned cat foods was greater for EA than NY when vitamin premix inclusion was not considered (12). This likely caused their thiamin contents to be similar even though the expectation was that inclusion of EA would result in similar thiamin content compared to the inclusion of BY instead.

This is the first experiment to assess the storage stability of thiamin in canned pet food. The storage length of 6 months represented the first 25% of a canned cat food's shelf life. During this time, canned cat foods may be transported from the manufacturing facility to a warehouse or distribution center before they are received by a retailer (21). While it is worthwhile to conduct an extended storage study to represent the entire shelf life of a canned cat food, this fell outside the scope of the present experiment.

The cubic relationship for the main effect of time was somewhat expected given the sensitivity of the high performance liquid chromatography methodology employed for thiamin analysis. For example, the relative standard deviation of thiamin analysis of canned cat food within select laboratories was 4.302%, whereas the value for riboflavin analysis in canned cat food was 1.687% (22). Measures can be taken to minimize variability in the assay, such as the use of amber glassware to prevent light degradation of the thiamin (23). Additionally, all samples in this experiment were analyzed by the same commercial laboratory to eliminate variation between laboratories as a contributing factor. Composite samples of three cans per treatment-timepoint combination were analyzed instead of single cans to minimize sampling variation. Therefore, it can be assumed that the variability observed is representative of the sensitivity of the assay and minimally confounded by other influences. This variability likely influenced the statistically observed cubic relationship between storage time and thiamin content. Other statistical observations likely influenced include the increase in thiamin content of the diets containing no vitamin premix and no yeast or EA compared to month 0, which would influence the statistical conclusions made regarding the interaction between storage time and the absence of vitamin premix. An extended storage study as suggested early in the manuscript may allow for a better picture of this main effect between thiamin content and storage time.

Storage of extruded pet food found that 80% of thiamin was retained after 6 months of storage when thiamin mononitrate was the sole thiamin source (13). This was similar to the 82.2% thiamin retention observed in the present experiment in the diet containing a standard vitamin premix with thiamin mononitrate and no yeast ingredient. This implied that supplemental thiamin degradation during storage is similar regardless of food format and could be confirmed by a future experiment evaluating the degradation of thiamin mononitrate during storage in food

processed by extrusion, canning, baking, and other processes. However, other researchers did not observe differences in thiamin content of commercial extruded diets for cats after 6 months of storage (24). Thiamin mononitrate and thiamin hydrochloride are the most common supplemental thiamin sources included in pet food formulations (23). Therefore, it can be assumed that those diets utilized one of those sources but cannot be confirmed as the authors did not disclose the ingredients in each diet. This could have significantly influenced thiamin retention and further stresses the importance of diet formulation as a factor in thiamin retention. Storage of canned rainbow trout and canned pollock identified greater retention in the rainbow trout than the pollock after 6 and 12 months of storage at 22°C (25). Thiamin intrinsic to meat may have less thermal stability compared to other ingredients. Thermally processed beef brisket retained roughly 33% of its post-processing thiamin content after 6 months of storage at 20°C, while thiamin retention for brown rice and split pea soup stored in the same conditions ranged from 85 to 95% (26). The other ingredients in the canned diet can influence thiamin retention even when meat is the primary ingredient. Care should be taken to minimize the oxidation level of fats and/or include antioxidants in the formula. For example, use of fresh fats and the inclusion of casein hydrolysate or rosemary extract improved thiamin retention in sterilized chicken meat during storage (27). Antioxidants were not included in canned cat food in this experiment to mimic a worst-case scenario. Additional fat inclusion was not necessary as the mechanically deboned low ash chicken (50.2% DMB crude fat), frozen pork liver (24.5% DMB crude fat), and ground chicken (46.2% DMB crude fat) (12) provided sufficient levels of crude fat to meet nutritional requirements of adult cats. Nevertheless, the addition of pork lard to chicken meat resulted in a longer half-life of thiamin during storage compared to the addition of soy or sunflower oil (27). It is possible that more thiamin could have been degraded if an unsaturated fat source was included in the diets, but this fell outside the scope of the present experiment.

The intent of this experiment was not to identify the mechanism behind observed differences in thiamin content of the canned cat foods. However, it is likely that the different thiamin forms present in thiamin mononitrate and the evaluated yeast ingredients greatly affected their storage stability. Previous researchers have identified thiamin binding proteins, which provided a protective effect on the thiamin during early stages of the yeast cells (28). The presence of thiamin binding proteins in the evaluated yeast ingredients could explain why diets containing them relatively maintained their thiamin content during storage. Confirmation of the presence of thiamin binding proteins in yeast ingredients fell outside the scope of this experiment, but could be a useful analysis when screening yeast ingredients as thiamin sources.

Conclusions

The present experiment identified 3 yeast ingredients that stabilized thiamin content in canned cat food during 6 months of storage in a commercial warehouse. In comparison, thiamin content of a diet containing a thiamin mononitrate from a vitamin premix decreased by 17.8% when stored in the same conditions. Further research is needed to discover how thiamin content is affected by prolonged storage to the end of shelf-life as well as how other formulation factors could affect thiamin stability. However, the data herein were promising and suggested that canned cat food formulas should consider including a yeast ingredient to promote better thiamin stability during storage.

Data availability statement

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

Author contributions

CA and AD designed the experiment. AD conducted the research, performed the statistical analyses, and wrote the manuscript. All authors revised and provided intellectual input on this manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

Author AD was employed by Simmons Pet Food, Inc. as a paid intern prior to conducting this research project. Authors BW and LL were employed by Simmons Pet Food, Inc., while this research project was conducted. Simmons Pet Food, Inc. manufactures canned foods for dogs and cats.

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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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2022.1090695/full#supplementary-material

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Fibroblast growth factor-21 (FGF21) analogs as possible treatment options for diabetes mellitus in veterinary patients

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Fibroblast growth factors (FGFs) are involved in numerous metabolic processes. The endocrine subfamily of FGFs, consisting of FGF19, FGF21, and FGF23, might have beneficial effects in the treatment of diabetes mellitus (DM) and/or obesity. The analog with the greatest potential, FGF21, lowers blood glucose levels, improves insulin sensitivity, and induces weight loss in several animal models. In this review we summarize recent (pre)clinical findings with FGF21 analogs in animal models and men. Furthermore, possible applications of FGF21 analogs for pets with DM will be discussed. As currently, information about the use of FGF21 analogs in pet animals is scarce.

KEYWORDS

glucose, metabolism, obesity, insulin, FGF, endocrine, diabetes

Introduction

Diabetes Mellitus (DM), i.e., persistent hyperglycemia, is a very common endocrine disorder caused by various etiologies, with interesting differences between species. For example, in dogs DM is most frequently due to (immune-mediated) destruction of pancreatic beta-cells, but may also be due to excessive circulating concentrations of insulin antagonistic hormones, such as cortisol and (progesterone-induced) growth hormone (1). In contrast, the majority of diabetic cats has a type of DM that is very comparable with type 2 DM in humans, in which both insulin resistance (induced by lifestyle factors such as obesity and physical inactivity) and β-cell destruction play an important role in the pathogenesis (2–5), but DM in cats is also frequently caused by hypersomatotropism due to a pituitary tumor (6). Subcutaneous injections with insulin, usually twice daily, are almost always needed in the treatment of DM (7), which has an impact on the (quality of) life of both pets and owners (8, 9). A study from Niessen et al. with over a thousand veterinarians even showed that ∼20% of cats diagnosed DM were euthanized within the first 2 years after diagnosis (9). Although remission of feline DM is possible, insulin administration in combination with dietary measurements and advices to increase physical activity quite often are insufficient to prevent hyperglycemic periods (7). Moreover, insulin treatment may also result in (life-threatening) hypoglycemic

events (4). Consequently, drugs that may help to better regulate pets with DM or may result in diabetic remission are urgently needed. Fibroblast growth factor 21 (FGF21) analogs may be interesting in this respect.

Fibroblast growth factors are extracellular signaling molecules with a plethora of functions, ranging from inducing proliferation to effects on glucose and lipid metabolism (10, 11). Although the amino acid homology between the various FGF members is limited, the domain structure is much more conserved. The conserved core region consists of 12-stranded β-sheets in the center of the around 200 amino acid long protein (Figure 1). The lack of a heparin-binding site (HBS) clearly discriminates FGF19, FGF21, and FGF23 from the other FGFs. As a consequence, FGF19, FGF21, and FGF23 cannot bind to endothelial membrane-bound receptors directly and this allows them to diffuse easily through the bloodstream. Therefore, FGF19, FGF21, and FGF23 do not act in an autocrine/paracrine fashion but function more as hormones in an endocrine mode of action. Binding to target cells requires the co-expression of α-Klotho or β-Klotho in addition to the classical FGF-receptors. The specific tissue expression of α-Klotho (for FGF23) and β-Klotho (for FGF19 and FGF21) explains why FGF23 acts upon the kidney/bone and FGF19 and FGF21 act on the liver/gallbladder and liver/adipose tissue, respectively (Table 1). Because of their effects on liver and adipose tissue, especially FGF19 and FGF21 may be interesting with regard to the treatment of diabetes mellitus.

FGF19 was discovered in 1999 from a human fetal cDNA library, based on the homology with probes for mouse FGF15 (13). The deduced amino acid sequence had 51% homology with the mouse FGF15. Upon binding to β-Klotho in conjunction with FGF-R1c/4, FGF19 stimulates hepatic glycogen synthesis and inhibits gluconeogenesis via its target genes glucose-6-phosphatase (G6Pase) and phosphoenolpyruvate carboxykinase (PEPCK). Furthermore, it inhibits bile acid production via the inhibition of CYP7A1 expression, a crucial enzyme in bile acid synthesis (14–16). Lastly, FGF19 inhibits hepatic lipogenesis.

One year after the discovery of FGF19, the same group identified FGF21, which had only 35% sequence identity with FGF19 (17). In adipose tissue, FGF21 increases the expression of GLUT1 (glucose transporter), thus facilitating glucose uptake (18). Serum and hepatic triglyceride levels were lower in FGF21-transgenic mice (19). This process is dependent on the expression of CD36, which is responsible for free fatty acid uptake (20). Finally, the metabolic effects of FGF21 include an increase in β-oxidation via increased expression of 3-hydroxyacyl-CoA dehydrogenase, carnitine palmitoyltransferase (CPT), peroxisome proliferator-activated receptor-gamma coactivator 1-α (PGC-1-α), and acyl-CoA oxidase (21).

The *in vitro* effects of FGF19 and FGF21 on metabolism suggest beneficial effects of FGF19 and FGF21 in the treatment of DM *in vivo*. The biological half-life of FGF19 is around 30 min, whereas FGF21 has a half-life of 2 h at maximum (22), which poses challenges in case these compounds are to be implemented in clinical settings. Therefore, analogs with enhanced stability have been developed. The longer half-life of FGF21 compared to FGF19 is most likely one of the reasons why most pharmaceutical companies have focused on the development of FGF21 analogs (23–26).

Most studies with the FGF21 analogs have been conducted in mice, monkeys or humans; studies in pets are scarce. Because the FGF21 analogs may have potential in the treatment of pets with DM, the aim of this study was to review the recent literature regarding the use of FGF21 (analogs).

Effects of FGF21 on metabolism

FGF21 has emerged as the preferred therapeutic target FGF for metabolic diseases such as non-alcoholic steatohepatitis (NASH), metabolic syndrome, and diabetes mellitus (DM). The preference for FGF21 over FGF19 and FGF23 is based on several observations. First, its preferential liver expression. Second, recombinant FGF21 stimulates glucose uptake in mouse 3Te-L1 adipocytes and primary human adipocytes and not in non-adipocytic cells (27). This observation prompted this group to investigate FGF21-effects in *ob/ob* and *db/db* mice, two classical models for obese and diabetic mice, respectively (27). FGF21 reduced plasma glucose and triglyceride concentrations to normal values up to 24 h after supplementation. GLUT1 mRNA and protein levels were increased by FGF21. Importantly, FGF21 did not induce hypoglycemia, cell proliferation, or increase body weight. These observations clearly established the potential of FGF21 as a regulator of aspects of the metabolic disturbances in obesity, NASH, and DM.

Subsequently, other metabolic improvements induced by FGF21 were reported such as a reduction of body weight, protection against high-fat diet-induced steatosis (lipid accumulation in hepatocytes), and improved serum cholesterol and insulin concentrations (19, 28–32). FGF21 stimulates the transformation of stem cells to adipocytes (18). FGF21 requires mature adipocytes to exert its metabolic effects. Fasting and a ketogenic diet induce FGF21 expression (19, 33). This enhanced

Abbreviations: DM, diabetes mellitus; BMI, body mass index; FGF, fibroblast growth factor; HBS, heparin binding site; G6Pase, glucose-6-phosphatase; PEPCK, phosphoenolpyruvate carboxykinase; GLUT, glucose transporter; CPT, carnitine palmitoyltransferase; PGC-1-α, peroxisome proliferator-activated receptor-gamma coactivator 1-α; NASH, non-alcoholic steatohepatitis; PPAR, peroxisome proliferator-activated receptor; VLDL, very low density lipoproteins; mTORC1, mammalian target of rapamycin complex 1; BAT, brown adipose tissue; WAT, white adipose tissue; UCP1, uncoupling protein 1; CNS, central nervous system; SGLT, sodium-glucose cotransporter; ROS, reactive oxygen species.

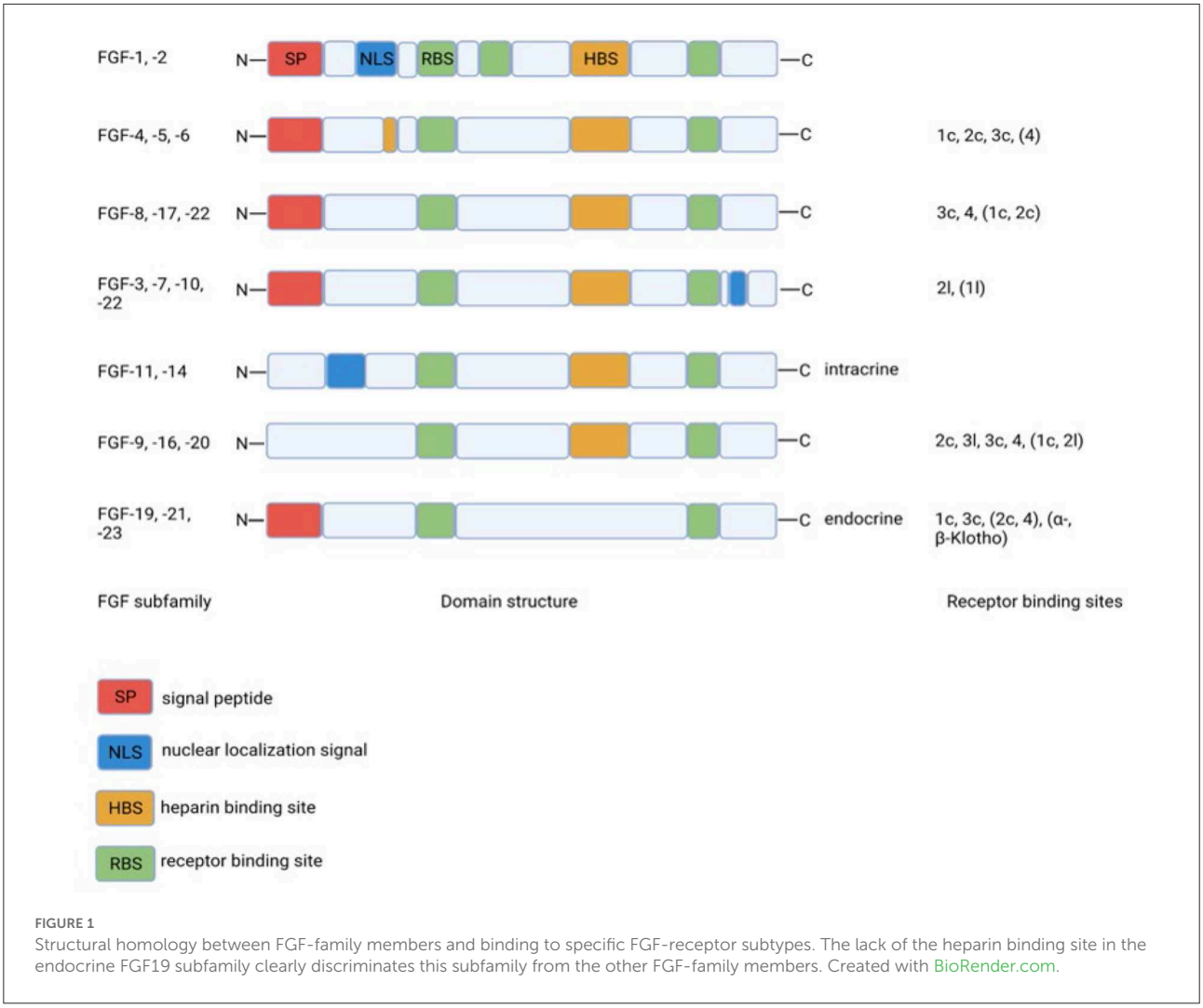

FIGURE 1
Structural homology between FGF-family members and binding to specific FGF-receptor subtypes. The lack of the heparin binding site in the endocrine FGF19 subfamily clearly discriminates this subfamily from the other FGF-family members. Created with BioRender.com.

TABLE 1 Overview of the FGF19 family members.

	FGF19	FGF21	FGF23
Subtypes of FGF-receptor binding	β-klotho, 1c, 4	β-klotho, 1c, 3c	α-klotho, 1c, 3c, 4
Produced in	The intestines	The liver	Bone
Affects	Liver, muscle, adipose tissue, CNS	Liver, muscle, adipose tissue, pancreas, CNS	Heart, intestines, kidney, bone
Effects	+ Glycogen synthesis	+ β-oxidation	+ Renal sodium excretion
	– Gluconeogenesis	+ Insulin sensitivity	– Vitamin D3 synthesis
	– Bile acid synthesis	+ TCA levels (12)	
	– Lipogenesis		

expression is mediated via peroxisome proliferator-activated receptor-alpha (PPAR-α) (15, 27, 34, 35). FGF21 levels are also increased in obese individuals and in patients with NASH (36). Apparently, this increase is not sufficient to revert the steatotic liver phenotype, despite the fact that FGF21 stimulates β-oxidation (37, 38). This enhanced β-oxidation is mediated via increased expression of hepatic protein (PGC-1α), resulting in lower plasma and hepatic lipid levels (12, 28, 29).

Part of the effects of FGF21 are mediated by PPAR-γ (30). Rosiglitazone is a stimulator of the PPAR-γ pathway which has been studied in conjunction with FGF21; the effects of FGF21 and rosiglitazone were studied both individually and

combined. Apart from their own effects, a synergistic effect could be demonstrated, which was a 10 fold higher expression of the insulin-independent GLUT1 in adipose tissue, as well as increased glucose uptake (18).

Studies showed a significant decrease in obesity in monkeys and mice upon FGF21 treatment. Furthermore, FGF21 decreased the number of VLDL-receptors (very low density lipoproteins) in the liver. These receptors transport fatty acids into the liver (39). Inhibition of this uptake leads to less fat in the liver and therefore less steatosis. FGF21 is known to increase insulin sensitivity in the liver. This increase could lead to more glycogen storage in the liver and more glucose uptake.

FGF21 represses the mammalian target of rapamycin complex 1 (mTORC1) in mice. This is a critical regulator in the pathogenesis of DM. FGF21 could therefore improve insulin sensitivity (40). This higher insulin sensitivity could also be the consequence of improved pancreatic β-cell function. Indeed, diabetic rodents had more glucose-induced insulin secretion upon FGF21 treatment (41).

One of the major effects of FGF21 is lowering hyperglycemia. This effect is mainly caused by increased glucose uptake, mostly by adipose tissue, as well as by reduced gluconeogenesis in the liver (27). The increased glucose uptake is thought to be the consequence of an increased expression of GLUT1, specifically in adipose tissue, as demonstrated in mice (18, 27, 42). Moreover, FGF21 combined with insulin sensitizes brown adipose tissue (BAT) for more glucose uptake, which is used for heat production, thus lowering blood glucose levels (43). FGF21 knockdown in mice showed an increase in both glycogenolysis and gluconeogenesis, indicated by increased G6Pase and PEPCK levels, suggesting that FGF21 has inhibitory effects on gluconeogenesis and glycogenolysis (44).

FGF21 is known to have weight losing potential by increasing energy expenditure in BAT. Mice receiving FGF21 supplements demonstrated higher oxygen intakes, lower respiratory quotients [*viz.* enhanced fat oxidation (45)], and small elevations of the core body temperature (28, 29, 46). Increased β-oxidation can contribute to weight loss and consequently reduce obesity development. The type of β-oxidation is BAT-mediated by uncoupling protein 1 (UCP1), which uncouples respiration from heat production. This means that no ATP is produced by the oxidation of fat, but the energetic fatty acids are merely used to produce heat (47). Moreover, FGF21 induces the expression of UCP1 in white adipose tissue (WAT), the so-called "browning process" which leads to enhanced thermogenesis, without ATP production, upon fat oxidation (48).

Altogether, FGF21 is clearly a key metabolic regulator in animals. It supports the stabilization of normoglycemic states, prevents the liver from steatosis, and could reduce the risk for obesity. Pets with DM present a challenge to regulate glycemic and lipid levels, and obesity. Therefore, it is conceivable to observe positive effects of FGF21 in diabetic animals.

FGF21 analogs in humans and animal models with DM

Based on the longer half-life of FGF21 compared to FGF19, and the metabolic effects of FGF21, FGF21 and more stable analogs have gained attention of several pharmaceutical and biotechnological companies like Lilly Research Laboratories (LY2405319), Pfizer (PF05231023), and Bristol-Myers Squibb [(PEG-)cFGF-21] to interfere in metabolic diseases. Each of these FGF21 analogs or stabilized constructs will be described with the emphasis on glucose and insulin lowering capacity, body weight reduction potential, and improvement of lipid profiles. This data is summarized in Table 2.

Naive FGF21 is not suitable to use within pharmaceutical preparations as it is a biophysically and thermally unstable protein. For that reason, a more potent variant of FGF21 was produced by DNA modification (LY2405319), which was proven to be much more stable and therefore more potent for pharmacological use in animals (54).

LY2405319 has been tested in mice (54), monkeys (49), and humans (50). The human patients were experiencing diabetes for an average of 7 years, whereas in the other species the obese and diabetic conditions were experimentally induced. In monkeys and humans, there was a significant improvement in fat composition in the blood on LY2405319 treatment, as triglycerides and total cholesterol/LDL-cholesterol decreased. This has not yet been studied in mice.

In mice and monkeys, there was significant weight loss, which was reversed within the wash-out periods in monkeys. The mice showed no change in total food intake, suggesting an intrinsic weight losing effect of LY2405319. In humans, there was a trend for weight loss, however food intake was not recorded. Therefore, further research is necessary to unravel the underlying mechanism of the observed weight loss effect. This could be psychologically driven by the central nervous system (CNS), due to a change in fat composition, or both.

Mice and monkeys showed significant decreases in blood glucose levels. High doses of LY2405319 did not induce hypoglycemia, which is an advantage compared to the current treatment with insulin, as overdosing insulin can result in hypoglycemic coma and death. In humans, only a trend toward normoglycemia was noted on LY2405319 treatment. Insulin levels did drop in all studies, suggesting that insulin resistance decreased as a result of LY2405319 treatment. This drop of insulin may also improve β-cell repair, however this has not yet been studied.

Antibodies against LY2405319 were demonstrated and analyzed in the monkeys, however the effects of LY2405319 were still seen throughout the study period, suggesting that the antibodies were not neutralizing. One monkey with an advanced stage of diabetes was included. It had extremely low levels of insulin, severe hyperglycemia, and low body weight. Treatment with LY2405319 did not result in normoglycemia, only a small,

TABLE 2 Summary of species-specific findings of FGF21 analogs.

FGF21 analog	Weight loss	Insulin levels ↓	Glucose levels ↓	[Lipid] improvement
LY (40, 49, 50)	MI & MO: yes HU: no	In all: yes	MI & MO: yes HU: no	HU & MO: yes
PF (22, 51, 52)	MO: yes HU: no	MI: yes Rest: no	MI: yes Rest: no	In all: yes
RGE (53)	MI and MO: yes	In all: yes	In all: yes	In all: yes
(PEG-)cFGF21 *Dog only* (40)	Yes	*	Yes	Yes

[Lipid] improvement: improved lipid levels in the blood.
* = insulin levels in dogs with primary DM are already very low.
MI, mice; MO, monkeys; HU, humans.

but not sufficient trend in glucose reduction was observed. Insulin levels had a small increasing trend. A possibility could be that insulin and LY2405319 work synergistically; one needs the other, and therefore there was only a small trend toward normoglycemia. However, this monkey did show positive changes in lipid composition, which is encouraging in the treatment of advanced diabetic patients.

In humans, treatment with LY2405319 did not show any side effects, however the need for daily injections is a limitation for its use, although it is only once daily compared to twice daily insulin injections. In conclusion, LY2405319 has promising possibilities for DM treatment, but further research is warranted.

PF05231023

A more recent FGF21 analog, PF05231023, consists of two modified FGF21 molecules, conjugated to an antibody. PF05231023 has been tested in mice (50), humans (55), monkeys (51, 52, 55), and rats (51). All these studies demonstrated lipid lowering effect. The monkeys and humans showed a decrease in triglycerides and an increase in HDL. The humans in the two different studies had some differences in outcome. One study showed a significant decrease in total cholesterol and LDL (51), whereas in the other study no significant changes were seen (56). This could be the result of a different injection rate, which was either once or twice weekly.

Monkeys had a significant lower caloric intake, resulting in a lower body mass (52). In addition, fewer pro-inflammatory markers were measured, indicative of reduced inflammation. In contrast, a study in rats demonstrated increased food intake on PF05231023, but no changes in body mass. The authors attributed this due to higher energy expenditure on FGF21. These rats got their injection intracerebroventricular, which suggests that FGF21 acts predominantly within the brain (56). However, the action of FGF21 on the CNS is still unclear, and subject for further research.

A human study with once weekly injections gave conflicting results. Triglyceride levels were significantly lowered, but inconsequently no changes in body mass were observed. Only the study in mice showed significant changes in glucose and insulin levels.

Some adverse effects were reported in humans and rats. Diarrhea was seen in about 30% of humans, which was accompanied by increased bowel movements. Some individuals showed an increase in bone markers that were indicators of bone resorption. This side effect was unexpected as to date, FGF23 and not FGF21 has been regarded as a bone-remodeling factor. Furthermore, humans and rats showed an increase in heart rate and blood pressure. Blood pressure went up with a maximum increase of 15 Hg, which is potentially harmful in DM patients with already high blood pressure. Mice showed sympathetic nerve activity in response to FGF21 injections. This could be the explanation for the increase in blood pressure and heart rate because of the stimulation of the sympathetic nervous system (57).

PF05231023 was injected once or twice a week, which is an advantage of PF05231023 when compared to LY2405319 which was used comparable to insulin in a twice daily regimen. However, PF05231023 was injected intravenously in these studies, not subcutaneously as LY2405319.

To conclude, PF05231023 has promising effects on the lipid metabolism profile and body mass. Unfortunately, no effect on blood glucose levels was seen. Side effects such as diarrhea, bone resorption, and increased heart rate and blood pressure have been reported in the studies, which is one of the reasons why PF05231023 treatment needs to be further optimized.

Fc-FGF21(RGE)

Fc-FGF21(RGE) is a novel FGF analog, with improvements made compared to their earlier analog [Fc-FGF21(RG)]. By this modification, there was less aggregation, proteolytic degradation, and a better affinity for the β-klotho receptor, allowing for once or twice weekly subcutaneous injections. RGE has been tested in mice and monkeys (53). Both species showed a decrease in glucose levels, insulin levels, triglycerides, and body weight, all of which were significantly better compared to the previous analog. Mice and monkeys had also a reduced food intake, which could be the reason for the observed weight loss.

Because of the better pharmacokinetics and -dynamics, RGE could be one of the most promising analogs. Moreover, no hypoglycemic episodes were reported in this study. The half-life of RGE was also increased compared to the previous analog.

Future studies on the use of RGE should be performed in humans or pets to see if similar results can be obtained.

(PEG)-cFGF21

To our knowledge, the first long-during study in diabetic dogs with an FGF21 analog was performed with PEGylated canine FGF21 (PEG-cFGF21). This was done in dogs who present primary DM, characterized by immune-mediated destruction of the pancreatic β-cells (58). PEG and cFGF21 were both able to lower glucose levels. Compared to treatment with insulin, which was able to maintain glucose levels normal for 2 h, cFGF21 and PEG maintained it for, respectively, 2 and 3 days. PEG had stronger effects compared to cFGF21 over time, suggesting PEG to be the better long-acting analog.

Triglyceride, total cholesterol, and LDL-levels were decreased in both analog groups, when compared to insulin. Furthermore, GLUT1 expression was increased in the liver, which could be indicative of concurrent increased expression of GLUT1 in adipose tissue which could be beneficial for DM patients. Moreover, expression of sodium-glucose cotransporter 1 (SGLT1) and SGLT2 decreased significantly. These transporters are used for (re)absorption of glucose from out of the kidneys (SGLT1) and intestines (SGLT2), back into the bloodstream. Fewer of these receptors results in lower blood glucose levels, which is beneficial in hyperglycemic patients.

The pancreas had decreased numbers of reactive oxygen species (ROS) and inflammatory markers, which points to less severe damage of the pancreas in diabetic patients. PPAR-γ was upregulated in adipose tissue, which could also lead to improved insulin sensitivity. The question remains whether these results in canine primary DM can be translated to cats with secondary DM, but the prospects on insulin levels are good and no adverse effects were reported so far.

Future perspectives

Before application of FGF21 in clinical practice, more research should be done on the underlying mechanism in diabetic patients such as regulation of SGLT in the kidneys, and the effect of FGF21 on the central nervous system, possible synergistic effects with insulin, long term effects, different application forms (oral, parenteral), and possible side effects. Ideally, these mechanisms and effects should be studied in clinical trials with the intended target species.

Final remarks

Extrapolation of results based on human and rodent, species that are omnivores, to the adaptive carnivores (i.e., dogs and cats) has some limitation especially regarding glucose and lipid homeostasis. Where men/mice have *de novo lipogenesis* (DNL) in the liver, in cats this occurs predominantly in fat tissue (59). This is probably due to the fact that cats are more strict carnivores whereas dogs are considered adaptive carnivores that are more used to thrive on an omnivorous diet, just like men and mice. Moreover in cats reduced food uptake will lead to recruitment of lipids from the fatty tissue to the liver. As the feline liver sometimes cannot cope with this sudden increase of lipid mobilization, even in a situation of starvation, cats may develop hepatic lipidosis. Dogs are also better able to cope with differences between nutrient abundance and starvation due to the feast and famine eating pattern of their ancestors (i.e., wolves). This sharply contrasts with mice and men. The details of the mechanistical species differences include the already mentioned differences in DNL and PPAR-activities.

In conclusion, FGF21 (and its analogs) has multiple favorable effects on metabolism, especially in humans with secondary DM. Paradoxically, FGF21 levels are elevated in obese or diabetic animals (60–62). When these obese mice were given more FGF21, they did not fully respond anymore. Two explanations can be made in this paradox. FGF21 could be harmful to the body and its metabolism, or obese patients could have a resistant state for FGF21. If FGF21 should have destructive effects, why did FGF21 demonstrate positive in almost all the animals in previous studies? Furthermore, FGF21 is physiologically present in the body, and therefore not likely to be harmful. The resistance hypothesis is thus more plausible. FGF21 was highly increased in mice that were fed a ketogenic diet. β-klotho- and FGFR1-receptors were reduced expressed in adipose tissue, whereas in the liver FGFR4-receptors were reduced expressed, with no change in β-klotho-receptor expression. Both observations led to an impaired signaling of FGF21 in ketogenic fed mice (33). This was also observed in obese mice, where the expression of β-klotho- and FGFR1-receptors reduced in adipose tissue, after a few weeks with the increasing level of obesity (63). On the contrary, overexpression of β-klotho receptors leads to more weight loss in obese animals and increased sensitivity to FGF21 (38).

Altogether, FGF21 might play a role in the treatment of pets with DM in the future, eventually combined with exercise instructions and dietary adaptations. FGF21 can tackle several important aspects of DM, by increasing insulin sensitivity, reducing hyperglycemia, improving the lipid metabolism profile of the blood, and inducing weight loss. To be a good alternative for, or an addition to insulin therapy, limitations and side effects should be reduced.

In 1922, insulin was firstly described by Banting and Best based on canine studies (64). It looks like one century later, a novel step to tackle DM is on the horizon.

Author contributions

DLvE drafted the manuscript as part of a BSc-thesis. RJC adapted this manuscript to the journals' requirements. RJC, HSK, DLvE, and LCP edited and supervised this manuscript.

All authors contributed to the article and approved the submitted version.

Conflict of interest

The authors declare 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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Predictive equations for dietary energy are improved when independently developed for dry and wet food which could benefit both the pet and the environment

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Introduction: Measuring energy availability through metabolizable energy feeding studies is the "gold standard" for establishing metabolizable energy concentration. However, predictive equations are often used to estimate metabolizable energy in dog and cat pet foods. The goal of this work was to evaluate the prediction of energy density and compare those predictions to each other and the energy needs of the individual pets.

Methods: Feeding studies used 397 adult dogs and 527 adult cats on 1,028 canine and 847 feline foods. Individual pet results for the estimate of metabolizable energy density were used as outcome variables. Prediction equations were generated from the new data and compared to previously published equations.

Results and discussion: On average the dogs consumed 747 kilocalories (kcals) per day (SD = 198.7) while cats consumed 234 kcals per day (SD = 53.6). The difference between the average prediction of energy density and the measured metabolizable energy varied from the modified Atwater prediction 4.5%, 3.4% (NRC equations), 1.2% (Hall equations) to the new equations calculated from these data at 0.5%. The average absolute values of the differences between measured and predicted estimates in pet foods (dry and canned, dog and cat) are: 6.7% (modified Atwater), 5.1% (NRC equations), 3.5% (Hall equations) and 3.2% (new equations). All of these estimates resulted in significantly less variation in the estimate of the food expected to be consumed than the observed variation associated with actual pet consumption to maintain body weight. When expressed as a ratio of energy consumed to metabolic body weight (weight in kilograms$^{3/4}$) the within species variation in energy consumed to maintain weight was still high as compared to the energy density estimates variance from measured metabolizable energy. The amount of food offered as the central point in a feeding guide, based on the prediction equations, would on average result in an average variance between 8.2% error in the worst case estimate (feline dry using modified Atwater estimates) and approximately 2.7% (the new equation for dry dog food). All predictions had relatively small differences in calculating food consumed when compared to the differences associated with the variation in normal energy demand.

KEYWORDS

dogs (*Canis lupus familiaris*), cats (*Felis catus*), energy predication, sustainability, environmental impact

Introduction

The use of commercially available pet foods provides for the nutritional needs of dogs and cats. The foods designed for meeting the complete nutritional needs of pets make this claim through testing or product analysis which adequately shows they are sufficient to meet the required nutritional needs. In order to formulate these foods an estimate of energy density is used with an estimate of energy requirements.

The estimate of the energy requirements and thus daily food intake offered is often based on an understanding of expected energy use associated with age and lifestyle (1). The estimates of energy density of the foods are then used to calculate the amount of food to be offered each day to each pet. Because energy density changes the amount of food consumed, the knowledge of energy density also drives the nutrient density of all other nutrients. The complete combustion of a pet food is used to measure the gross energy concentration (1) while metabolizable energy (ME) is either measured through a feeding study or by estimation through use of calculations based on measured concentrations of gross energy, moisture, protein, fat, ash and fiber. The standard for measuring ME is a feeding study where the energy lost in the feces and urine is subtracted from the gross energy of the food to calculate the available ME for use by the pet. The Association of American Feed Control Officials (AAFCO) (2) has suggested feeding protocols which when applied determine the energy density of dog and cat foods. The prediction equations for estimating energy density include the modified Atwater equations suggested by AAFCO (2) which are the result of modifications for dogs (3) and for cats (4). These factors do not change for the energy digestibility of the foods but have been shown to reasonably be used as estimates for commercial dog and cat foods from 85 to 75% energy digestibility (5). However, as many pet foods have energy digestibility above 85% there has been a number of other energy estimate equations suggested (6–8). The analytical basis used in these predictions requires endpoints with standard analysis that are highly repeatable. These equations have started with gross energy (GE) and crude fiber (9). The equations of the NRC (1) are such equations and are based on using the gross energy of the food as a starting point and then modifying that estimate by factors associated with dietary fiber (either crude fiber or total dietary fiber) as well as dietary protein concentration. The equations of Hall et al. (7) use gross energy and then modify that energy density by factors associated with dietary concentration of moisture, protein, fat, ash and crude fiber. Depending on the foodstuffs and foundationally the energy digestibility of the food different equations are best at predicting measured energy density (10). Because of limits on available data, the equations most used have been developed to estimate energy density in both dry and wet foods. As ingredients and energy digestibility are often different between dry and wet pet foods there is a reasonable possibility of improved equations tailored to each form. Because pet foods are often formulated and sold without measuring energy density through feeding studies they have energy estimates that may result in nutrients either being under formulated or over formulated as nutrient concentration is scaled to dietary energy density. The best practice of feeding pets is to adjust energy intake through changing food offering (which maintains an optimal body composition). If dietary energy density is overestimated than the initial feeding guide will underestimate food use, as food intake is adjusted up to maintain body weight there is a subsequent overconsumption of nutrients. Therefore, if energy density is not correctly known there can be an over intake of nutrients and subsequent increased excretion leading to an increased environmental footprint. There is therefore a constant desire to improve dietary energy prediction in order to optimize feeding guides, enhance the balance of nutrients to energy and improve the environmental footprint of pet food. This study uses 1,875 digestibility studies to evaluate the energy density predictions of the modified Atwater factors, the NRC (crude fiber) equations, the Hall equations, and generates new equations associated with a subset of these data. The data were separated into two groups with approximately 30% of the data randomly assigned to a group which was not used to generate the new prediction equations but reserved as a group to test the equations accuracy.

Materials and methods

The studies reported here were completed over a period of 24 years. There were 397 healthy adult dogs and 527 healthy adult cats included in these studies (Table 1). There were 6,155 individual canine energy digestibility data points used and 4,828 feline individual results used. The digestibility studies were conducted by following AAFCO methodology where energy intake is measured through measuring food intake while non-metabolizable energy is measured through fecal energy output with urinary energy loss calculated through calculation of urinary nitrogen (2). In short, these studies consist of two phases, in the first phase there is a 7 day period to allow the dogs and cats to become acclimated to the food as well as to adjust food intake in order to maintain weight as needed. The second phase is the next 5 days (120 h) which is used for total fecal collection. During this second phase food offered is held constant (at the amount determined in phase one to maintain weight). The length of the two periods allows sufficient time for control of energy intake to maintain weight (period 1) and enough fecal collection so that timing of bowel movements does not excessively change estimates of digestibility (period 2). All pets had access to water at all times and daily food offerings of the amount which maintains body weight.

The Institutional Animal Care and Use Committee, Hill's Pet Nutrition, Inc., Topeka, KS, USA (Permit numbers CP13 and CP14) after review approved these studies. All cats were immunized against the feline panleukopena virus, feline calicivirus, viral rhinotracheitis and rabies. All dogs used in these studies were immunized against canine distemper, adenovirus, parvovirus, bordetella, and rabies. All dogs and cats were healthy and had no signs of systemic disease. This was concluded on the basis of urinalysis, serum biochemical analysis, complete blood count and physical exams all evaluated on a yearly basis in an annual exam. The dogs were allowed to exercise in groups in indoor runs and housed individually. Cats were also individually housed and had indoor runs where daily interaction with other cats and exercise was allowed. Both cats and dogs were housed with access to natural light and with varied seasonal changes. Both cats and dogs had enrichment activities and opportunities for socialization. Enrichment included interactions and play with toys, other cats or dogs and with animal care workers.

The test means (using test as the experimental unit) of a subgroup of these data were previously used to explore the effect of ingredient source on digestibility (11). Less than 20% of these data (using test as the experimental unit) were part of the data set used to estimate each species energy digestibility equation for the combined dry and wet foods (7). Initial feeding amounts were based on either previously measured energy density with similar formulae, manufactures statements as to energy density, energy density based on Hall et al. (7), or on modified Atwater predictions (3, 4).

The canine and feline foods varied in gross energy, energy digestibility, moisture, protein, fat, ash and crude fiber as reported in Tables 2, 3 for cats and dogs, respectively. The foods represent the wide spectrum of available foods including foods designed to aid in the management of disease, foods designed for specific life stages or conditions, and foods designed to meet the needs of all life stages. The main ingredients generally were animal proteins

TABLE 1 Means (and standard deviations) of pet weight, age, and energy use.

	Weight (kg)	95% Confidence interval	Age (years)	95% Confidence interval	Energy intake kcalories/day	95% Confidence interval	Energy to weight ratio[a]	95% Confidence interval
Dogs[b]	11.5 (3.01)	11.3–11.7	6.2 (2.68)	6.1–6.3	747 (198.7)	737–757	120 (23.9)	119–121
Cats[c]	5.1 (1.22)	5.0–5.2	6.6 (2.67)	66.5–6.7	234 (53.6)	232–236	70 (14.6)	69–71

[a]Values are kilocalories/(Weight in kg)$^{3/4}$.
[b]There were 397 dogs in these studies (were 64 intact females, 26 intact males, 162 neutered males and 145 spayed females).
[c]There were 527 cats in these studies (61 intact females, 15 intact males, 227 neutered males and 224 spayed females).

(meats, meat meals, eggs), grains and high carbohydrate ingredients (rice, corn, wheat, sorghum, potato, quinoa), and plant proteins (corn gluten meal, rice protein concentrate, soybean either as a meal or an isolate) with oils (animal fat, soybean oil, fish oil, flax oil) and vitamins and minerals. This study used the measurements of gross energy, moisture, protein, crude fat, ash, and crude fiber to predict energy density. The measurement of these dietary factors was completed as previously outlined (2) using the methods of the Association of Analytical Communities (AOAC) by a commercial laboratory. Food analytical measurements for energy, moisture, protein, fat, crude fiber and ash were performed as outlined by AAFCO (2). Food composition of the experimental foods was determined by a commercial laboratory (Eurofins Scientific, Inc., Des Moines, IA) using Association of Analytical Communities methods (moisture—AOAC 930.15; protein—AOAC 2001.11; fat—AOAC 954.02; fiber—AOAC 962.09; and ash—AOAC 942.0).

A group of responses (∼30% of total by random allocation) were assigned as a check group which were not used to establish the new prediction equations. The group of 70% of the responses was used to generate new equations based on species and dietary form (dry or wet). The calculations of variation from measured energy density for all prediction equations were completed to evaluate the accuracy of the energy estimates. The previously established energy prediction statements follow. Gross energy and the estimates are expressed as kilocalories per kilogram. Protein, fat, moisture ash, and crude fiber are expressed as grams/100 grams. Fiber and fat are both the result of the AOAC methods described earlier and are often stated as "crude fat" or "crude fiber."

Canine ME (Kcal/kg) estimates:

$$(modified\ \ Atwater) = 35 * protein + 85 * fat + 35 * (100 - fat - protein - moisture - fiber - Ash)$$

$$(Hall) = 575 + (0.8166) * GE - (20.616) * protein + (12.086) * fat - (6.076) * moisture - (52.766) * fiber$$

$$(NRC) = 10 * [((91.2 - (1.43 * fiber))/100) * (GE/10) - (1.04protein)]$$

Feline estimates:

$$(modified\ \ Atwater) = 35 * protein + 85 * fat + 35 * (100 - fat - protein - moisture - fiber - Ash)$$

$$(Hall) = -541 + (0.923) * GE - (4.216) * protein + (14.686) * fat + 4.806 * moisture - 44.31 * fiber$$

$$(NRC) = 10 * [((87.9 - (0.88 * fiber))/100) * (GE/10) - (0.77 * protein)]$$

The statistical analysis performed used SAS 9.4 (SAS Institute, Cary, NC, USA). The absolute value of the difference between predicted and measured metabolizable energy (ME) was calculated. These differences were then compared among prediction equations by using PROC MIXED in SAS within the dry and wet food categories of each species, using individual pet identification as a random variable. When the differences were not normally distributed, log values of the differences were used for mean separation. A $p <$

TABLE 2 Characteristics of cat foods tested.

	Dry cat foods ($n = 506$)					Wet cat foods ($n = 341$)				
	Mean	Standard deviation	95% Confidence interval	Median	Range	Mean	Standard deviation	95% Confidence interval	Median	Range
Moisture (%)	6.50	1.25	6.4–6.6	6.40	2.4–11.1	77.9	2.78	77.7–78.1	78.4	65.8–84.7
Protein (%)	33.3	4.53	33.1–33.5	32.8	22.8–57.8	8.54	1.69	8.45–8.63	8.30	5.0–15.0
Fat (%)	18.0	3.91	17.8–18.2	18.6	3.1–31.7	5.03	1.55	4.95–5.11	5.00	2.0–13.8
Crude fiber (%)	3.01	2.63	2.89–3.13	2.0	0.2–14.9	1.04	0.81	1.00–1.08	0.80	0.1–5.4
Ash (%)	5.45	1.47	5.38–5.52	5.20	3.5–30.1	1.35	0.27	1.34–1.36	1.30	0.6–2.5
Gross energy[a]	5,106	249.8	5,095–5,117	5,142	4,009–5,815	1,245	182.4	1,235–1,254	1,205	844–2,172
Metabolizable energy (ME)[a]	4,199	354.5	4,183–4,215	4,247	2,786–4,871	984	176.9	975–994	969	603–1,840
Within test ME standard deviation[b]	121.8	55.5	119.3–124.3	113.6	0.0–528.6	36.6	21.3	35.4–37.8	32.4	0.8–204.7
Digestible energy (%)	87.2	3.99	87.0–87.4	88.0	74.1–94.0	83.9	5.26	83.6–84.2	85.3	61.4–92.8

[a]Values (kcals/kg) are from tested ME using the AAFCO protocols.

[b]Values (kcals/kg) are the within test standard deviation reflecting the variation within each individual test.

TABLE 3 Characteristics of dog foods tested.

	Dry cat foods ($n = 711$)					Wet cat foods ($n = 317$)				
	Mean	Standard deviation	95% Confidence interval	Median	Range	Mean	Standard deviation	95% Confidence interval	Median	Range
Moisture (%)	8.41	1.06	8.37–8.45	8.40	3.5–12.2	77.0	3.86	76.9–77.1	76.5	63.8–84.4
Protein (%)	22.5	5.41	22.3–22.7	22.3	9.9–54.2	5.46	1.36	5.41–5.51	5.20	1.90–11.3
Fat (%)	14.8	3.91	14.6–15.0	14.4	6.7–35.9	4.10	1.73	4.04–4.16	3.70	0.6–9.8
Crude fiber (%)	3.66	3.37	3.53–3.79	2.40	0.5–21.8	1.17	0.90	1.14–1.20	0.90	0.1–4.6
Ash (%)	5.24	1.13	5.20–5.28	5.10	2.3–14.6	1.28	0.30	1.27–1.29	1.20	0.6–2.5
Gross energy[a]	4,693	247.7	4,684–4,702	4,670	3,987–6,123	1,176	223.3	1,167–1,184	1,159	438–1,990
Metabolizable energy (ME)[a]	3,808	348.5	3,793–3,819	3,851	2,453–4,933	928	225.9	920_936	896	216–1,713
Within test ME standard deviation[b]	76.0	37.1	74.6–77.4	69.4	11.7–331.6	22.1	13.3	21.6–22.6	19.2	1.0–124.9
Digestible energy (%)	86.1	5.28	85.9–86.3	87.6	59.4–95.4	83.1	5.95	82.9–83.9	84.5	57.6–97.8

[a]Values (kcals/kg) are from tested ME using the AAFCO protocols.

[b]Values (kcals/kg) are the within test standard deviation reflecting the variation within each individual test.

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0.05 was used as cut off for significance. Regression analysis was used to generate co-factor coefficients for each species and within the wet or dry categories were generated using PROC MIXED in SAS. Besides the actual dietary factors measured the analysis of the possible predictor factors included the square of each of the factors and the complete list of one way interactions. The addition of a new factor to those considered for coefficients was evaluated through PROC STEPWISE in SAS. If the factor addition was significant ($p < 0.05$) and if it improved the r-square of the overall prediction ($p > 0.005$) then it was included. In order to remain in the model the factor also had to be significant in the PROC MIXED analysis for generation of the coefficients. An overall evaluation of the predictions was performed using species type and check status (used to develop the model or held for independent evaluation) as independent variables. Colinearity was evaluated by using the variance inflation factor (VIF) indicator generated by PROC REG in SAS according to the methods previously defined (12). The VIF (which is $1/(1\text{-}R_i^2)$) where R_i^2 is the coefficient of determination for the regression of the *i*th independent variable on all other independent variables increases as the colinearity increases which is related to the instability of the estimate of the coefficients. There is no formal determining maximum for VIF although higher values suggest reduced confidence in the coefficients. However, for prediction purposes the coefficients are less important than the final prediction value (13).

Results

The dietary intakes of the daily metabolizable energy for dogs and cats are reported in Table 1. There is an increased energy use (when scaled to weight in kilograms to the ¾ power) in dogs as compared to cats. However, both species have similar and significant variation of pet to pet energy use with one standard deviation 20% of the mean for dogs and 21% for cats (when energy is expressed as kcals/weight$^{3/4}$).

The observed values for concentrations of the macronutrients and energy of the foods used are reported in Tables 2, 3. These values were the results of the variation of pet foods tested and were not selected for any specific values or central tendencies. The mean dry matter energy densities for cat food were (4,490 kcal/kg dry and 4,452 kcal canned) and dog food were (4,155 kcal/kg dry and 4,034 canned). The mean energy digestibility of the dog and cat, dry and canned foods, are also shown in Tables 2, 3. The energy in dry foods was more digestible than canned and the gross energy in the cat food was more digestible than that in dogs. By using previously published methodology (14) the maximum total error acceptable for comparable methodologies can be calculated. In short, this method requires an estimate of the maximum allowed imprecision, the maximum allowed inaccuracy, and total error which is calculated from within animal and between animal variation. This analysis relates to the acceptance of the values of the comparative method and to those of the original method. The maximum total errors for energy density calculated from Tables 2, 3 for dogs are 3.9% for dry and 8.1% for wet food. For cat food the maximum total errors are 3.9% for dry and 7.6% for wet food. The measured ME concentrations of dry and wet cat and dog foods and the estimated ME concentrations of the predictive equations are shown in Tables 4, 5, respectively. The new equations based on species and dietary form (dry or wet) are as follows:

Canine ME (Kcal/kg) estimates:

$$\begin{aligned}(New\ Dry) &= 48.7 + (0.934) * GE - (7.21) * moisture - (50.19) \\ &* protein + (64.9) * fat - (41.2) * fiber - (13.4) * ash \\ &+ (0.00736) * GE * protein - (0.0126) * GE * fat \\ &- (3.323) * fiber * ash \\ (New\ Wet) &= -307.7 + (1.072) * GE + (1.29) * moisture + (9.85) \\ &* protein - (4.90) * fat - (46.1) * fiber + (10.49) * ash \\ &- (0.0182) * GE * protein + (8.134) * fat * fiber \\ &- (25.06) * fiber * ash\end{aligned}$$

Feline estimates:

$$\begin{aligned}(New\ Dry) &= -505.5 + (0.9233) * GE - (0.432) * moisture \\ &- (0.3719) * protein + (13.1) * fat - (12.65) * fiber \\ &+ (14.57) * ash - (0.402) * protein * fiber \\ &- (0.7567) * protein * ash - (1.794) * fiber * fat \\ (New\ Wet) &= -419 + (0.9462) * GE + (4.26) * moisture - (10.79) \\ &* protein + (4.59) * fat - (48.69) * fiber + (10.79) * ash\end{aligned}$$

These previously published and new equations were evaluated for comparative benefit in predicting ME by comparing the means of the absolute values of the difference between the predicted and measured ME. The feline dry and wet food energy density estimates were most accurate with the new equations followed by the Hall equation. Both of these estimates were better than the NRC estimates which in turn were superior to the modified Atwater estimates (Table 4).

The dog food energy estimates for dry food are best using the newly derived equation, which was followed by the NRC equation, followed by the Hall equation while the modified Atwater equation had the worst estimates of energy density for dry dog food. For the canine wet foods the best estimate of ME was through using the newly derived equation, followed by the Hall equation which was followed by the modified Atwater equation, while the NRC prediction had the most variance from the measured ME (Table 5).

The prediction values listed in Tables 4, 5 all had significant slopes ($p < 0.01$) when regressed to measured energy digestibility. There was an overestimation of ME when energy digestibility was low and an underestimation when energy digestibility was high in all equations. The intercepts of energy digestibility for the feline dry and wet forms for modified Atwater prediction were 75.4 and 78.3%; Hall prediction intercepts were 86.8 and 81.0%; NRC prediction intercepts were 83.2 and 87.4%; The new feline prediction intercepts were 87.1 and 85.9%; dry and canned, respectively. In the dog the intercepts for modified Atwater predictions were 78.2 and 82.6%; Hall prediction intercepts were 92.1 and 90.4%; NRC prediction intercepts were 85.7 and 91.9%; The new canine prediction intercepts were 89.5 and 82.6%; dry and canned respectively. The scatter plots of each estimate of energy density as plotted against measured ME are shown in Supplementary Figures 1–16. Because there was not always a difference between the variance of the prediction equations in all of the above groupings an overall evaluation was conducted. This showed that in the data from which the new predictions were calculated, as well as in an independent analysis with the data not included in the development of the equations, the variations were modified Atwater>Hall>NRC>New with each prediction method being different from all others.

TABLE 4 Feline tested and estimated metabolizable energy (Kilocalories/kg).

	Dry cat foods ($n = 2,115$)[π]				Wet cat foods ($n = 1,253$)[π]			
	Mean	Standard deviation	Median	Range	Mean	Standard deviation	Median	Range
Metabolizable energy (ME)[1]	4,199	375.1	4,272	2,687–5,124	996	187.7	973	124–1,921
Modified Atwater prediction[2]	3,875	270.3	3,941	2,880–4,406	952	162.9	922	600–1,786
Modified Atwater variance[3]	347[a]	181.5	339	0–1,182	70[a]	52.8	62	0–476
Hall prediction[4]	4,190	337.9	4,265	3,011–5,062	986	190.9	953	592–1,897
Hall variance	133[c]	117.0	106	0–1,286	44[c]	42.8	34	0–467
NRC prediction[5]	4,095	276.6	4,163	3,082–4,716	1,027	159.5	995	685–1,484
NRC variance	170[b]	123.9	152	0–1,214	53[b]	53.8	38	0–560
New prediction[6]	4,198	333.2	4,283	3,007–4,951	997	178.1	970	620–1,819
New prediction variance	128[c]	114.2	102	0–1309	40[d]	42.1	30	0–495
	Dry cat foods ($n = 859$)[Ω]				Wet cat foods ($n = 601$)[Ω]			
Metabolizable energy (ME)[1]	4,198	372.1	4,253	2,836–5,027	970	158.9	963	609–1,422
Modified Atwater prediction[2]	3,883	264.4	3,909	3,160–4,458	928	143.5	905	665–1,286
Modified Atwater variance[3]	347[a]	193.5	340	0–1,247	61[a]	41.0	57	0–207
Hall prediction[4]	4,202	332.6	4,226	3,362–4,906	959	163.3	937	627–1,403
Hall variance	138[c]	145.8	109	0–1,611	38[c]	28.8	34	0–192
NRC prediction[5]	4,104	270.3	4,118	3,422–4,688	1,005	137.0	981	764–1,366
NRC variance	173[b]	143.5	110	0–1,501	52[b]	46.8	39	0–272
New prediction[6]	4,208	326.5	4,224	33,982–4,891	972	153.4	954	643–1,387
New prediction variance	137[c]	143.8	1,101	0–1,648	35[c]	27.5	29	0–169

[π]These data (group 1, n = 2,115 dry; 1,253 wet) were randomly chosen as the data set to be used to generate the new prediction equations.

[Ω]These data (group 2, n = 859 dry; 601 wet) were randomly chosen as a check data set independent of the data used to generate the new prediction equations.

[1]ME and all numbers in the table are kcals/kg.

[2]Modified Atwater energy = 85*fat + 35*NFE + 35 * protein.

[3]Variance is the absolute value of the difference between the measured ME and the prediction.

[4]Hall et al. prediction equation ME defined in the paper.

[5]NRC crude fiber prediction equation ME defined in the paper.

[6]New prediction equations which were generated from the 2,115 cat data set (dry foods) and 1253 cat data set (wet foods).
ME defined in the paper.

[a,b,c,d]Means with different superscripts in the same column and group are different $p < 0.01$.

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TABLE 5 Canine tested and estimated metabolizable energy (kilocalories/kg).

	Dry dog foods ($n = 2,843$)$^{\pi}$				Wet dog foods ($n = 1,326$)$^{\pi}$			
	Mean	Standard deviation	Median	Range	Mean	Standard deviation	Median	Range
Metabolizable energy (ME)[1]	3,820	345.1	3,861	1,951–4,819	926	230.6	896	112–1,779
Modified Atwater prediction[2]	3,638	240.7	3,658	2,750–4,440	920	197.0	913	572–1,662
Modified Atwater variance[3]	216[a]	141.6	202	0–1,485	59[b]	85.7	41	0–1,043
Hall prediction[4]	3,889	302.5	3,924	2,723–4,679	941	226.0	908	442–1,732
Hall variance	114[b]	108.3	90	0–1,671	35[c]	46.0	25	0–480
NRC prediction[5]	3,814	296.1	3,872	2,561–4,625	994	203.6	966	355–1,712
NRC variance	106[c]	102.5	84	0–1,701	73[a]	58.4	58	0–525
New Prediction[6]	3,849	316.1	3,897	2,637–4,649	925	224.7	893	231–1,706
New prediction variance	82[d]	82.6	66	0–1,172	22[d]	19.5	17	0–156
	Dry dog foods ($n = 1,416$)$^{\Omega}$				Wet dog foods ($n = 570$)$^{\Omega}$			
Metabolizable energy (ME)[1]	3,778	371.6	3,835	2,472–4,986	926	215.5	875	475–1,578
Modified Atwater prediction[3]	3,619	291.2	3,642	2,666–4,721	927	191.8	900	603–1,490
Modified Atwater variance[4]	205[a]	143.5	189	0–1,176	51[b]	40.1	44	0–245
Hall prediction[5]	3,855	354.0	3,900	2,634–4,877	943	219.6	892	546–1,689
Hall variance	124[b]	106.9	95	0–728	33[c]	30.6	26	0–265
NRC prediction[6]	3,778	357.7	3,838	2,447–4,693	999	187.9	973	622–1,689
NRC variance	109[c]	101.2	83	0–916	77[a]	58.6	63	0–319
New prediction[7]	3,814	366.9	3,863	2,499–4,731	926	214.2	879	499–1,672
New prediction variance	89[d]	81.8	75	0–603	31[c]	29.0	25	0–268

$^{\pi}$ These data (group 1, $n = 2843$ dry; 1326 wet) were randomly chosen as the data set to be used to generate the new prediction equations.

$^{\omega}$ These data (group 2, $n = 1416$ dry; 570 wet) were randomly chosen as a check data set independent of the data used to generate the new prediction equations.

[1] ME and all numbers in the table are kcals/kg.

[2] The average of the absolute value of the individual pets estimate as compared to the 6 pet mean.

[3] Modified Atwater energy = 85*fat + 35*NFE + 35*protein.

[4] Variance is the absolute value of the difference between the measured ME and the prediction.

[5] Hall et al. prediction equation ME defined in the paper.

[6] NRC crude fiber prediction equation ME defined in the paper.

[7] New prediction equations which were generated from the 2,843 dog data set and 1,346 dog data set (wet foods).

[a,b,c,d] Means with different superscripts in the same column and group are different $p < 0.01$.

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Discussion

These studies show that the AAFCO digestibility technique has a robust ability to measure dietary metabolizable energy over a broad spectrum of energy concentrations in dog and cat foods. This conclusion is based on the standard deviation (expressed as coefficient of variance) as an estimate of the within study variation which was always below 4%. This shows low biological variability in the test which leads to the reasonable conclusion that the test is repeatable with a robust ability across quite variable energy densities. As the analytical variance is equally low these CV% reflect a repeatable and valuable assay. If there were no constraints in resources, the biological measurement of ME is clearly an excellent and superior technique allowing both optimum nutrient to energy ratios and limiting the environmental overload resulting from imbalanced nutrition. The equations using the food analysis to evaluate measured metabolizable energy in this study take different approaches to estimating energy density. The modified Atwater equation uses coefficients that estimate the energy in carbohydrate (as represented by the amount of food which is not moisture, protein, fat, ash or crude fiber), protein and fat. This has a great potential for bias (and subsequent error) as the digestibility for each macronutrient increases above what was used to generate the coefficients. The underestimated ME estimated by using the modified Atwater coefficients in highly digestible pet food has been repeatedly shown (7, 15–17). Intuitively, using the extra information associated with gross energy is a significant enhancement as compared to the modified Atwater factors. However, all equations are limited by the data set from which they are derived and may be enhanced by increasing information describing the food whose energy density is being estimated. In this evaluation the equations which used gross energy were all superior to the modified Atwater estimates which do not use it. All estimates of ME have at their base an estimate of the foods energy digestibility and all equations have error associated with the prediction vs. actual energy digestibility. When the relationship between the delta of the predictions from the measured ME (the prediction estimate subtracted from measured ME) was regressed against measured energy digestibility the modified Atwater prediction underestimated energy concentration in dry food when energy digestibility was above 78.2 (canine) and 75.4% (feline). As the average pet food in this analysis exceeded these energy densities this relationship partially explains the underestimated energy density observed using the modified Atwater prediction. The NRC equations start with the measured gross energy and then estimate the loss of energy associated with changes in dietary fiber and protein. These prediction equations are also influenced by energy digestibility. In the case of these equations the energy digestibility intercepts for dry food (85.7% canine, 83.2% feline) were closer to the actual energy digestibility in these data. The Hall equations were also influenced by energy digestibility with the intercept (92.1 canine and 86.8% feline) showing a computational bias toward a more correct answer with higher digestible energy foods as compared to either the NRC equations or the modified Atwater equation. Finally, the newly derived equations have intercepts in the dog (86.5% and 83.0% dry and wet, respectively) and feline (87.2% and 84.2%, dry and wet, respectively). These also show the bias toward better predictions for foods with higher energy digestibility. This discussion on the relationship of the prediction to actual energy digestibility highlights a weakness of all prediction equations which is that they are tuned to the data set from which they are derived. It is interesting in this case that the data set used to derive the equations and the group used to check the derived equations resulted in similar and precise average deltas from the measured ME (~3% of ME). This suggests they are robust equations. However, it also could suggest that both groups had similar foods with similar ingredients and energy digestibility, thus providing a good match for the derived equations.

The errors associated with both imprecision and inaccuracy of prediction equations are summed in the absolute value of the difference between the estimated and the measured metabolizable energy. This analysis allows the variance to be analyzed on an individual pet basis which increases the variation estimate from what is estimated from the prediction equation and the means of the group in which the food was tested. The individual pet data also allows a calculation of a maximum acceptable difference for comparison of methods. The maximum allowable total error calculation has previously reported and is a function of both within test and between test variations (14). When looking at the data from this perspective, the equations generated from the modified Atwater equations exceed the maximum total error for both canine and feline dry products. All other equations have equal or less than the calculated total error maximums. However, because there is still significant error associated with the prediction equations (Tables 4, 5) none of them achieve the goal of making the measured metabolizable energy a redundant measure. It remains then to evaluate these equations with respect to their specific uses.

Previous research (15) has reported that the overall best equations for estimating energy density were the NRC equations that use total dietary fiber as compared to the equations that used crude fiber. This current study cannot evaluate the improvements that may be associated with using total dietary fiber as it was not measured in enough of these studies to allow for a meaningful comparison. Unfortunately, because the analysis done previously (7, 15, 16) was completed using the digestibility study as an experimental unit (as compared to the individual pet) it is not directly possible from those evaluations to establish an acceptability of error using the variation associated with the within test pet to pet variation in the metabolizable energy tested evaluation. Also, the knowledge of the variation of the individual pet energy need (as well as the individual's ability to digest energy) informs the evaluation of metabolizable energy and this was not previously reported in these studies.

When using estimates of energy density for feeding guides, the variation between each pet associated with energy use is of significantly greater importance than the variation from any of these estimates of energy density. For example in this analysis an average dog of 11.5 kg consumed 747 kcals/day. A standard deviation change in energy use results in a change of 198.7 kcals per day. If the pet food contains the energy density of 3,806 kcals/kg the grams change difference based on this energy use is 52 grams. The mean variance of the worst canine estimate (modified Atwater dry food) has a mean error of 216 and a standard deviation of 141.6 kcals/kg. This example then estimates that a standard deviation of increased error added to the average of the worst estimated ME changes the energy density by 358 kcals/kg food. This calculates to a change of food intake of <20 grams/day for the 11.5 kg dog. This illustration shows that the variation associated with the energy estimates of the pet food is significantly smaller than the variation associated with energy

demand of the pet. The conclusion therefore is that the measurement or the estimate of the energy use for each individual pet must be completed by establishing the food required for weight maintenance for each pet. So, the feeding amount is reasonably established by a starting point using any of these methods to establish energy density and then refined for each pet. This is done by feeding, weighing, changing food offering, and weighing again until energy consumed is equal to energy used. It has been reported that most dog owners in Canada used this method to establish the correct amount of food to be offered daily (8).

However, the value of knowing the energy density is established nutritionally because nutrients are scaled to energy density. This is done to avoid both underfeeding and overfeeding nutrients. The prediction variance can lead to both under formulating or over formulating specific nutrients. For example if using the modified Atwater prediction for feline dry foods the food would be calculated to contain ~ 93% of the actual ME density. Therefore, on a calorie basis the food would have a >7% under formulation of protein. If using the NRC equation for wet dog food the formulation would have a calculated energy density more than 3% above the actual energy density. In this case, on a calorie basis the food would have a 3% over formulation of protein. There is a significant improvement in energy density prediction using the formulas which were created not only for each species but also for the dry and wet forms. In this case the average variation from measured metabolizable energy was 3.2%. It's not only the average change between the predictions and the measured energy density that has value. The reduced variation (of the delta between observed and measured ME) observed in the new species and form specific equations impart a value in reducing both the over and under formulation of nutrients. Therefore, using these new equations could enhance optimal nutrition through the improved nutrition to energy ratios. For example, again using protein for a pet that is on the lower end of energy consumed to maintain weight, another 7% decline in protein consumed (because of the error associated with predicting energy density) could come close or even surpass the normal nutritional over formulation used to assure delivery of the minimum protein required. Similarly, nutritional overfeeding could be a problem for a pet that is consuming a high amount of calories on a daily basis where excess nutrients would be consumed. For example the increased fecal ammonia and branched chain fatty acids of over feeding highly available phosphorus has been reported (17). Similarly, overfeeding protein has been shown to have increased fecal ammonia and branched chain fatty acids (18). These examples show there can be negative effects from overfeeding of nutrients and this is exacerbated by incorrectly estimating energy density.

The two most significant issues influencing the sustainability and environmental impact of pet food are nutrient composition and ingredient selection (19). Nutrient composition is directly the result of expected energy density. Therefore, from an environmental standpoint incorrectly estimating energy density is of significant concern. Overestimating energy density can be one of the causes of an increased supplementation of nutrients of concern such as phosphorus, protein and metals (e.g., iron and copper). Although each species has a different carbon footprint for protein output, and the use of proteins not competing for human consumption for protein supplied in pet food is significant, it is still correct that protein is the most expensive nutrient from both an ecological and economic perspective. Using protein as an example–the best delivery of appropriate protein is done through measuring ME. However, the improvement of energy density estimate through using the new equations would reduce protein used and subsequent protein waste as compared to the other equations evaluated in this study. Achieving a nutritional optimum for protein supplementation is not an easy task. It involves not simply the protein to energy ratio but also knowledge of digestibility and the amino acid profile of the protein in the food (protein quality). The combination of the nutritional need and the protein quality of the food allow the calculation of the optimum amount delivered to the pet. This optimum is influenced by food intake (as we've noted above) and also genetic variation, pet health, life stage and lifestyle. Nevertheless, a correct understanding of the energy density of the food improves the protein nutrition delivered independently of these other factors. This applies to phosphorus, iron and copper as well. In a global environmental impact of pet food evaluation (20) it was concluded that pet food (which was estimated to be produced at 2.66 x 10^{10} kilograms per year) had an environmental impact associated of 0.8–1.2% of global agricultural land use. It is apparent that a better calculation of metabolizable energy density allowing an improvement in the protein to energy ratio delivered in the pet food will be a part of the sustainability of pet foods in the future. Currently, the dry matter basis concentration of protein in pet food is 31.4% for dry and 40.8% for wet (21). Although the mix of dry and wet is not known a conservative estimate of protein use in pet food exceeds 1 x 10^{10} kilograms. Clearly, even a 1 or 2% change as a result of better estimates of energy density is a significant environmental benefit. Regarding minerals, an improvement in formulation based on an improved energy density calculation is perhaps less impactful than that of protein. However as seen in swine food (22) an improvement in the nutritional supplement of excess zinc and copper is an environmental benefit.

This study is limited because it was a retrospective analysis. This limitation restricted the opportunity to use coefficients of interest as food analysis was completed for other purposes (and misses some factors which could be quite impactful for estimating energy density). Although there is a reasonable spectrum of variable foods represented another limitation is that the generated equations have the central tendency of foods used. In this data set the foods have a higher energy digestibility than many foods in the market place. This limits the accuracy of the estimated energy density, especially for lower digestible foods. Future research to evaluate more dietary variables and the effect of specific ingredients could further improve prediction equations.

Conclusion

The new equations, based on both species and product form could be of value in providing optimum nutrition while simultaneously reducing the unneeded over formulation and enhance the environmental footprint of pet food.

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 Hill's Pet Nutrition Institutional Animal Care and Use Committee.

Author contributions

DJ designed and oversaw the experiment. MJ and DJ analyzed, summarized the data, and wrote and edited the manuscript. Both authors have read and agreed to the published version of the manuscript.

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Conflict of interest

MJ is employed by Hill's Pet Nutrition Inc., and DJ was employed by Hill's Pet Nutrition Inc.

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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.2023.1104695/full#supplementary-material

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Effects of dietary camelina, flaxseed, and canola oil supplementation on inflammatory and oxidative markers, transepidermal water loss, and coat quality in healthy adult dogs

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Introduction: Camelina oil contains a greater concentration of omega-3 (*n*-3) a-linolenic acid (C18:3n-3; ALA) than omega-6 (*n*-6) linoleic acid (C18:2n-6; LA), in comparison to alternative fat sources commonly used to formulate canine diets. Omega-3 FAs are frequently used to support canine skin and coat health claims and reduce inflammation and oxidative stress; however, there is a lack of research investigating camelina oil supplementation and its effects on these applications in dogs. The objective of this study was to evaluate the effects of camelina oil supplementation on coat quality, skin barrier function, and circulating inflammatory and oxidative marker concentrations.

Methods: Thirty healthy [17 females; 13 males; 7.2 ± 3.1 years old; 27.4 ± 14.0 kg body weight (BW)] privately-owned dogs of various breeds were used. After a 4-week wash-in period consuming sunflower oil (*n*6:*n*3 = 1:0) and a commercial kibble, dogs were blocked by age, breed, and size, and randomly assigned to one of three treatment oils: camelina (*n*6:*n*3 = 1:1.18), canola (*n*6:*n*3 = 1:0.59), flaxseed (*n*6:*n*3 = 1:4.19) (inclusion level: 8.2 g oil/100 g of total food intake) in a randomized complete block design. Transepidermal water loss (TEWL) was measured using a VapoMeter on the pinna, paw pad, and inner leg. Fasted blood samples were collected to measure serum inflammatory and oxidative marker concentrations using enzyme-linked immunosorbent assay (ELISA) kits and spectrophotometric assays. A 5-point-Likert scale was used to assess coat characteristics. All data were collected on weeks 0, 2, 4, 10, and 16 and analyzed using PROC GLIMMIX in SAS.

Results: No significant changes occurred in TEWL, or inflammatory and oxidative marker concentrations among treatments, across weeks, or for treatment by week interactions. Softness, shine, softness uniformity, color intensity, and follicle density of the coat increased from baseline in all treatment groups ($P < 0.05$).

Discussion: Outcomes did not differ ($P > 0.05$) among treatment groups over 16-weeks, indicating that camelina oil is comparable to existing plant-based canine oil supplements, flaxseed, and canola, at supporting skin

and coat health and inflammation in dogs. Future research employing an immune or exercise challenge is warranted, as the dogs in this study were not subjected to either.

KEYWORDS

omega-3, omega-6, canine nutrition, skin and coat health, flaxseed oil, canola oil, camelina oil

Introduction

Dogs are unable to produce the omega-6 (*n*-6) linoleic acid (C18:2n-6; LA) and the omega-3 (*n*-3) α-linolenic acid (C18:3n-3; ALA), endogenously, and as such, these must be obtained in the diet (1). Omega-3 fatty acids (FAs) in particular have been linked to numerous health benefits, including a reduction in inflammation and oxidative stress, and improved skin and coat health properties, which are directly associated (2–7).

There is a competitive relationship between the *n*-6 and *n*-3 FA pathways for the use of the Δ5- and Δ6-desaturase and elongase enzymes needed to convert LA and ALA into longer chain FAs. Consequently, a balanced dietary *n*-6:*n*-3 ratio is needed to ensure sufficient conversion to longer chain FAs in both pathways. Specifically, and most notably, LA is converted into arachidonic acid (AA), and ALA is converted into eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) (8). Both AA and EPA and DHA are parent compounds for the production of pro- and anti-inflammatory eicosanoids, respectively. An increase in endogenous *n*-6 AA results in a prothrombotic, pro-constructive, and pro-inflammatory state, whereas increased EPA and DHA give rise to resolvins, which are anti-inflammatory and pro-resolving. Greater concentrations of *n*-6 FAs and a higher *n*-6:*n*-3 ratio allow for greater conversion of *n*-6 FAs to AA and more pro-inflammatory effects. In contrast, greater concentrations of *n*-3 FAs and a lower *n*-6:*n*-3 ratio allow for increased production of EPA and more anti-inflammatory effects (9). As a result, excessive amounts of *n*-6 FAs and a high *n*-6:*n*-3 ratio promote the pathogenesis of many inflammatory, autoimmune, and dermatological disorders, whereas greater concentrations of *n*-3 FAs and a low *n*-6:*n*-3 ratio exert suppressive effects (10).

In order to formulate canine diets to meet the ideal *n*-6:*n*-3 ratio of between 5:1 and 10:1, *n*-3 rich ingredients are typically required (11). Two oils commonly used to increase *n*-3 inclusion in canine diets are fish oil, as a result of its high levels of EPA and DHA (180 mg EPA, 120 mg DHA/1,000 mg of oil provided in the most common fish oil capsules in the United States today, however, doses vary widely between supplements), and flaxseed oil, due to its favorable *n*-6:*n*-3 ratio of 1:4.19 (12–15). However, large-scale fish oil production required to meet the demands of the growing pet food industry is not environmentally sustainable long-term, and the high abundance of ALA in flaxseed oil makes it susceptible to oxidation, making its use in commercial diets difficult (12, 15). Additionally, flaxseed crops are sensitive to various climates, diseases, and pests, making both of these options less than desirable (12, 14, 15). Alternative animal-based (beef, 1:0.05; milk, 1:0.07; eggs, 1:0.05) and plant-based (canola, 1:0.59; corn, 1:0.01; soybean, 1:0.12; and sunflower oil, 1:0.00) lipid sources commonly used in canine diet formulations all have higher concentrations of *n*-6 FAs rather than *n*-3 FAs (15–17). This leaves room in the market for an alternative plant-based oil source that is economically and environmentally sustainable, with good shelf-stability and a favorable concentration of *n*-3 FAs that could contribute to achieving the ideal *n*-6:*n*-3 ratio in canine diets.

The oil seed camelina (*Camelina sativa*) is considered a low-input, high-yield crop due to its short growing season and resistance to various seasons, climates, and soil types (18–21). The product of this robust crop, camelina oil, provides a rich source of *n*-3 FAs as a result of its desirable *n*-6:*n*-3 ratio of 1:1.8 (22). Additionally, camelina oil contains high concentrations of tocopherols and polyphenols, which have been associated with improved skin and coat health due to their antioxidant properties (22). Due to camelina oil being naturally high antioxidants as well as having a slightly lower concentrations of *n*-3 FAs in contrast to flaxseed oil, it's shelf-stability is better by comparison (23).

Additional data from this study suggests camelina oil to be safe for canine consumption (24). The inclusion of oil supplements in canine diets is often associated with claims of maintenance or support of skin and coat health, but currently there is no data directly comparing the effects of camelina oil supplementation to the effects of other oils approved for use in pet foods on markers of skin and coat health and inflammation. The objective of this study was to compare the effects of dietary camelina oil supplementation to those of flaxseed oil and canola oil supplementation on skin and coat health and inflammatory and oxidative markers in healthy, adult dogs. Outcomes include changes in oxidative and inflammatory biomarkers and coat quality. Additionally, skin barrier function and integrity was assessed by measuring transepidermal water loss (TEWL). Authors hypothesize that camelina oil (*n*-3:*n*-6 = 1:1.8) is comparable, flaxseed (*n*-3:*n*-6 = 1:4.19) and canola oil (*n*-3:*n*-6 = 1:0.59) in terms of its effects on oxidative and inflammatory markers, coat quality, and TEWL.

Materials and methods

Animals and housing

This experiment was approved by the University of Guelph's Animal Care Committee (AUP #4365) and was carried out in accordance with national and institutional guidelines for the care and use of animals. Thirty client-owned, adult (7.2 ± 3.1 years) dogs of mixed sex (17 females: 16 spayed, one intact; 13 males: 10 neutered, three intact), weight (27.4 ± 14.0 kg) and

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TABLE 1 Mean age, mean body weight, breeds, and male:female and neutered:spayed:intact ratios of 30 client-owned dogs enrolled in a research trial investigating the effects of three oil supplements (camelina, canola, flaxseed) on transepidermal water loss, inflammatory and oxidative markers, and coat quality over a 16-week period.

Treatment	Mean age (years)[a]	Mean BW (kg)[b]	Breeds	Male:female	Neutered:spayed:intact
CAM	7.8	25	Miniature dachshund Havanese Mix, unknown Mix, Australian shepherd/collie Mix, boxer whippet Standard poodle Norwegian elkhound Labrador retriever (3)	2:8	2:7:1
FLX	7.7	27	Miniature dachshund Pekingese Mix, sled dog/unknown Mix, border collie/sheltie Mix, husky/pointer Great dane Standard poodle Bernese Labrador retriever (2)	6:4	5:4:1
OLA	6.05	28	Mix, mastiff/boxer King Charles cavalier spaniel Mix, samoyed/collie Sheltie German shepherd Barbet Standard poodle Bernese Labrador retriever (2)	6:4	4:4:2

[a]Mean age of dogs on week 0 of research trial; units = years.
[b]Mean body weight of dogs on week 0 of research trial; units = kilograms.
BW, body weight; CAM, camelina oil; FLX, flaxseed oil; OLA, canola oil.
Treatment oils were provided at an inclusion level of 8.2 grams of oil per 100 grams total dietary intake.

breed participated in this study (Table 1). All dogs were deemed healthy based on their previous medical history as well as a pre-study physical examination performed by a licensed veterinarian, complete blood count (CBC), and serum biochemistry profile. During the recruitment process, dogs were excluded if they had any skin conditions, received any pro- or anti-inflammatory medications 2-months prior to baseline samples, had abnormalities on their physical examination, CBC, or serum biochemistry, or were younger than 2 years of age. Dogs were housed at their owners' homes for the duration of the study, they followed their usual daily routines. Pet owners were instructed to provide no supplements, medications, antibiotics, antifungals, antiparasitics, or topical creams without notifying the researchers. Prior to week 10, dog #10, consuming FLX, withdrew from the study due to circumstances unrelated to the research trial or treatment diet.

Dietary treatments

Over a 4-week wash-in period, all dogs were acclimated to a dry extruded commercial kibble (SUMMIT Three Meat Reduced Calorie Recipe, Petcurean, Chilliwack, BC, Canada; Table 2), sunflower oil (SA Kernel-Trade, Kuiv, Ukraine; Table 3), and beef-based treats (Beef Tendersticks, The Crump Group, Brampton, ON, Canada; proximate analysis: metabolizable energy 3039 kcal/kg; crude protein minimum 65%; crude fat minimum 5.1%; crude fiber maximum 4.0%; moisture max 9.56%). Oil was included in the diet at 8.2 grams of oil per 100 grams of total food intake, bringing the total dietary lipid content to 20% on an as-fed basis. Treats were included in the diet up to 2.5 grams per 100 grams total intake, and the remaining proportion of the diet was provided as kibble. During the wash-in period and throughout the study, daily portions of

food, oil, and treats were pre-weighed by researchers and provided to the owners in 2-week intervals to be offered to dogs daily at a frequency determined by the owner. To avoid the occurrence of lipid peroxidation, owners were instructed to mix the oil with the food immediately before feeding. Any leftover kibble, oil, and/or treats were returned to researchers and subsequently weighed and recorded. Dogs were initially fed to meet their estimated maintenance energy requirements (110 kcal ME × kg $BW^{0.75}$), and BW was recorded every 2 weeks starting at baseline. Each dog's food allotment was then adjusted accordingly to maintain baseline BW throughout the study. No abnormal observations were reported by owners throughout the 16-week study period in terms of diet tolerance (i.e., vomiting, stool quality, halitosis, etc.).

TABLE 2 Proximate analysis, metabolizable energy, omega-6 and omega-3, and linoleic and docosahexaenoic acid content of a commercial extruded kibble[a] on an as-fed basis, fed to 30 client-owned dogs during a skin and coat health trial over a 16-week period.

Nutrient profile	As fed basis
Moisture (%)	8.00
Crude protein (%)	21.0
Nitrogen-free extract (%)	52.0
Crude fiber (%)	2.80
Crude fat (%)	9.00
Omega 6 (%)	2.00
Omega 3 (%)	0.20
Linoleic acid (%)	1.90
Docosahexaenoic acid (%)	0.01
Ash (%)	7.10
Metabolizable energy (kcal/kg)	3,324

[a]Chicken meal, whole brown rice, whole white rice, barley, oatmeal, chicken fat (preserved with mixed tocopherols), peas, lamb meal, salmon meal, natural chicken flavor, whole dried egg, sunflower oil, rice bran, flaxseed, dried kelp, dicalcium phosphate, potassium chloride, choline chloride, sodium chloride, calcium carbonate, vitamins (vitamin A supplement, vitamin D3 supplement, vitamin E supplement, niacin, L-ascorbyl-2- polyphosphate (a source of vitamin C), d-calcium pantothenate, thiamine mononitrate, beta-carotene, riboflavin, pyridoxine hydrochloride, folic acid, biotin, vitamin B12 supplement), minerals (zinc proteinate, iron proteinate, copper proteinate, zinc oxide, manganese proteinate, copper sulfate, ferrous sulfate, calcium iodate, manganous oxide, selenium yeast), DL-methionine, glucosamine hydrochloride, chondroitin sulfate, yeast extract, *Yucca schidigera* extract, dried rosemary.

Study design

This study was conducted using a randomized complete block design (RCBD) with repeated measures. Following the 4-week wash-in period, dogs were blocked by breed, age, and BW and groups were randomly assigned to one of 3 treatment oils: camelina oil (CAM) ($n = 10$; eight females; two males), flaxseed oil (FLX) ($n = 10$; five females; five males), or canola oil (OLA) ($n = 10$; four females; six males). The sunflower oil used during the wash-in was replaced with either CAM, FLX, or OLA, and feeding continued as described for 16 weeks. Both OLA and FLX were chosen as control groups for this study as they are commonly used to formulate canine diets and provide a source of *n*-3 FAs.

Blood collection

Dogs were fasted for a minimum of 10 h overnight and blood samples were collected *via* cephalic venipuncture using a syringe (Becton, Dickinson and Company, Franklin Lakes, NJ, USA). Of the collected blood, 5 mL was put into a serum vacutainer (Becton, Dickinson and Company, Franklin Lakes, NJ, USA). Blood was allowed to clot and was centrifuged at 7,200× *g* for 15 min using an accuSpin Micro 17 centrifuge (Thermo Fisher Scientific, Waltham, MA, USA). Then, the serum aliquots were frozen at −80°C until later analysis.

Inflammatory and oxidative markers

Serum samples were analyzed for prostaglandin E_2 (PGE_2) (Canine Prostaglandin E_2 ELISA Kit MBS013017, MyBioSource, Vancouver, BC) and junction plakoglobin (JUP) (Canine Junction Plakoglobin ELISA Kit MBS104997, MyBioSource, Vancouver, BC) using commercially available ELISA (Enzyme-linked immunosorbent assay) kits. Samples were run in duplicate according to the manufacturer's instructions. Serum glycosaminoglycan (GAG) (dimethyl methylene blue) and nitric oxide (NO) (Griess Reaction; Molecular Probes, Eugene, OR) concentrations were determined using spectrophotometric assays (26, 27). Serum NO and GAG samples were analyzed as previously described by MacNicol et al. (28).

TABLE 3 Analyzed fatty acid profiles of camelina oil, canola oil, flax oil, and sunflower oil fed to 30 client-owned dogs top dressed on commercial kibble during a skin and coat health trial over a 16-week feeding period.

Parameter	Sunflower[a]	Canola[b]	Flaxseed[b]	Camelina[b]
Saturated fatty acids (%)	9.61	6.50	8.20	9.50
Monounsaturated fatty acids (%)	14.1	63.8	16.6	35.2
Polyunsaturated fatty acids (%)	76.3	29.7	75.2	55.3
Omega 6 (%)	76.2	18.6	16.5	19.8
Omega 3 (%)	0.04	11.1	58.6	35.4

[a]Numerical values from Kostik et al. (25) and only represent generic sunflower oil, not the brand used for this study.
[b]Samples analyzed in duplicate by SGS Canada Inc. (Guelph, ON, Canada), average values reported.
Burron et al. (24).

Skin barrier function

Skin barrier function and integrity were assessed by measuring TEWL, which is defined as the amount of water that passively evaporates through skin to the external environment due to a water vapor pressure gradient on both sides of the skin barrier and is commonly used to characterize skin barrier function and integrity (29, 30). On weeks 0, 2, 4, 10, and 16, TEWL was measured using a VapoMeter® SWL-3 (Delfin Technologies Ltd, Kuopio, Finland), according to the manufacturer's instructions. Since privately-owned dogs were used, it was not feasible to shave multiple patches for TEWL measurements, and as a result, researchers chose three body sites with little hair to measure TEWL, including: the right paw pad, right pinna, and right inner thigh. Ten measurements were taken per body site and the average was used for analyses. Once the averages were calculated, any values above or below the average by 50 g/m^2/h or more were considered outliers and removed. All dogs were brought to the University of Guelph by their owners on collection days to ensure environmental conditions during collections remained consistent. All measurements were carried out by a single operator, in the same order of body sites, and in a climate-controlled room to maintain consistency between samples and to avoid variation in VapoMeter® readings due to temperature and humidity fluctuations (29). Room conditions were stable at 22–23°C ambient temperature and 44–50% ambient relative humidity. The evaporation rate value is calculated in grams of water per square meter per hour (g/m^2/h). All dogs were behaviorally acclimated to the use of the VapoMeter®, the researchers involved in sample collection, and the collection room, prior to the first sample day to minimize stress, thereby reducing variation in measurements. If dogs were wet due to weather upon arrival they were dried with a towel, to reduce variation further.

Coat quality

Two researchers blinded to treatment were trained to perform a subjective coat assessment on weeks 0, 2, 4, 10, and 16 using a 5-point Likert scale (under Supplementary material). A Likert scale was used to measure the softness, shedding, dander, shine, spring, softness uniformity, color, color uniformity, and follicle density of the coat. Follicle density was assessed on the center of the back of the dogs by scoring the thickness/amount of hair coming from individual follicles. To increase consistency among dogs given different management practices in each household, all dogs were bathed 2 weeks prior to each assessment and owners were instructed to keep dogs dry and to not brush or groom them during this period.

Statistical analysis

Data are presented as mean ± SD unless otherwise stated. All statistical analyses were performed using the PROC GLIMMIX of SAS Studio® software (v.9.4., SAS Institute Inc., Cary, NC, USA). Dog was the experimental unit, and treatment, TEWL site, and sex, and age were treated as fixed effects (age and sex data not presented). Week was treated as a repeated measure. An analysis of variance (ANOVA) was performed to assess the effects of treatment on inflammatory and oxidative marker concentrations, TEWL, and coat scores. When the fixed effects were significant, the means were separated using Tukey–Kramer adjustments. Significance was declared at a $P \leq 0.05$. Trends were declared at $P \leq 0.10$.

Results

Inflammatory and oxidative markers

Prostaglandin E_2

There were no differences among treatments ($P = 0.973$), across weeks ($P = 0.397$), or for treatment by week interactions ($P = 0.987$) (Table 4). Additionally, no differences were observed due to sex ($P = 0.937$) or age ($P = 0.274$).

Junction plakoglobin

There were no differences among treatments ($P = 0.969$), across weeks ($P = 0.249$), or for treatment by week interactions ($P = 0.913$) (Table 4). No differences were observed due to sex ($P = 0.914$) or age ($P = 0.743$).

Glycosaminoglycan

There were no differences among treatments ($P = 0.208$), across weeks ($P = 0.995$), or for treatment by week interactions ($P = 0.915$) (Table 4). Concentrations of GAG tended to be greater in males compared to females ($P = 0.078$). There were no differences observed due to age ($P = 0.329$).

Nitric oxide

There were no differences among treatments ($P = 0.648$), across weeks ($P = 0.359$), or for treatment by week interactions ($P = 0.729$) (Table 4). No differences were observed due to sex ($P = 0.226$) or age ($P = 0.424$).

Transepidermal water loss

Of the 4,440 individual TEWL measurements collected throughout the study period, 18 were considered outliers and removed [D = Dog, W = Week; Paw pad: D6W2(CAM), D8W16(FLX)(2 values), D9W16(FLX), D17W4(CAM), D18W2 (FLX), D18W4(FLX)(2 values), D23W10(CAM), D23W16(CAM); Inner ear: D5W4(OLA), D5W10(OLA), D12W10(OLA); Inner leg: D6W2(CAM), D6W10(CAM), D12W0(OLA), D16W0(FLX), D29W0(FLX)]. These outliers could often be attributed to changes in the environment, leading to signs of stress or excitement in the dogs (i.e., researchers entering and leaving the room, noises occurring outside of the sample room, and in the case of some outliers these samples were taken near the end of the collection period and the dogs would become impatient, no longer wanting to remain in the same spot for samples).

TABLE 4 Serum prostaglandin E_2, junction plakoglobin, glycosaminoglycan, and nitric oxide concentrations of healthy adult dogs supplemented one of three treatment oils[a] on weeks 0, 2, 4, 10, and 16 of a skin and coat health trial, presented as lsmeans ± standard error.

	Week					*P*-values		
	0	2	4	10	16	Treatment	Week	Treatment*week
Prostaglandin E_2 (pg/mL)								
CAM	0.88 ± 1.45	2.77 ± 1.45	3.49 ± 1.45	2.35 ± 1.45	2.32 ± 2.33			
OLA	3.07 ± 1.34	3.07 ± 1.38	2.41 ± 1.44	2.82 ± 1.44	2.80 ± 1.39	0.9734	0.3965	0.9868
FLX	2.55 ± 1.23	4.07 ± 1.28	3.07 ± 1.34	3.44 ± 1.28	3.15 ± 1.33			
Junction plakoglobin (ng/mL)								
CAM	8.73 ± 1.08	9.38 ± 1.08	8.56 ± 1.11	8.65 ± 1.08	7.82 ± 1.08			
OLA	10.09 ± 1.01	9.60 ± 1.01	9.51 ± 1.01	9.96 ± 1.01	7.39 ± 1.09	0.9693	0.2487	0.9133
FLX	8.94 ± 0.94	10.97 ± 0.94	10.78 ± 0.94	9.34 ± 0.97	8.35 ± 1.02			
Glycosaminoglycan (μg/mL)								
CAM	4.43 ± 0.73	4.73 ± 0.73	4.23 ± 0.73	4.91 ± 0.80	3.97 ± 0.76			
OLA	3.03 ± 0.73	4.34 ± 0.73	4.47 ± 0.72	4.17 ± 0.76	3.74 ± 0.72	0.2083	0.9945	0.9147
FLX	4.33 ± 0.66	4.50 ± 0.66	4.82 ± 0.69	4.85 ± 0.69	4.04 ± 0.78			
Nitric oxide (μM/mL)								
CAM	2.20 ± 5.50	9.30 ± 5.50	4.82 ± 5.62	8.34 ± 5.60	10.90 ± 5.64			
OLA	4.31 ± 5.05	7.19 ± 5.05	5.85 ± 5.05	9.26 ± 5.05	10.15 ± 5.18	0.6476	0.3587	0.7288
FLX	11.70 ± 4.58	12.76 ± 4.58	19.56 ± 4.72	13.74 ± 4.72	16.34 ± 4.72			

[a]Treatment oils: CAM, Camelina; OLA, Canola; FLX, Flaxseed oil; Data presented as mean ± standard error; *n* for each treatment group on weeks 0, 2, 4: CAM = 10, OLA = 10, FLX = 10, and weeks 10, 16: CAM = 10, OLA = 10, FLX = 9.

TABLE 5 Mean transepidermal water loss (TEWL) values (g/m^2/h) of the right paw pad, right pinna, and right inner thigh of healthy adult dogs supplemented one of three treatment oils[a] on weeks 0, 2, 4, 10, and 16 of a skin and coat health trial, presented as lsmeans ± standard error.

		Week					*P*-values		
Treatment	Site	0	2	4	10	16	Trt	Site	Week
CAM	Paw pad	92.57 ± 8.80	98.97 ± 8.80	88.28 ± 8.80	83.98 ± 8.80	92.7 ± 8.80			
OLA	Paw pad	88.27 ± 8.85	86.95 ± 8.85	76.32 ± 8.85	71.38 ± 8.85	67.56 ± 8.85			
FLAX	Paw pad	99.43 ± 8.79	109.51 ± 8.79	100.37 ± 8.79	87.38 ± 9.21	88.46 ± 9.21			
CAM	Pinna	14.03 ± 8.80	12.27 ± 8.80	18.78 ± 8.80	14.47 ± 8.80	16.68 ± 8.80			
OLA	Pinna	14.43 ± 8.85	15.84 ± 8.85	16.87 ± 8.85	24.43 ± 8.85	18.40 ± 8.85	0.7261	<0.0001	0.7375
FLAX	Pinna	9.10 ± 8.79	12.69 ± 8.79	12.13 ± 8.79	13.27 ± 9.21	9.92 ± 9.21			
CAM	Inner thigh	23.11 ± 8.80	23.56 ± 8.80	18.2 ± 8.80	17.52 ± 8.80	22.93 ± 8.80			
OLA	Inner thigh	16.86 ± 8.85	15.72 ± 8.85	18.18 ± 8.85	17.32 ± 8.85	21.23 ± 8.85			
FLAX	Inner thigh	15.7 ± 8.79	13.44 ± 8.79	16.36 ± 8.79	14.30 ± 9.21	16.51 ± 9.21			

[a]Treatment oils: CAM, Camelina; OLA, Canola; FLX, Flaxseed oil; Data presented as mean ± standard error; *n* for each treatment group on weeks 0, 2, 4: CAM = 10, OLA = 10, FLX = 10, and weeks 10, 16: CAM = 10, OLA = 10, FLX = 9.

There were no differences among treatments ($P = 0.726$), across weeks ($P = 0.738$), or for treatment by week interactions ($P = 0.996$). Additionally, there were no differences for site by week ($P = 0.378$), or sex ($P = 0.274$) (Table 5). However, there were differences observed among sites ($P < 0.0001$), in that TEWL values for the paw pad were greater than those of the pinna or inner thigh. Additionally, there was a trend observed in age ($P = 0.072$), in that senior dogs (11–14 years; $n = 3$) tended to have lower mean TEWL values compared to young (2–4 years; $n = 7$), young adult (5–7 years; $n = 9$), and adult dogs (8–10 years; $n = 9$).

Coat quality

Softness

There were no differences among treatments ($P = 0.539$), for treatment by week interactions ($P = 0.757$), or due to age ($P = 0.479$), week by age (0.338) or week by sex ($P = 0.738$) interactions. However, there were differences observed across weeks for pooled data ($P = 0.005$) in that softness was greater on week 10 and 16 compared to week 0, and greater on week 10 compared to week 2. Week 4 was not different from any other time points

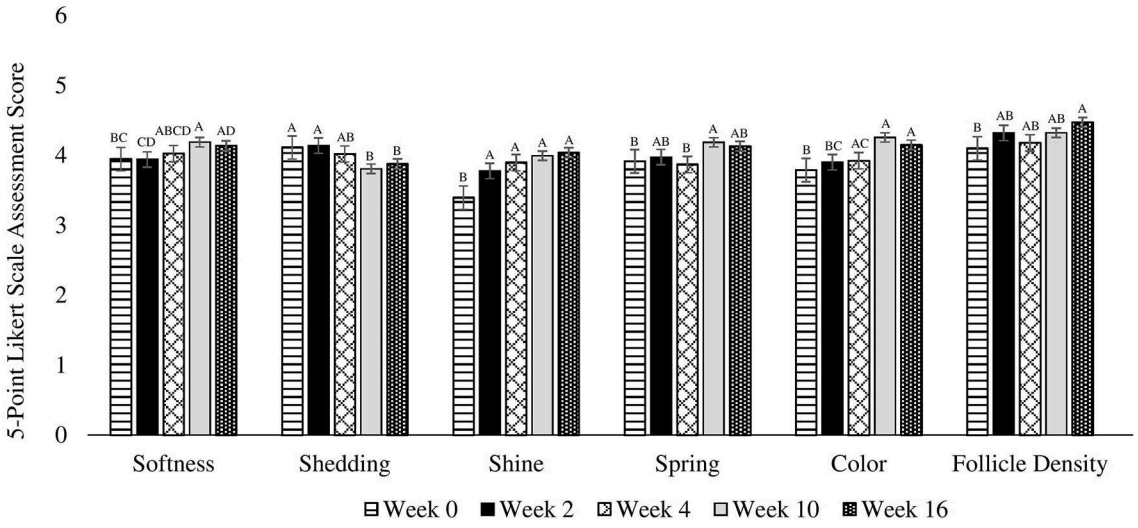

FIGURE 1
Mean coat quality assessment scores completed using a 5-point Likert scale on 30 client owned healthy adult dogs fed one of three treatment oils (camelina oil, canola oil, flaxseed oil) and commercial kibble. [A,B,C,D]Bars without a common letter differ significantly ($P < 0.05$).

(Figure 1). Additionally, softness was greater in females compared to males ($P = 0.026$).

Shedding

There were no differences among treatments ($P = 0.882$), due to age (0.894) or sex ($P = 0.760$), or for treatment by week ($P = 0.444$), week by age ($P = 0.302$), or week by sex ($P = 0.514$) interactions. For pooled data across weeks, shedding was greater on weeks 0 and 2 compared to weeks 10 and 16 ($P = 0.004$). Week 4 was not different from any other time points (Figure 1).

Dander

There were no differences among treatments ($P = 0.648$), due to age ($P = 0.114$) or sex ($P = 0.349$), across weeks ($P = 0.129$), or for treatment by week ($P = 0.869$), week by age ($P = 0.171$), or week by sex ($P = 0.163$) interactions (Figure 1).

Shine

There were no differences among treatments ($P = 0.815$), due to age ($P = 0.945$), or sex ($P = 0.191$), or treatment by week ($P = 0.998$), week by age (0.992), or week by sex ($P = 0.375$) interactions. However, there were differences across weeks for pooled data ($P < 0.0001$) in that shine on weeks 2, 4, 10, and 16 was greater than at week 0 (Figure 1).

Spring

There were no differences among treatments ($P = 0.918$), due to age ($P = 0.663$) or sex ($P = 0.401$), or for treatment by week ($P = 0.397$), week by age ($P = 0.773$), or week by sex ($P = 0.997$) interactions. However, there were differences across weeks for pooled data ($P = 0.014$) in that spring was greater on week 10 compared to week 4 and 0. There were no differences on weeks 2 and 16 (Figure 1).

Softness uniformity

There were no differences among treatments ($P = 0.969$), due to age ($P = 0.860$) or sex ($P = 0.132$), or for treatment by week ($P = 0.799$), week by age ($P = 0.996$), or week by sex ($P = 0.142$) interactions. However, a trend was observed across weeks for pooled data ($P = 0.065$) in that softness uniformity tended to be greater on week 16 compared to week 0. Weeks 2, 4, and 10 were not different from any other time points (Figure 1).

Fur color

There were no differences among treatments ($P = 0.323$), due to age ($P = 0.770$) or sex ($P = 0.546$), or for treatment by week ($P = 0.567$), week by age ($P = 0.345$), or week by sex ($P = 0.954$) interactions. However, there were differences across weeks for pooled data ($P < 0.0001$) in that color was higher on weeks 4, 10, and 16 compared to week 0. Additionally, color was greater on week 10 and 16 compared to week 2. Furthermore, color tended to be higher on week 10 compared to week 4 (Figure 1).

Fur color uniformity

There were no differences among treatments ($P = 0.541$), due to age ($P = 0.893$) or sex ($P = 0.911$), across weeks ($P = 0.362$), or for treatment by week ($P = 0.291$), week by age ($P = 0.787$), or week by sex ($P = 0.910$) interactions (Figure 1).

Follicle density

There were no differences among treatments ($P = 0.873$), due to age ($P = 0.795$) or sex ($P = 0.854$), or for treatment by week

($P = 0.670$), week by age ($P = 0.846$), or week by sex ($P = 0.299$) interactions. However, there were differences across weeks for pooled data ($P = 0.027$) in that follicle density was greater on week 16 compared to week 0. Weeks 2, 4, and 10 were not different from any other time points (Figure 1).

Discussion

The purpose of this study was to assess the effects of camelina oil supplementation on skin and coat health compared to canola and flaxseed oil, two oils currently used to formulate canine diets. The results presented herein suggest no differences in TEWL, coat quality, or the inflammatory and oxidative markers assessed due to treatment over the 16-week period.

Inflammatory and oxidative markers

In the current study, concentrations of GAG tended to be higher in males compared to females. Studies in humans by (1) Larking (31) and (2) Claassen and Werner (32) found that, similar to the present study, females have lower concentrations of GAG. Claassen and Werner analyzed GAG in thyroid cartilage while Larking measured GAG excretion in the tissue. Since GAG is a marker of cartilage turnover, Claassen and Werner attribute their findings to greater cartilage turnover in males, while Larking accredits their findings to the males in their study having a greater mean height (31, 32). It is possible that the female dogs in the present experiment had a smaller average height and lower cartilage mineralization than the males, which contributed to the lower concentration of circulating GAGs observed. However, height and cartilage mineralization were not measured in the present study. Furthermore, the observation made in our study was only a tendency; this, combined with the dearth of work carried out in dogs and lack of equal distribution of male/female, intact/neutered/spayed dogs in the current study make it difficult to form any cogent conclusions. Future research should investigate this relationship further using a dog model.

No significant changes were observed in PGE_2, JUP, GAG, or NO concentrations over the 16-week study period. It is possible that the stability of these concentrations across time and among treatments is attributed to the lack of exercise or immune challenge experienced by the dogs on the current study. It is well-established that both exercise and immune challenges result in a wide range of physiological and biochemical adaptations, the magnitude of which is directly related to the intensity and duration of the exercise or immune challenge encountered (33–36). This wide range of physiological and biochemical adaptations include changes in inflammatory and oxidative biomarker concentrations (28, 33).

Dogs and horses both experience increased PGE_2 concentrations following exercise. In horses, NO and GAG concentrations increase following exercise and compared to baseline, but no change was observed in dogs (28, 33). Pearson et al. attribute these results, similar to previous findings, to variations in NO production depending on exercise intensity, suggesting that it is possible that the lack of changes observed in NO concentration in the current study is due to the low intensity of the exercise experienced by the dogs (33). Markers like PGE_2, NO, GAG, and JUP are often upregulated during times of immune challenge/disease (37–40). A myriad of studies completed in humans suggest no effects of *n*-3 PUFA supplementation on inflammatory or immune markers in healthy individuals (41–43). As an example, Pot et al. found that supplementing fish oil and sunflower oil to healthy individuals had no effect on chemokine, cytokine, or cell adhesion molecule concentration compared to baseline (41). Healthy individuals, similar to the canine subjects of our study, generally have low levels of circulating inflammatory markers. Thus, the chance that low levels of inflammation are reduced even further by an intervention with oil is very small and difficult to measure. The dogs of the present study were healthy upon recruitment and on every sample period based on a veterinary examination, as well as CBC and biochemistry analysis, indicating a lack of immune response that would elicit an inflammatory response. Additionally, the dogs did not participate in any intense exercise prior to or on sample days, and thus had no known reason to elicit any exercise stress induced response impacting markers of inflammatory or oxidative stress. For safety and animal care purposes, no procedures with the potential to cause harm to the animals, like an inflammatory or immune challenge, can be carried out in client-owned dogs. Additionally, the objective of the present study was to determine how these three oils compare to one another in terms of their effects on these biomarkers to gauge their use in dog food formulations for typical pets, not to evaluate their performance following an exercise or immune challenge. Future studies should compare the effects of these three oils and their performance following exercise and immune challenge.

Transepidermal water loss

In the present study, mean TEWL values were significantly greater when measured on the paw pad compared to the inner leg and inner ear. This is likely the result of the tubular, unbranched eccrine glands that open directly onto the skin of the paw pads and noses of canines. These glands allow sweat to be released from these areas, contributing to the water-loss detected by the VapoMeter, and thereby likely contributing to greater TEWL values compared to the inner leg and pinna (44). Additionally, TEWL values were found to be lower in senior dogs compared to young, young adult, and adult dogs. Similar findings have been observed in other canine and human studies and although the exact mechanism behind these observations is unclear, there are various theories (45, 46). The thickness of the stratum corneum and flattening of corneocytes increases with age, while natural moisturizing factors, stratum corneum hydration, and epidermal lipid synthesis are reduced (47–53). Additionally, the density of dermal capillaries decreases with age, which may lower skin temperature and in turn decrease water diffusion (51, 54). All of these findings provide examples of mechanisms that increase the path length and resistance of a water molecule and subsequently contribute to lower TEWL in older individuals, and in agreement with the present study.

Coat quality

Spring and follicle density increased significantly from baseline. This is likely due, at least in part, to the growth of winter coats as the study began at the end of summer and went into the winter (September–January). Dogs have a light summer undercoat that is shed before a thick winter undercoat grows in, which could explain the increase in spring and follicle density. This further supports the observation of the present study in that shedding was greater in all dogs at the beginning of the study at weeks 0 and 2, compared to weeks 10 and 16.

Softness, shine, and color of the dogs' coats increased from baseline. This is likely a result of the dogs consuming an increased amount of *n*-3 FAs following baseline, which can be further metabolized into EPA and DHA, though with limited efficiency. Supplementation of fish oil, a rich source of EPA and DHA, was found to improve skin and hair coat quality in dogs from baseline based on a clinical score, with maximal improvement occurring after 8 weeks (55). The positive effects on skin and coat health are thought to be due to an increase in EPA and DHA in the erythrocyte membrane, along with increased total lipids in the hair shaft (55). The same study observed that following supplement withdrawal, skin and coat health clinical scores remained the same for 1 month and began to deteriorate following the second month (55). Although we did not take measurements on week 8, we did take measurements on week 10, and this is where we saw the largest improvement (i.e., softness, shedding, shine, spring, and color). This is most likely due to the increase in ALA, which is the parent compound of EPA and DHA, the dogs received from their treatment oil (CAM 1:1.8, FLX 1:4.19, OLA 1:0.59) in comparison to the wash-in sunflower oil (1:0). It is important to note that our study had no negative control group, since the absence of an oil supplement would alter all macronutrient intakes and our aim was to compare to existing approved oil supplements. As a result it cannot be ruled out that the observed changes in coat quality may be a result of the placebo effect. Future studies should consider employing a control group fed no oil supplement to rule out the possibility of the placebo effect impacting observations.

All dogs in the current study were considered healthy, with no known dermatological conditions or skin disorders. The coats of these dogs were in relatively good condition at baseline, and future research should investigate these oil supplements and their effects on skin and coat health in dogs with poor skin and coat quality as a result of conditions like atopic dermatitis. It is important to note that ectoparasites, particularly fleas in dogs, can negatively impact skin and coat health (56). In this study, although complete blood count and biochemistry values were assessed, and physical examinations were performed by a licensed veterinarian prior to study recruitment and throughout the entire trial, diagnostic and preventive control in terms of ectoparasites was not considered, and this is a limitation of this study. Authors recommend future studies consider using more specific techniques as inclusion criteria when recruiting participants in order to ensure the absence and prevention of parasites and their potential impact on skin and coat health.

Conclusion

In conclusion, camelina oil is comparable to canola and flaxseed oil in terms of its effects on skin barrier function, coat quality, and the circulating inflammatory and oxidative markers measured in the current study when fed to healthy adult dogs, subjected to no physical or immunological challenge, and observed for 16-weeks. Canola and flaxseed oil are commonly used in canine food formulations. Flaxseed oil specifically has the ability to support skin and coat health claims, making camelina oil a potential alternative plant-based oil source with high concentrations of ALA that could contribute to achieving the ideal *n*-6:*n*-3 ratio in canine diets, while supporting skin and coat health claims.

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 Guelph Animal Care Committee. Written informed consent was obtained from the owners for the participation of their animals in this study.

Author contributions

AS and WP: conceptualization and funding acquisition. AS, WP, and DM: methodology. TR, SB, KP, and CG: study conduct. TR: formal analysis and writing—original draft preparation. TR, SB, DWM, CG, KP, LT, DM, WP, and AS: writing—reviewing and editing. All authors have read and agreed to the published version of the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

AS is the Champion Petfoods Chair in Canine and Feline Nutrition, Physiology and Metabolism and additionally consults for Champion Petfoods. AS has received various honoraria

and research funding from various pet food manufacturers and ingredient suppliers and was a former employee of P&G Petcare and Mars Petcare.

The remaining authors declare 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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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2023.1085890/full#supplementary-material

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Long-term nutritional management of an obese German Spitz with paroxysmal dyskinesia, calcium oxalate urolithiasis, and suspected pancreatitis—A case report

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Background: To our knowledge, this is the first description of long-term nutritional management in a dog with paroxysmal dyskinesia.

Case summary: An obese 9-year-old, male entire, German Spitz was presented for dietary management after being diagnosed with calcium oxalate urolithiasis and suspected pancreatitis. Since he was seven years old, the dog has had a history of neurological signs, which were thought to be epileptic seizures. He was treated with phenobarbital and potassium bromide and was clinically controlled. For his nutritional advice, aiming to minimize one of the most important risk factors for the diseases, a weight loss program was started and successfully executed. However, 10 months later, the dog restarted presenting neurological episodes at a high frequency (3x/week). Based on videos and the characteristics of the neurological signs, the dog was diagnosed with paroxysmal dyskinesia. To investigate the role of gluten intake on this patient's neurological signs, a dietary trial with a commercial hypoallergenic diet (gluten-free; hydrolyzed protein) was followed. During the 3 months of the dietary trial, four neurologic episodes related to food indiscretion occurred. Upon the decrease in neurological episodes, the anti-seizure drugs were slowly discontinued. During this period, the dog presented only two neurologic episodes that were related to the days that the anti-seizure drugs were decreased. For 4 months the dog remained episode-free. However, a change in the dog's diet to another gluten-free diet (higher fat) led the dog to vomit and experience another neurologic episode. Once the dog was back to the previous gluten-free diet, it clinically improved, and no other clinical signs were reported by the client during the next 5 months.

Conclusion: Although a relationship between gluten and paroxysmal dyskinesia cannot be confirmed, the dog's improvement after the nutritional management and the removal of the anti-seizure therapy is supportive of dietary association.

KEYWORDS

dog, nutrition, neurological signs, gastrointestinal signs, bladder stones

1. Introduction

Canine paroxysmal dyskinesia (PD) is a movement disorder characterized by recurrent episodes of abnormal and involuntary self-limiting movements (1). Although it is not a life-threatening disease, the neurologic episodes can last minutes or hours (1) and may be distressing for the owners.

Over the last few years, researchers have been studying possible dietary causes of PD (2–6). Some case reports have shown clinical improvement in Border-Terriers after being fed gluten-free diets (4, 6). However, long-term nutrition follow-up has never been reported. The focus of the presented case is the nutritional management of a patient with multiple diseases: PD, obesity, calcium oxalate (CaOx) urolithiasis, and possible pancreatitis.

2. Case description

A 9-year-old, male entire, German Spitz was first presented to its regular veterinary surgeon with lower urinary tract symptoms (Month -4). Bladder stones were detected and surgically removed. The stones were sent to an external laboratory and were diagnosed as CaOx (10% CaOx monohydrate and 90% CaOx dihydrate). The dog's medical history also indicated occasional neurological episodes since it was 3 months old (Month -108), which were thought to be epileptic seizures. By the age of seven years (Month -28), the frequency of the neurological episodes increased but was controlled with phenobarbital (7 mg/kg ideal body weight [iBW] BID) and potassium bromide (23 mg/kg iBW BID) as anti-seizure drugs (ASD). The dog was episode-free for 2 years (Figure 1). Due to the use of the medication and its increased-related risk of pancreatitis (7–9), commercial wet and dry low-fat diets (diet 1; estimated total daily fat intake: 2.4 g/kg$^{0.75}$) were given until CaOx stones were diagnosed. The dog was also occasionally fed rice and yogurt as a treat. After removal of the CaOx stones, the dog was put on dry and wet diets designed to prevent uroliths (diet 2; estimated daily fat intake: 5.8 g/kg$^{0.75}$) (Month -4).

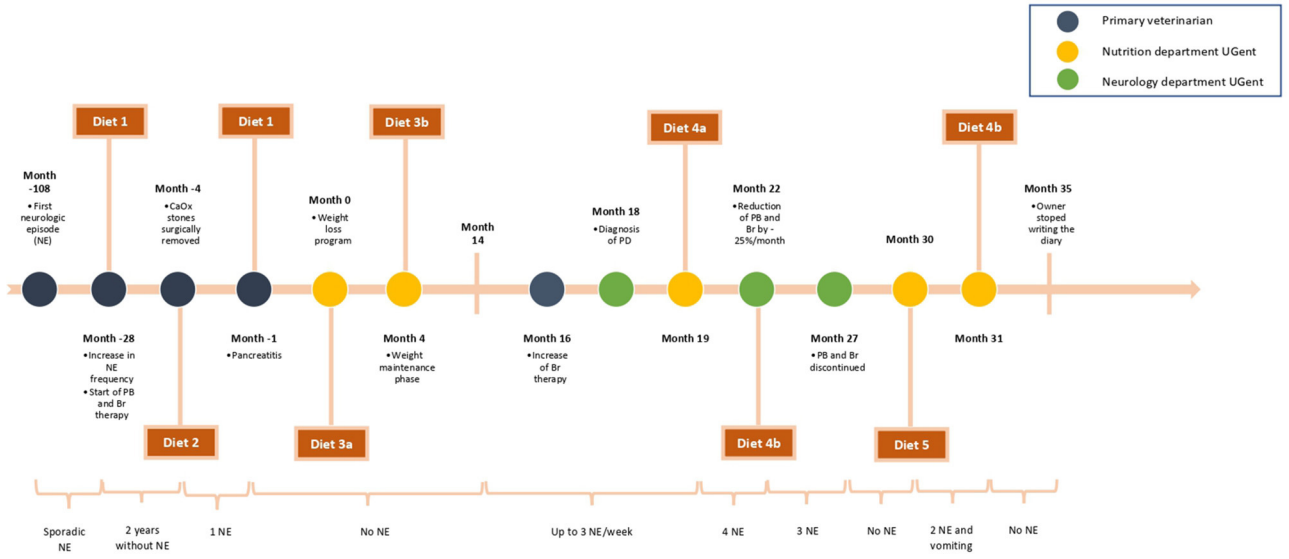
Three months later (Month -1), the dog was hospitalized due to abdominal pain, and gastrointestinal and neurologic complaints. The owner reported that since diet 2, the dog had low fecal consistency and darker feces color. Blood work revealed mild anemia, high serum alkaline phosphatase (406 U/L; reference range 23–212 U/L), serum amylase (>2,500 U/L; reference range 500–1,500 U/L), and serum lipase (>6,000 U/L; reference range 200–1,800 U/L) values. As pancreatitis was suspected, low-fat dry and wet diets were resumed (diet 1). The dog was then referred to the Faculty of Veterinary Medicine of Ghent University for its nutritional management (Month 0).

On initial physical examination, the dog was weighing 4.4 kg and had a body condition score (BCS) of 8/9 with an estimated iBW of 3.4 kg. Cranial abdominal pain and mild periodontitis were noticed. The dog was fed a mixture of 100 grams of wet food per day and *ad libitum* dry food. The wet food was given as a meal and also to facilitate the dog's medication intake.

Considering the dog's physical examination, clinical and feeding (Table 1) history, risk factors (10, 11) (Table 2), and key nutrients (10) (Table 3) for each disease (obesity, chronic pancreatitis, and CaOx), a weight loss regimen was advised. The selection of the diet aimed to prevent CaOx by feeding a low relative supersaturation (RSS) diet, prevent pancreatitis by reducing the fat intake, and safely reduce the dog's body weight (BW). The prescribed weight loss diet (diet 3a; 2.4 g fat/kg$^{0.75}$) was given at 70kcalxkg iBW$^{0.75}$ and consisted of a mixture of commercial dry and wet food and, upon the owner's request, two options of vegetables (zucchini or cucumber) (5% energy intake) were advised to be fed in a food dispenser toy. Four months later (Month 4), the dog weighed 3.5 kg and had a BCS of 5/9 and the mean weight loss rate was determined at 1.72% of BW per week. To keep the dog at a stable weight, the energy intake was then slowly increased to up to 15% (diet 3b; 2.8 g fat/kg$^{0.75}$), and the dog maintained its weight without presenting gastrointestinal, neurological, or low urinary tract symptoms hereafter.

Ten months later (Month 14), the neurological signs increased to three episodes per week and were unresponsive to a higher dose of potassium bromide (23 mg/kg iBW in the morning and 46 mg/kg iBW in the evening), the dog was then referred to the neurology service of the same Faculty (Month 18). The episodes were mainly occurring during rest and lasted for approximately one to 3 min. During the episode, the dog was conscious and able to respond to its owner calling its name, but the episodes could not be stopped by distraction. Videos of the episodes (Supplementary material) showed dystonia-like movements (stiffness and cramping of ≥1 muscle group) mainly of the limbs and neck. No autonomic signs were reported. After an episode, the dog was immediately normal again. The first episode was often followed by a second one within minutes. Clinical and neurological examination revealed no abnormalities and recent blood tests did not show metabolic causes for the increased number of episodes. In addition, serum concentrations of ASDs were constantly monitored over the years and were within therapeutic levels. Based on the videos of the neurological episodes, PD was suspected. Brain MRI was not performed as the dog was normal in-between episodes for ≥6 years, suggesting no structural brain disease. Aiming to investigate the relationship between diet and neurological episodes, an elimination diet trial was started (Month 19). A combination of moderate-fat dry and wet hypoallergenic, gluten-free diets with low RSS (diet 4a; 3.3 g fat/kg$^{0.75}$) was introduced. There was no prior test for anti-gliadin and anti-transglutaminase A2 antibodies and to evaluate the effect of the diet on the neurologic symptoms, the ASD therapy was not altered at first, and the owner was advised to keep a diary of the neurologic episodes (Supplementary Table S1).

Three and a half months later (Month 23), the dog came for a follow-up consultation in which the owner reported four PD episodes after the onset of the diet trial. Two of those episodes were observed when a commercial dog treat (Dentastix®) was fed, and the other two were associated with accidental ice cream intake. Since there was an overall improvement in the PD episode frequency (Supplementary Table S1), a slow decrease in ASD was started. To avoid withdrawal epilepsy by the quick reduction of the ASD, the drugs were decreased by 25% per month. At that moment, the dog's BW had increased by 10% and BCS was evaluated as 6/9, therefore the calorie intake was decreased by 4%. Additionally, the owner requested a treat option, and a hypoallergenic, low-RSS, moderate-low-fat (3.62 g fat/100 kcal) treat was



NE = neurologic episode(s); PB = phenobarbital; Br = potassium bromide; PD = Paroxysmal Dyskinesia; CaOX = calcium oxalate

Diet 1: Royal Canin Gastrointestinal Low Fat dry and wet

Diet 2: Royal Canin Urinary S/O Small Dog and Trovet Canine Renal & Oxalate RID wet

Diets 3a,b: Royal Canin Satiety Small Dog dry and wet

Diets 4a,b: Royal Canin Multifunction Urinary + Hypoallergenic and Hill's Prescription Diet z/d Canine wet + Hill's Hypoallergenic Treats

Diet 5: Royal Canin Multifunction Urinary + Hypoallergenic and Hill's Prescription Diet z/d Feline wet + Hill's Hypoallergenic Treats

FIGURE 1
Timeline showcasing most important events, neurological episodes (NE), anti-seizure drugs and dietary changes.

combined with the diet as 4.4% of the dog's daily calorie intake (diet 4b; 3.1 g fat/kg$^{0.75}$).

During the first 2 months of the reduction of the ASD, the dog presented two PD episodes; the first one, 3 days after the first reduction of the medical treatment (Month 23); and the second one, one day after tapering the medication (Month 24). In the last month (Month 27) of the reduction of the medication the dog again presented a PD episode, but this was short-lived and since then the dog had remained symptom-free.

Three months later (Month 30), due to the unavailability of the prescribed commercial wet food, the owner contacted the nutrition service requesting another commercial wet food option. After careful evaluation of the commercial diets available on the market, no option was found to be suitable for all the criteria (moderate-low fat, low RSS, hypoallergenic and gluten-free). To have the lowest impact on the diet, a hypoallergenic and gluten-free wet food from the same brand, but specifically made for cats, was advised for a short period and only until the initially prescribed canine wet food was available again. Both wet food products had the same ingredient list, but they differed in nutrient composition. Since the cat's wet food was higher in fat, the dry and wet food ratio was changed, aiming at 3.5 g fat/kg$^{0.75}$ (diet 5). Moreover, the owner was informed that the prescribed diet could change the targeted urinary pH and therefore, should not be used in the long term. Immediately after starting the new diet, the owner reported that the dog had two PD episodes followed by vomiting. The cat's wet food was then removed from the feeding plan and the previously advised diet (diet 4b) was resumed. Since then, no other neurological, gastrointestinal, or urinary clinical signs have been observed (Month 35). After that, the follow-up contact was reduced, but the owner was contacted again in Month 37 and small neurologic episodes were reported, those were short-lived and the owner did not record the dates. The timeline with all the events can be seen in Figure 1.

3. Discussion

Obesity is considered a predisposing factor for uroliths (12). The current obese patient developed CaOx stones, which were surgically removed and prevented by nutritional management as stated in literature (13–15). However, after the CaOx treatment, the patient presented a neurologic episode after remaining PD episode-free for 2 years and was hospitalized with presumed pancreatitis. Although the advised diet (diet 2) had low RSS, aiming for the decrease of the CaOx supersaturation, it also had higher fat content (+245%) than the dog's previous diet (diet 1). Due to the patient's history of use of ASD and its possible associated risk of pancreatitis (7–9), the higher fat intake was likely the cause of the gastrointestinal symptoms. Nonetheless, although serum amylase and serum lipase activity concentrations have limited value in the diagnosis of pancreatitis, due to their low sensitivity and specificity (16–18), based on the dog's medical history, symptoms, and physical examination, pancreatitis was

TABLE 1 Comparison between key nutrients (expressed in g/kg$^{0.75}$) and ingredients of the patient's diets throughout its life.

	Nutrient levels (adult dogs)			Advised diets by primary veterinarian		Advised diets by the nutritional service from the University of Ghent				
	Minimum by NRC	Recommended by NRC	Recommended by FEDIAF	Diet 1[a]	Diet 2[b]	Diet 3a[c]	Diet 3b[c]	Diet 4a[d]	Diet 4b[d]	Diet 5[e]
kcal ME/kg$^{0.75}$			80	120*	120*	71	81	81	78	78
kJ ME/kg$^{0.75}$			335	500*	500*	296	341	341	326	325
Nutrients (g/kg$^{0.75}$)										
Protein	2.62	3.28	4.95	8.34	6.38	8.87	10.07	4.41	4.20	5.11
Fat		1.80	1.51	2.36	5.79	2.41	2.79	3.28	3.13	3.51
Calcium	0.06	0.13	0.14	0.36	0.21	0.21	0.24	0.13	0.13	0.13
Phosphorous		0.10	0.11	0.25	0.18	0.16	0.19	0.11	0.11	0.12
Sodium	0.01	0.03	0.03	0.13	0.27	0.08	0.10	0.23	0.19	0.24
Magnesium	0.01	0.02	0.02	35.53	13.50	0.19	0.22	10.71	0.11	0.10
Other										
Gluten-containing ingredient				Wheat and grains	Wheat	Wheat and grains		No		No
Urinary claim				-	Low RSS and pH-increasing effect disclaimer	Low RSS		Low RSS		Low RSS
Hypoallergenic with hydrolyzed protein				No	No	No		Yes		Yes

Due to different energy intakes over time, the evaluation of the diets was made in g/kg$^{0.75}$.

*Estimated energy intake based on FEDIAF, 2021 for > 7-year-old adult dog.

[a]Royal Canin Gastrointestinal Low Fat dry and wet.

[b]Royal Canin Urinary S/O Small Dog and Trovet Canine Renal & Oxalate RID wet.

[c]Royal Canin Satiety Small Dog dry and wet.

[d]Royal Canin Multifunction Urinary + Hypoallergenic and Hill's Prescription Diet z/d Canine wet + Hill's Hypoallergenic Treats.

[e]Royal Canin Multifunction Urinary + Hypoallergenic and Hill's Prescription Diet z/d Feline wet + Hill's Hypoallergenic Treats.

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TABLE 2 List of risk factors for each associated disease that were taken into account for this patient's nutritional recommendation.

	Obesity	Calcium oxalate stones	Chronic pancreatitis	Paroxysmal dyskinesia
Drug	Treatment with phenobarbital		Treatment with phenobarbital	Treatment with phenobarbital
Medical conditions		Obesity	Obesity	
		Chronic pancreatitis		
Animal odds		Gender: male	Male	
		Breed predisposition (4%)		
		Age: older (8.5 average)	Age: older	The onset of symptoms at an early age (<1 year)
Dietary factors	High fat	Homecooked diet	High fat	Possible correlation to gluten-containing diets
	High energy dense diets	High protein	Low protein	
	Free-choice feeding	High sodium, vitamin D, and magnesium: hypercalciuria		
	Treats	High vitamin C: Hyperoxaluria		
		Deficiency of pyridoxine: Hyperoxaluria		
		Low magnesium, phosphorous, and calcium		

Hand et al. (10).
Lowrie and Garosi (11).

TABLE 3 Key nutrients and their aimed values for the associated diseases.

Key nutrients	Obesity	Calcium oxalate stones	Chronic pancreatitis	Paroxysmal dyskinesia
Energy density	<3.4 kcal/g or 14.2 kJ/g	-	-	-
Water	Increased	High: to decrease urine supersaturation	High: due to dehydration	-
Protein	High: >25% DM	Avoid excess: 10–18% DM	Moderate to low: 15–30% DM	Hydrolyzed; gluten-free
Fat	Low: <9–14% DM	-	Low: <10–15% DM	
Fiber	High: 12–25% DM	High: to decrease intestinal absorption and urinary excretion of Ca	-	-
Calcium (Ca)	-	Avoid excess: 0.4–0.7% DM	-	-
Phosphorous (P)	0.4–08% DM	Avoid deficiency: 0.3–0.6% DM	-	-
Ca:P		Ratio: 1.1:1–2:1	-	-
Sodium	0.2–04% DM	Moderate restriction: <0.3% DM	-	-
Magnesium		Moderate: <0.04–0.15% DM	-	-
Vitamin C	> 100 mg/kg	Avoid supplements and food containing vitamin C	-	-
L-carnitine	>300 ppm	-	-	-
Oxalate	-	Avoid high oxalic acid sources	-	-

%DM, percentage of dry matter.
Hand et al. (10).

strongly suspected. Additionally, CaOx preventive diets should be cautiously used whenever it is combined with medical treatment since the urinary pH can influence the rate of excretion of several drugs, including phenobarbital. The pH alkalinization increases urinary excretion of this drug and shortens its elimination half-life (19), decreasing its efficacy. Moreover, higher sodium intake increases potassium bromide excretion, decreasing its effectiveness (20). Therefore, the occurred neurological episode was potentially related to the lower efficiency of ASD after the dietary change.

Due to all the nutritional concerns and the risk factors from the different diseases (obesity, suspected pancreatitis, and CaOx), a

weight loss program was firstly aimed at and successfully achieved without overlooking pancreatitis and CaOx stones. Changes in diet and body composition (obesity or weight loss) alter the pharmacokinetics of phenobarbital and adjustments in dose may be needed (21). At that moment the dog was well controlled on its PD episodes, therefore, no changes in the medical and nutritional therapy were advised. One year later, a neurological evaluation was performed after the increase in the PD episodes' frequency, followed by an unsuccessful attempt to increase the ASD dosage, and PD was diagnosed.

Canine PD is a movement disorder characterized by recurrent episodes of abnormal and involuntary self-limiting movements (1, 22). Although reports on dogs have increased in the past years, it remains important to differentiate it from epileptic seizures (11). In this case, the dog was first assumed to have epilepsy, and this led to long-term treatment with ASD. Most PD cases do not respond to ASD (5, 23–25), and only a few cases have been described to reduce the episodes' frequency upon medical treatment (26, 27). However, the presented patient remained episode-free for 2 years during the ASD therapy and this was potentially the reason for its misdiagnosis. The diagnosis of PD is based on the owner's description and video evaluation of the events. The most important differences between epileptic seizures and PD episodes are the preservation of consciousness, the longer duration of episodes (<1 min vs. >1–5 min), and the lack of a post-ictal phase in PD cases (1). All of these features were seen in the reported case and confirmed the diagnosis of PD. In veterinary medicine, the most recognized form of PD episodes is paroxysmal non-kinesigenic dyskinesia, where the episodes are occurring spontaneously at rest (1). This type was suspected in the presented case (1, 28–31). Videos of the neurologic episodes showed episodes of dystonic movements. Dystonia-like movements are often seen in dogs with PD and are defined as sustained or intermittent involuntary contractions of a group of muscles producing abnormal movements, postures, or both (1, 20, 22).

PD has been described in different breeds (1), including the paroxysmal gluten-sensitive dyskinesia (PGSD) in Border Terriers, which often display concurrent dermatological and/or gastrointestinal signs (3–6, 11). Evidence shows partial or complete resolution of episodes after feeding the dogs with a gluten-free diet (2–6), however, scientific evidence is low. Although a positive response to gluten-free diets was seen in those case reports (2–6), a complete diagnosis of adverse reaction to food following the gold standard was not performed. For its diagnosis, serological tests (immunologic response against transglutaminase A2 and gliadin) were previously described as diagnostic markers for PGSD in Border Terriers (6), however, those tests were not formally validated for other dog breeds and are still limited in veterinary medicine. Additionally, although those markers may be an interesting tool, their results should be cautiously interpreted as they indicate if the animal was or was not previously in contact with gluten and it should not be assumed that high levels of those markers are diagnostic of PGSD. Nonetheless, it can be a useful marker to monitor compliance with a gluten-free diet therapy by testing it before and after diet change (6). For that reason, up to now, a diet trial is considered the gold standard to diagnose adverse reactions to food (32), including an adverse reaction to gluten. This trial involves the resolution of the symptoms after an elimination diet trial, followed by the reoccurrence of the symptoms after a challenge trial with the prior diet (32). For the elimination trial, a commercial or home-prepared novel protein diet or a commercial hydrolyzed protein diet should be consistently fed to the dog for 12 weeks or as soon as the symptoms ameliorate (33). However, the evaluation of neurological improvement can be difficult in a short period since animals with PD can stay several weeks/months without presenting another episode (34). Therefore, a long-term follow-up and a consistent diary can help interpret the results. Despite the lack of scientific evidence on the role of gluten on PD, in the current case, it was suggested to the owner to follow a gluten-free elimination diet trial as a first treatment approach and to re-evaluate the dog's symptoms after its completion. Due to the history of CaOx and the difficulty in controlling urinary pH with home-cooked diets, a commercial gluten-free diet with low RSS was preferred and recommended. In addition, to limit the risks of cross-contamination that over-the-counter gluten-free products may impose (35), a gluten-free hypoallergenic commercial diet with hydrolyzed protein was advised.

Although the standardized diagnostic trial, including the challenge phase, was not formally completed, in the current case, improvement of the symptoms was seen and the onset of the episodes after food indiscretion with a treat containing gluten was used as an accidental challenge. This was in agreement with previously PGSD documented cases (3–6, 11), as an improvement in the dogs' neurologic symptoms was observed after putting them on a gluten-free diet. Nonetheless, after those dogs (2–4, 6) clinically improved, there was no challenge with a gluten-containing diet, consequently, no confirmation of the suspected diagnosis.

In this case, although a causal relationship between a gluten-free diet and the management of its neurological symptoms is not 100% confirmed, the patient's clinical improvement and deterioration after an unintended challenge are supportive of a diet association. The re-challenge of the dog with gluten, documenting the recurrence of symptoms, would be the standard way to prove it. Nonetheless, it might not be strong enough to support such association, since the dog also presented symptoms after changing to another gluten-free diet (diet 5). Although the owner was convinced that the dog did not eat any other food besides the advised diet, food indiscretion cannot be entirely excluded, as it was observed at the beginning of the dog's diet trial (treat feeding with commercial pet food treats and human food). As stated before, the use of the serological tests (immunologic response against transglutaminase and gliadin) would have been a good option to ensure that the dog was being exclusively fed a gluten-free diet (6), however, since the dog was not tested before the start of the diet, testing the dog at this moment would be unreliable. Nonetheless, the increase in the fat intake from 3.1 to 3.5 g/kg$^{0.75}$ could have been the cause of the vomiting and subsequently, the associated stress (5, 28, 29, 36) could have triggered the neurologic signs. The same symptoms were also noticed when the dog was hospitalized with suspected pancreatitis, presenting a PD episode after remaining episode-free for 2 years. Although we have previously mentioned that this PD episode could have been due to a decrease in ASD efficiency, it is also possible that this episode was triggered by the

stress from the onset of gastrointestinal symptoms. This could be explained by the link between the nervous system and gut (gut brain-axis) (37, 38), and the possible interaction between those and the exocrine pancreas (39, 40). Finally, after a few months of neurological episodes remission, the owner was contacted again and reported the occurrence of short-lived episodes, but since the owner's commitment to keeping a diary was low, no associations could have been evaluated at this point.

Some important factors should be emphasized in the presented case report. Firstly, this patient was misdiagnosed and was long-term treated for epilepsy, increasing the risk of chronic pancreatitis (7–9, 20). Secondly, for patients with several medical conditions, nutrition management should be aimed at an individual basis, taking into consideration medical history, treatment, dietary history, and risk factors of each disease. In this case, after the removal of the CaOx stones, a general diet for uroliths prevention (diet 2) was advised by the primary veterinarian, which was not meeting all the criteria (key nutrients) for the prevention of pancreatitis and the treatment of obesity. Due to the patient's increased risk of pancreatitis, the use of an inadequate diet with a high-fat content (diet 2) was the possible cause of the gastrointestinal symptoms. Finally, although it has been hypothesized that gluten intolerance/allergy is associated with the onset of neurologic symptoms, this has not been proven yet. Other dietary proteins could be possible culprits as advised diets for PD patients are generally not only gluten-free but also hypoallergenic. In future investigations, it might be interesting to combine the use of serological tests (immunologic response against transglutaminase A2 and gliadin) with an elimination diet trial. The use of both diagnostic tools can lead to a better understanding of the etiology of the disease.

To the authors' knowledge, this is the first case report of PD in a German Spitz as well as the first PD case with a complete nutritional history and a long follow-up. Despite several limitations that a case report imposes, as well as the lack of information regarding the etiology of the disease, it was interesting to see the clinical improvement of this patient after nutritional management. This showed the importance of a precise nutrition anamnesis, a detailed nutritional evaluation of the previous diets, the continuous follow-up of the patient, the owner's compliance, and the adaptation of diet and/or nutrient intake for the prevention of clinical symptoms, especially for patients suffering from several medical conditions.

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.

Ethics statement

Ethical review and approval were not required for the animal study because this case report describes the clinical treatment of a privately owned animal. Written informed consent was obtained from the owners for sharing the videos and the description of this case.

Author contributions

CB was the veterinarian directly managing the nutrition issues of this case with NR-S's support. MHer was the veterinarian directly managing the neurologic symptoms of this case with FV's support. MHes and SB supervised and mentored CB. CB wrote the report with assistance and feedback from MHer, NR-S, FV, SB, and MHes. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2023.1054251/full#supplementary-material

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Rice protein concentrate is a well-accepted, highly digestible protein source for adult cats

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Introduction: The use of rice protein concentrate (RPC) as a protein source in cat food is uncommon. Therefore, this study aimed to determine the acceptability and digestibility of foods formulated to contain increasing levels of RPC to support its inclusion in foods for adult (non-gravid, non-lactating) cats.

Methods: Increasing levels of RPC (0, 7, 14, and 28%) were formulated into test foods fed to 24 cats in a Latin square design with 15-day periods and no washout between periods. Food intake and fecal scores were measured to determine the acceptability of test foods. Fecal output was measured on days 11–15. Food and fecal samples from day 15 of each period were analyzed for nutrient composition to calculate the macronutrient digestibility of the test foods. Analysis of variance and orthogonal contrasts were used to assess the effects of RPC inclusion on food intake, fecal output, fecal scores, and macronutrient digestibility.

Results: The results showed that as-fed (AF), dry matter (DM), and gross energy (GE) intake increased with increasing RPC levels ($p>0.05$). Fecal output, both as-is and DM, was unaffected by RPC inclusion ($p>0.05$); however, fecal scores increased linearly with increasing RPC inclusion ($p<0.001$). Furthermore, true protein and apparent DM, GE, and carbohydrate (NFE) digestibility increased linearly with RPC inclusion ($p<0.05$). Apparent fat digestibility was high for all test foods but was unaffected by RPC inclusion ($p=0.690$).

Discussion: Overall, the inclusion of RPC was well-accepted, improved fecal characteristics, and increased the apparent and true macronutrient digestibility compared to the control. Therefore, this study demonstrated that RPC can serve as a high-quality and acceptable protein source for adult cats.

KEYWORDS

macronutrient digestibility, rice protein concentrate, novel ingredient, feline, cat

1. Introduction

A consistent supply of good quality protein with uniform amino acid content is required to formulate high-quality foods designed to meet the nutritional needs of cats. To this end, sources of plant proteins, such as corn gluten meal, wheat gluten, pea protein, and potato protein, are already widely used and accepted in commercial cat foods (1). The inclusion of plant proteins in food can reduce the requirement for animal protein sources and may have a less negative impact on the environment (2).

From a clinical standpoint, cats with food intolerances, gastritis, enteritis, or other chronic conditions need a source of protein that is palatable, maximally digestible, and that they can tolerate (3). As a result, therapeutic foods for these indications often utilize alternative or hydrolyzed protein sources to reduce the likelihood of adverse food reactions (4–6). Since the digestibility of plant proteins has been observed to be comparable to that of animal proteins in feline foods (1), it is worth investigating the inclusion of concentrated plant proteins such as rice protein concentrate (RPC) in feline foods formulated for these conditions.

Rice protein concentrate is sourced from rice or rice brokens, which are co-products of milling husked rice to produce white rice. To prepare RPC, rice is first soaked in water and then ground; protein is then solubilized and insoluble starch is decanted. The protein is then recovered by precipitation and dried to form a concentrated protein powder (7). The resulting RPC is a high-quality protein source that provides an amino acid (AA) content comparable to that of other commonly used protein sources in pet foods (7, 8). Hydrolyzed rice protein and rice protein concentrate have been investigated and are currently used as alternatives to cow's milk or soy protein in infant formulas. They are considered to be safe, viable alternatives for infants with cow's milk protein allergy (9–11). Rice protein concentrate has also been evaluated as a protein source in the diets of weaned pigs and farmed fish, like rainbow trout and sea bass. These investigations report that RPC could be used as a replacement for other protein sources, such as dried whey and fish meal, without adverse effects on health or growth performance (12–16). These studies also highlighted an important limitation of RPC as a protein source, which is that it may be lacking in some essential amino acids, such as lysine, thus emphasizing the importance of including other protein or amino acid sources as needed to ensure that amino acid requirements are met.

However, information regarding the use of RPC in feline foods is limited. Although the digestibility of cat and dog foods containing RPC and other plant-based proteins has been investigated (1), the effects of increasing RPC levels on acceptability and digestibility in cats are unknown. This study aimed to determine the acceptability, metabolizable energy, and macronutrient digestibility of feline foods containing increasing levels of RPC. The digestibility of macronutrients in the RPC ingredient itself was estimated using regression analyses. It was hypothesized that the inclusion of RPC would not negatively affect the acceptability or macronutrient digestibility of the test foods.

2. Materials and methods

2.1. Humane consideration

This study was conducted with the approval of Hill's Pet Nutrition Institutional Animal Care and Use Committee (IACUC) and in accordance with Hill's Global Animal Welfare Policy. At no time was any animal subjected to procedures expected to cause pain or distress.

2.2. Animals

Cats of at least 1 year of age, fully grown, in good health, and with a known weight were considered for inclusion in this study. Cats were excluded from the study if they had been diagnosed with a chronic disease, including, but not limited to, kidney disease, cancer, hyperthyroidism, and diabetes. Cats enrolled in the study were subsequently removed from the study if they: (1) experienced excessive weight loss (>1.5% per week); (2) stopped eating for 2 days or ate less than 50% of the food offered for 3 days; or (3) were subsequently diagnosed with any secondary systemic disease as described in the 'exclusion criteria' mentioned above. All cats were immunized against Rabies (rabies), Felid alphaherpesvirus 1 (viral rhinotracheitis), Feline caliciviridae (feline calicivirus), and Feline panleukopenia (feline panleukopena virus). None of the cats had chronic systemic disease, as evaluated by annual urinalysis, serum biochemical analyses, complete blood count, and physical examination. The cats were housed individually, owned and maintained by Hill's Global Pet Nutrition Center, and treated in accordance with Hill's Global Animal Welfare Policy. While individually housed, the cats had access to group socialization and interaction with animal care technicians and toys. The study design did not interfere with the daily routines of the animals. The cats were housed in temperature-controlled facilities with access to natural light.

Twenty-six domestic shorthair cats (15 spayed females, 11 neutered males; 5.1 ± 0.9 kg body weight; 7.7 ± 2.2 years of age) were initially enrolled in this study, and a summary of their signalment is shown in Table 1. Two cats were removed from the study: one was removed because of a health concern that required an antibiotic regimen (deemed by the attending veterinarian to be unrelated to the test food), and the cat selected to replace the first cat was subsequently removed from the study after poor eating as outlined above. Thus, a total of 24 cats completed the study and were included in the statistical analysis. Following enrollment, the cats were fed once daily to maintain their ideal body weight based on the daily metabolizable energy requirements of adult cats at maintenance, calculated as $(70 \times BW^{0.75}) \times 1.2$, and the food intake was manually recorded each day by an animal care technician. Water was provided *ad libitum*.

2.3. Study design

This study used the American Association of Feed Control Officials (AAFCO) quantitative collection protocol (17) with a minimum of six adult cats. This protocol consists of two phases. The pre-collection phase lasts for at least 5 days and allows the cats to become acclimated to the test food. Food intake is then adjusted as needed to ensure that weight is neither gained nor lost. The next five-day phase is used for total fecal collection. Food offerings remain unchanged during the fecal collection phase (based on the amount needed to maintain weight from the earlier phase).

TABLE 1 Signalment for each panel of cats.

Signalment	Overall	By panel			
		1	2	3	4
Animals	26	6	6	6	8[1]
Male	11	3	2	4	2
Female	15	3	4	2	6
Body weight, kg	5.1 ± 0.9	5.5 ± 0.8	4.7 ± 0.4	5.1 ± 0.6	5.0 ± 1.3
Average age, yr	7.7 ± 2.2	7.5 ± 1.0	8.8 ± 3.2	8.0 ± 2.4	6.8 ± 1.3

[1]Includes two cats removed from the study whose data were not included in the statistical analysis.
Data are represented as absolute counts or as mean ± standard deviation.

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In this study, 24 adult male and female cats were grouped into four panels ($n = 6$ cats per panel) that were arranged in a 4 × 4 Latin square design. Each panel was fed each test food for 15 days with no washout between periods. Body weight (BW) was measured on days 1, 8, and 15 during each period. Fecal scores were recorded on days 10–15 using a 6-point scale (1 = liquid diarrhea, 2 = soft feces lacking form, 3 = soft, moist, but formed feces, 4 = firm, well-formed feces, 5 = hard, segmented feces, and 6 = constipation with no feces). Fecal scores of 3–5 were considered normal. Feces were collected from each cat individually using non-absorbent beads (Providence House Manufacturing, Inc., Seal Rock, OR, USA) in a litterbox and were weighed on days 11–15. Cats were individually fed once daily with access to food for approximately 22 h each day, and their food intake was recorded. Food and fecal samples from day 15 were analyzed for nutrient composition and subsequent calculation of digestibility.

2.4. Study foods

The study foods were manufactured using a Wenger X-115 extruder (Wenger Manufacturing, Inc.; Sabetha, KS, USA) according to the manufacturing procedures outlined in Supplementary Table 1. Test foods were formulated to meet or exceed AAFCO requirements for adult cats at maintenance, with the major ingredients and nutrient profiles of the study foods are summarized in Tables 2, 3, respectively. The analyzed nutritional composition of the RPC included in the test foods on a dry matter basis (DMB) is provided in Supplementary Table 2 and compared to grade A whole large eggs (18) as an indicator of protein quality.

2.5. Sample analysis

Measurements of ash, crude fiber, fat, protein, moisture, dry matter, and gross energy were completed for both food and fecal samples by a commercial laboratory (Eurofins Scientific, Inc., Des Moines, IA, USA) using official methods of analysis published by AOAC International (19).

TABLE 2 Select ingredients included in test foods containing increasing levels of rice protein concentrate.

	Rice protein concentrate inclusion			
Ingredient, %	0%	7%	14%	28%
Hydrolyzed chicken liver and heart	49.4	39.0	27.1	6.1
Rice, brewers	31.0	34.1	38.4	45.3
Rice protein concentrate	0.0	7.0	14.0	28.0
Taurine	0.2	0.2	0.2	0.2
Soybean oil	3.8	3.8	3.8	3.8
Coconut oil	2.4	2.4	2.4	2.4
Vitamin premixes[1]	0.4	0.4	0.5	0.4
Mineral premixes[2]	0.3	1.2	1.8	1.9

[1]Vitamin premixes included rice hulls as a carrier for this blend. The individual vitamin compounds included vitamin A, vitamin D, vitamin E, thiamine, riboflavin, pyridoxine, vitamin B12, ascorbic acid, niacin, pantothenic acid, folic acid, biotin, and beta-carotene.
[2]Mineral premixes included calcium carbonate as a carrier for this blend. The individual mineral compounds included ferrous sulfate, zinc oxide, copper sulfate, manganese oxide, calcium iodate, and sodium selenite.
Values are shown as percent of the total recipe. All test foods were formulated to meet or exceed AAFCO requirements for adult cats at maintenance (17).

The apparent digestibility of dry matter (DM), fat, gross energy (GE), and carbohydrates (nitrogen-free extract or NFE) was calculated as follows:

$$\text{Apparent digestibility}(\%) = \left[(\text{intake} - \text{fecal output}) / \text{intake}\right] \times 100 \quad (1)$$

To correct for endogenous metabolic fecal protein (i.e., fecal protein of non-dietary origin), an endogenous protein correction of 63 mg nitrogen/kg weight to the ¾ power suggested by Kendall et al. was used (20). This value is in the range of estimates for fecal metabolic protein for both the dog and cat (21, 22). Therefore, true protein digestibility was calculated by subtracting an estimate of the metabolic protein contained in the feces from the measured fecal protein concentration. This was calculated as follows:

$$\text{True protein digestibility}(\%) = \left\{\left[\text{protein intake} - (\text{fecal protein} - \text{endogenous metabolic protein})\right] / \text{protein intake}\right\} \times 100 \quad (2)$$

2.6. Statistical analysis

An investigation revealed an error in the fat analysis of the 28% RPC food sample collected during the first period of the study (3.1% crude fat compared to the mean of 12.2% crude fat from the three samples of the 28% RPC food collected during the other three periods of the study). This resulted in an artificial reduction in the subsequent calculation of apparent fat digestibility for that period. The error could not be rectified because the sample had already been destroyed. Therefore, data regarding fecal fat content from the first period for the panel consuming 28% RPC were removed from the analysis and were not included in the apparent fat digestibility calculations, as they were >3 standard deviations from the mean. All remaining data were analyzed using analysis of variance (ANOVA) of a linear model in R (v. 4.0.3) (23), including the fixed effects of diet, period, panel, and all associated interactions. The fixed effect panel was not significantly different ($p > 0.05$) and was removed from the model along with all associated interactions. Orthogonal contrasts were used to determine the linear, quadratic, or cubic relationships. The macronutrient digestibility of 100% RPC was predicted using linear regression analysis. The effects were considered significant when $p \leq 0.05$. The results were presented as mean ± standard deviation or standard error, as appropriate.

3. Results

3.1. Food intake and fecal characteristics

The mean food intake and fecal characteristics are presented in Table 4. Neither the period nor the interaction of treatment by period affected body weight, food intake or fecal characteristics ($p > 0.05$). Mean food offered (63.4 ± 5.1 g as-fed/d) and BW (5.1 ± 0.3 kg) were

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TABLE 3 Analyzed nutrient composition and energy content of test foods containing increasing levels of rice protein concentrate (RPC) on a dry matter basis.

			Rice protein concentrate inclusion			
Nutrient	AAFCO minimum[1]	Unit	0%	7%	14%	28%
DM		%	90.2	92.3	92.1	93.3
Calories		kcal/kg	4,542	4,960	4,828	4,718
Protein (crude)	26.0	%	32.1	33.4	30.8	33.7
Fat (crude)	9.0	%	19.5	19.0	16.0	12.2
Fiber (crude)		%	2.1	2.7	2.4	3.0
Total dietary fiber		%	5.2	5.3	5.2	5.2
Methionine + cysteine	0.40	%	1.0	1.3	1.2	1.4
Phenylalanine + tyrosine	1.53	%	2.5	2.7	2.6	3.0
Arginine	1.04	%	1.8	2.2	2.1	2.5
Histidine	0.31	%	0.7	0.8	0.7	0.8
Isoleucine	0.52	%	1.3	1.5	1.3	1.2
Leucine	1.24	%	2.8	2.8	2.5	2.6
Lysine	0.83	%	2.2	2.1	1.7	1.4
Threonine	0.73	%	1.4	1.4	1.3	1.2
Tryptophan	0.16	%	0.4	0.5	0.5	0.5
Valine	0.62	%	1.7	1.9	1.7	1.8
Taurine	1,000	ppm	4,000	4,000	3,200	2,400
Calcium	0.6	%	0.8	0.8	0.8	0.8
Phosphorous	0.5	%	0.8	0.8	0.8	0.7
Potassium	0.6	%	0.9	1.0	0.9	0.8
Sodium	0.2	%	0.4	0.4	0.4	0.3

[1]All test foods were formulated to meet or exceed AAFCO requirements for adult cats at maintenance (17).

similar between treatments ($p = 0.278$ and 0.365, respectively). As-fed (AF), DM, and GE intakes increased with RPC inclusion compared to control ($p = 0.040$, 0.017, and 0.040, respectively) but were similar between RPC inclusion levels ($p = 0.880$, 0.957, and 0.879, respectively). Fecal output, both as-is and DM, was unaffected by the inclusion of RPC ($p = 0.267$ and 0.685, respectively). With increasing RPC inclusion, fecal moisture decreased linearly ($p = 0.001$), whereas fecal scores increased linearly ($p < 0.001$). However, all mean fecal scores were considered normal for all treatments.

3.2. Macronutrient digestibility

The apparent macronutrient digestibility and true protein digestibility of test foods with increasing levels of RPC are presented in Table 5. Neither the period nor the interaction of treatment by period affected digestibility of any macronutrients ($p > 0.05$). Except for apparent fat digestibility ($p = 0.690$), the digestibility of all macronutrients increased linearly with increasing RPC inclusion ($p < 0.05$).

3.3. Predictions of digestibility of RPC

The test food recipes were designed with increasing levels of RPC (0, 7, 14, and 28%), with adjustments to the amount of hydrolyzed chicken liver and heart, and rice in the recipe to create test foods with similar levels of total protein. This allowed a prediction to be made regarding the apparent macronutrient digestibility of 100% RPC (Table 6). The predicted value in adult cats was high, with predicted dry matter digestibility above 85%, predicted fat digestibility above 90%, and predicted protein and NFE digestibility above 95%. Since there was no effect of RPC on apparent fat digestibility, the R^2 for predicted apparent fat digestibility was low ($R^2 = 0.07$), indicating it is a poor model for estimating the apparent fat digestibility of 100% RPC.

4. Discussion

4.1. Characteristics of RPC

Rice protein concentrate can be considered a high-quality protein source because it is an excellent source of essential AAs. Compared to Grade A large whole eggs, which may be considered the gold standard in protein quality and digestibility for both human and pet nutrition (8, 24), the nutritional composition of the RPC used in this study meets or exceeds the DM AA content of whole eggs in all but two AAs: lysine and cysteine (10). It is common for plant-based proteins to be deficient in at least one essential amino acid (8, 25), further emphasizing the importance of ensuring that foods utilizing plant proteins such as RPC are formulated to be complete and balanced, which has been recommended when

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TABLE 4 Mean intake, fecal output, and fecal scores of cats consuming increasing levels of rice protein concentrate.

	Rice protein concentrate					*p*-Value					
Item	0%	7%	14%	28%	SE	Treatment	Linear	Quadratic	Cubic	Period	Treatment × period
Body weight, kg	5.1	5.1	5.1	5.1	0.27	0.365	0.053	0.108	0.371	0.781	0.808
Intake											
Intake, g AF/d[1]	54.0[a]	58.8[b]	60.7[b]	61.1[b]	1.01	0.040	0.880	0.104	0.974	0.618	0.129
Intake, g DM/d	49.2[a]	54.7[b]	55.9[b]	57.2[b]	1.04	0.017	0.957	0.082	0.717	0.118	0.124
GE Intake, kcal/d	270.3[a]	294.0[b]	302.1[b]	305.6[b]	5.05	0.040	0.879	0.104	0.974	0.142	0.129
Output											
Fecal output, g as-is/d	40.6	39.4	36.2	34.5	1.28	0.267	0.171	0.743	0.816	0.267	0.103
Fecal output, g DM/d	13.9	14.2	13.1	13.1	0.40	0.685	0.540	0.819	0.518	0.316	0.809
Fecal moisture, %	65.7[a]	63.8[b]	63.7[b]	61.9[c]	0.40	0.001	0.002	0.852	0.534	0.402	0.811
Fecal score	3.9[a]	4.2[b]	4.3[b,c]	4.6[c]	0.08	<0.001	<0.001	0.274	0.087	0.720	0.088

[1]AF = as fed; DM = dry matter; GE = gross energy.
[a-c]Means within a row with different letters indicate a difference at $p \leq 0.05$.
Fecal scores were determined using a 6-point scale (1 = liquid diarrhea, 2 = soft feces lacking form, 3 = soft, moist, but formed feces, 4 = firm, well-formed feces, 5 = hard, segmented feces, and 6 = constipation with no feces). Fecal scores of 3–5 were considered normal.

TABLE 5 Total tract apparent and true macronutrient digestibility in cats consuming increasing levels of rice protein concentrate.

	Rice protein concentrate					*p*-Value					
Digestibility, %	0%	7%	14%	28%	SE	Treatment	Linear	Quadratic	Cubic	Period	Treatment × Period
Apparent DM	80.1[a]	82.7[b]	84.3[b]	85.1[b]	0.62	0.008	0.029	0.478	0.845	0.678	0.465
True protein	90.3[a]	92.4[a,b]	93.7[b]	95.8[c]	0.61	0.003	0.018	0.815	0.536	0.676	0.462
Apparent fat	91.0	91.0	91.7	89.8	0.34	0.289	0.690	0.193	0.231	0.228	0.989
Apparent carbohydrate (NFE)	83.5[a]	89.0[b]	90.5[b,c]	92.8[c]	0.97	< 0.001	<0.001	0.131	0.135	0.834	0.256
Apparent GE	83.6[a]	86.0[b]	87.0[b]	87.8[b]	0.51	0.008	0.038	0.364	0.622	0.625	0.571

[a-c]Means within a row with different letters indicate a difference at $p \leq 0.05$.

other plant protein sources have been investigated for use in companion animal foods (25). For example, all four test foods in this study were supplemented with taurine at the same level (0.2% of the recipe), but inclusion of brewers rice and RPC in test foods (at the expense of hydrolyzed chicken liver and heart) reduced the taurine content of the 14 and 28% foods as neither brewers rice nor RPC contains no taurine. However, all AA levels in these test foods, including taurine, exceeded the minimum recommended allowance of the National Research Council (26) and the AAFCO minimum (17) for adult cats at maintenance as a result of the supplemental taurine added to the test foods.

4.2. Food intake and fecal characteristics

Overall, the study foods were well-accepted by all cats. While the amount of food offered was similar between treatments, AF and DM intakes increased with RPC inclusion compared with those in the

TABLE 6 Prediction of apparent macronutrient digestibility of 100% rice protein concentrate.

Nutrient	Linear equation	SE	Adjusted R^2	Predicted digestibility, %
Dry matter	$Y = 83.03 + 0.038x$	1.61	0.57	86.8
True protein	$Y = 93.01 + 0.040x$	1.44	0.65	97.0
Fat	$Y = 90.89 - 0.0066x$	1.25	0.07	90.2
Carbohydrate	$Y = 88.94 + 0.065x$	1.84	0.78	95.5

control food, which resulted in greater gross energy intake when cats were fed RPC-inclusive foods. Since cats were fed to maintain their ideal body weight, this may indicate a preference for foods with higher levels of RPC, which may be of particular interest because cats are often picky eaters (27). However, the increased energy intake did not translate into an increase in BW. This was most likely a result of the short experimental period (15 d). A similar effect was reported by Detweiler et al. (28), who reported lower food intake but no change in BW when cats were fed an experimental food formulated with beet pulp as the main source of dietary fiber. One notable limitation of the current study is that neither body condition score nor muscle condition score were assessed. It would be prudent in future work to include such measures in addition to BW in order to assess the potential of RPC to affect lean body mass or fat mass. The increase in intake also did not translate into an effect on fecal output (for as-is or DM). These data were consistent with the increase in DM digestibility with increasing RPC inclusion in the test foods. The fecal scores, while considered normal (stool scores of 3–5 on a 1–6 scale) for all test foods throughout the study, increased linearly with increasing RPC inclusion, which is consistent with the corresponding decrease in fecal moisture. The increased fecal score was likely due to high digestibility, reduced hydrolysate content, and slightly higher crude fiber content in the recipes containing RPC. While an increase in dietary fiber is associated with increased fecal output (29–31), it is likely that the fiber differences in these test foods were not substantial enough to impact as-is fecal output as seen in these other studies.

4.3. Macronutrient digestibility

All study foods were highly digestible, with the digestibility of all macronutrients above 80%. Except for apparent fat, the digestibility of all macronutrients increased linearly with increasing RPC inclusion. Apparent fat digestibility was high (>90%) in all test foods and was unaffected by RPC inclusion level, though this may be a result of the error in food sample analysis that resulted in the removal of one 28% RPC period's apparent fat digestibility data. This can be avoided in future work through the closer inspection of results and retention of samples to re-analyze if necessary. While the predictive equation for apparent fat should not be used due to the absence of an effect of RPC on apparent fat digestibility as well as the poor fit of the linear regression predictive equation for fat digestibility, the predicted macronutrient digestibility of 100% RPC was high for all other macronutrients (above 85% for DM and above 95% for both protein and carbohydrate). The hydrolyzed chicken ingredient was specifically chosen as the other primary protein source because hydrolyzed meat ingredients are considered highly digestible protein sources and are commonly used in foods for cats with food sensitivities as proteins with lower molecular weight (< 10,000 daltons) are less likely to elicit an immune response (32). Thus, it is interesting that replacing the hydrolyzed chicken ingredient with RPC resulted in higher protein digestibility. However, all test foods, including the control that had the hydrolyzed chicken ingredient as the primary protein source, demonstrated true protein digestibility above 90%. These results do not indicate that hydrolyzed meat ingredients are poorly digestible, but rather that molecular weight of an ingredient is not the only factor determining the digestibility of a protein source. These data are consistent with previous investigations on the digestibility of plant and animal proteins in feline foods, in which protein digestibility in cats increased with the percentage of dietary protein from plant-based sources (1). As with AA content of the ingredient, RPC may also be comparable to whole eggs in terms of protein digestibility. Previous work has shown that when used in dog food at 20% of total crude protein, an egg-inclusive food had a total tract apparent protein digestibility of 91.2% (33).

In conclusion, adult cats were fed foods containing increasing levels of RPC up to 28% of the total recipe, and it was demonstrated that RPC could serve as a protein source in cat foods owing to its high digestibility and protein quality. Overall, the inclusion of RPC was well-accepted by cats, improved fecal characteristics, and increased the apparent and true macronutrient digestibility compared with the control food that did not contain RPC. Thus, RPC may serve as a complementary protein source in feline foods considering its AA content, good digestibility, and excellent taste acceptance, all of which make it appropriate for use in therapeutic foods indicated for cats with food sensitivities. Future directions include the investigation on the effect of RPC inclusion on lean body mass in 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 Hill's Pet Nutrition Institutional Animal Care and Use Committee.

Author contributions

KG and SP: conceptualization. KG, SP, and CS: methodology, writing—review and editing. EM: formal analysis, project administration, and writing—original draft preparation. KG and CS: investigation. EM and KG: data curation. KG: supervision. All authors contributed to the article and approved the submitted version.

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Conflict of interest

EM, SP, and CS are employed by Hill's Pet Nutrition, Inc. KG is a former employee of Hill's Pet Nutrition, Inc. The authors declare that this study received funding from Hill's Pet Nutrition, Inc. The funder was provided an opportunity to review the manuscript for appropriate animal welfare documentation but was not involved in the study design, collection, analysis, interpretation of data, the writing of this article, or the decision to submit it for publication.

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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.2023.1168659/full#supplementary-material

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Determining the protocol requirements of in-home cat food digestibility testing

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In-home cat food digestibility testing has the potential to yield data that are highly representative of the pet population for which the food is intended. However, no standardized and validated in-home digestibility test protocols are currently available. Such protocols for in-home testing should address key factors that explain variation in cat food digestibility values and here we investigated the required period of adaptation, fecal collection and sample sizes. Thirty privately-owned indoor housed cats of various breeds (20♀ 10♂, 5.9±3.9 yr, 4.5±1.3 kg) received a relatively low and high digestible complete dry extruded food with the marker titanium (Ti) dioxide. Foods were given in a cross-over design of 2 periods of 8 consecutive days each. Owners collected feces daily for the determination of daily fecal Ti concentrations and digestibility of dry matter, crude protein, crude fat, and gross energy. Data originating from 26 cats were analyzed as mixed models and broken line regressions to investigate the required adaptation and fecal collection period. Bootstrap sampling was used to assess the impact of increasing the number of fecal collection days and sample size on the precision of the digestibility estimates. Feces were collected on 347 out of 416 study days (16 days/cat; 26 cats), implying the necessity for multiple collection days to account for cats not defecating every day. Cats showed stable fecal marker concentrations from day 2 onwards when fed the low digestible food and from 3 onwards when fed the high digestible food. Digestibility values were stable from day 1, 2 or 3 onwards, depending on the test food and nutrient. Increasing the number of fecal collection days from 1 to 6 days did not result in more precise digestibility estimates, whereas increasing the number of animals from 5 to 25 cats did. For future in-home digestibility tests of cat food, the findings support a minimum of 2 adaptation days and 3 fecal collection days. Appropriate sample sizes depend on the test food, the nutrient of interest, and the acceptable margin of error. The findings of this study support the protocol development for future in-home digestibility testing of cat foods.

KEYWORDS

adaptation, fecal collection, sample size, protocol requirements, in-home test

1. Introduction

Commercial cat foods are the main source of nutrients of Western pet cats (1, 2), making the nutritional quality of such foods of paramount importance. To evaluate the quality of formulations, ingredients, and processing technologies, pet food companies routinely conduct digestibility testing especially on the final product. Digestibility testing of cat foods is almost exclusively conducted at dedicated facilities that employ a limited number of cats housed under

standardized conditions. The latter differs greatly from the actual living conditions of cats in households, making the results on food digestibility being less representative for the pet cat population for which the foods are intended. Findings are expected to be more representative if these are obtained with pet cats housed at their home environment, i.e., 'in-home' (3, 4). In-home testing can not only provide information on nutrient digestibility in cats with different characteristics (e.g., sex, neuter status, age, breed, food history) and living conditions (e.g., housing, eating pattern, activity), but also on important parameters related to food quality as perceived by owners. Protocol requirements for in-home cat food digestibility testing, such as study duration (adaptation and fecal collection) and study population (number of cats and cat characteristics) likely differ from those commonly used (5, 6) in dedicated facilities. For example, the increased variety in test subjects and relatively uncontrolled test conditions will impact the precision of the determined digestibility values.

The current protocol requirements for experimental studies on cat food digestibility provided by AAFCO (5) and FEDIAF (6) include a minimum of six healthy, fully grown cats over 1 year of age, individually housed for 10 days with 5 days of adaptation and 5 days of fecal collection. The required length of the adaptation period to assess digestibility values may vary between nutrients and energy. Digestibility values of four cats measured over days 4–7, 8–14 and 15–21 (pooled faecal samples per cat) were similar for dry matter (DM), energy, fat and N-free extract (7). A longer adaptation period is, however, supported by differences for crude protein digestibility and wet feces output for days 8–14 compared to days 15–21 (7). The optimal period of adaptation warrants further investigation as in a recent study with dogs ($n = 53$) it was found that a 1-day adaptation period suffices to reach constant digestibility values (8). The digestive system adapts rapidly and digestive enzyme activity and microbiota composition changes within a few hours after changes in the amount and type of dietary protein, carbohydrate or lipid (9–11). Having several fecal collection days allows to smooth out day-to-day variation in fecal composition, and the pooling of fecal samples of pigs has been shown to result in more precise digestibility estimates (12). The day-to-day variation in digestibility estimates did not show in the group of pet dogs and the inclusion of multiple days did not increase the precision of digestibility estimates (8).

The use of an indigestible marker is a more practical approach than quantitative feces collection when conducting in-home digestibility studies (3). In the case an indigestible marker is used, the adaptation period is not only important to allow the animal's digestive system to adapt to the test food (9), but also to ensure a constant marker excretion in the feces (13). The time to reach the latter is determined by the gastrointestinal transit time, which might vary between cats in-home. Even when controlling for diet, environment and genetic background, individual cats show high variability in gastrointestinal transit time (14) and vary between young (26.5 ± 5.8 h) and older cats (35.7 ± 14.1 h) (15), and between fasted (28.9 h, range 18.4–90.9 h) and fed state (46.6 h, range 15.4–109.4 h) (14). Housing conditions, meal size, and meal frequency are all likely to impact gastrointestinal transit time as these influence gastric emptying rate (16, 17). As in-home digestibility testing is conducted under less controlled conditions, the greater variability compared to testing in dedicated feline facilities would require a larger number of cats to achieve the same level of precision as obtained with six cats prescribed within the AAFCO (5) and FEDIAF (6) protocols. Factors such as age, body condition, food history, feeding level and owner (non) compliance may impact on digestibility estimates (18–27), and in a heterogenous study population of pet cats such factors raise variation in food digestibility estimates. The consequences for minimal sample sizes for in-home studies are yet to be determined.

The present in-home study assesses the degree of variation in food digestibility values in privately-owned cats across 8 days, with the aim to determine the minimal period of adaptation and fecal collection, as well as the required number of cats. The findings support protocols for in-home cat food digestibility testing.

2. Experimental methods

2.1. Study design

In-home digestibility tests were conducted with privately-owned cats in which owners fed their cat two different dry extruded foods and collected their cat's feces on a daily basis. A cross-over design was used with two consecutive 8-day feeding periods and the start food was alternated across participants based on the order of entering the study (i.e., owner 1 Food A, owner 2 Food B, owner 3 Food A, owner 4 Food B, etc.). Participation in the study occurred from February to May 2021 and participants started the in-home digestibility study on different days during this period.

The study was approved by the Animal Welfare Body of Wageningen University (Wageningen, Netherlands) and did not qualify as an "animal experiment" according to the Dutch Experiments on Animals Act (2014). An inform consent was signed by the cat owners before the start of the study and the surveys used in this study adhered to the guidelines for privacy and data handling of Wageningen University & Research. The completion of surveys did not interfere significantly with normal daily life of the cat owners, and the questions were not psychologically burdening, thereby, exempting the surveys from approval by the ethics committee according to the guidelines of Wageningen University Medical Ethics Review Committee (Medisch Ethische Toetsingscommissie van Wageningen University, METC-WU).

2.2. Participants

Dutch cat owners were recruited through online advertisements. Candidate participants completed an online questionnaire (Microsoft Forms) on predominantly demographics and cat characteristics. Cat owners were eligible when they were willing to provide the test food as the sole source of nutrition to their cat, collect the cat's feces and control both its individual food intake and place of defecation through the use of a dedicated litterbox. Mainly owners of single- and indoor-housed cats met these criteria. Cats were eligible when older than 1 year, not pregnant or lactating and healthy (no medication, no intestinal upsets over the past 3 months, free of previously diagnosed chronic diseases, no food allergies or intolerances). Cats described by the owner as a "difficult eater" received a sample of the test food (1 daily portion) prior to the study and entered the study only when readily accepting the test food. Owners rated their cat's pickiness in the online questionnaire on a

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5-point scale from a very difficult eater (score 1) to and an easy eater (score 5) with scores <3 being identified a difficult eater. The cat owners that satisfied the inclusion criteria entered the study and the number of cats entering the study was determined by the available resources needed to run the study (e.g., time, funding, logistics, sample processing, chemical analyzes).

The participants received a brochure containing information about the study and received further explanation in person about how to perform the tasks during a visit when materials were delivered. The latter included daily food portions, lime and quartz sand cat litter (Catsan Hygiëne Plus, Catsan™), feces collection bags, feces information bags, a freezer container, a mini freezer (Primo DV2-WS, Primo Elektro, Herentals, Belgium) if requested, and a diary. The diary included the Waltham Feces Scoring Chart (28) and owners were instructed on how to score feces for consistency. During the study, owners were contacted by the researcher at least once by email and could contact the researcher by email and/or phone any time.

2.3. Foods and feeding

The two test foods were commercial dry extruded cat foods (Jonker Petfood BV, Waalwijk, Netherlands) that differed in ingredient quality and inclusion and in nutrient composition such that it established a contrast in nutrient digestibility. Food formulations (Table 1) met the nutritional guidelines of FEDIAF for adult cats (6) and included TiO_2 (Hombitan FG, Venator Germany GmbH, Duisburg, Germany) as an indigestible marker. The particle size distribution of TiO_2 was determined by the Mastersizer 3,000 (Malvern Panalytical BV, Almelo, Netherlands) and was devoid of nanoparticles [<100 nm (29)]. Dry ingredients and the marker were mixed for 60 s in a paddle shift mixer (Forberg F60, Forberg International AS, Oslo, Norway), followed by extrusion using a co-rotating double screw extruder (Baker Perkins MF50, Baker Perkins, Manor Drive, United Kingdom), oven-drying at 45°C overnight, vacuum coating with pre-heated (60°C) poultry fat and liquid digest, and mixing (Dinnissen 305, Dinnissen BV, Sevenum, Netherlands) with powder digest at the research facilities of Wageningen University & Research (Wageningen, Netherlands).

The foods were formulated to contain either 16.0 MJ/kg ME (food A) or 14.2 MJ/kg ME (food B) and were fed at maintenance energy requirements (314 kJ × kg $BW^{0.67}$; FEDIAF, 2021). Feeding levels were discussed with the owner prior, or when requested, during the study and adjusted where appropriate. The cats were fed following the feeding schedule they were used to (i.e., meals or *ad libitum*), but were all instructed to start the study in the morning on the first day. Cat owners were provided with daily food portions, instructed to only provide the test food to their cat, and to carefully collect and store leftovers each day. Water was instructed to be provided *ad libitum*.

TABLE 1 Ingredient composition and analyzed chemical composition and energy contents of the relatively high (Food A) and low (Food B) digestible dry extruded cat foods.

Component	Food A	Food B
Ingredient composition (g/kg)		
Wheat	–	350
Wheat-semolina	–	150
Rice	260	–
Linseed oil	5.00	–
Beet pulp	30.0	–
Poultry fat	109	57.9
Salmon oil	10.0	–
Digest	30.0	7.49
Fish meal	310	–
Greaves	–	140
Meat bone meal	–	120
Premix	14.0	12.0
Barley	–	100
Keratin protein	–	50.0
Potato	184	–
Choline chloride	1.00	2.50
Fibers	5.00	10.0
Rice protein	41.5	–
Titanium dioxide	1.00	1.00
Chemical constituents (g/kg as-is)		
Dry matter	931	937
Crude ash	65.4	63.1
Organic matter	935	937
Nitrogen	49.3	47.4
Crude protein	308	297
Crude fat	109	149
Starch	348	263
Total dietary fiber	103	191
Titanium	0.55	0.53
Energy (MJ/kg as-is)		
Gross energy	19.5	20.5
Metabolizable energy*	14.3	13.4

*Calculated using NRC, 2006 (66).

2.4. Feces and data collection

The cat owners were asked to daily collect their cat's feces from the litter box with as little contamination as possible. Due to feasibility reasons, owners collected feces mostly once a day. The collection might not have been directly after their cat's defecation, resulting in fresh and non-fresh feces samples being collected within a 24 h timeframe each day. Single feces samples were collected with the collection bag and placed in an information bag with a label containing the cat's name and the collection date and time. Storage occurred at −18°C in a freezer of the owner or one that was temporarily provided. Feces were translocated to Wageningen University & Research within 2 weeks after an owner had completed the study and stored at −20°C pending further processing and chemical analyzes.

Cat owners were requested to fill in a daily diary providing information on consumption of the test foods and (accidental) other items, as well as feces characteristics (number of defecations; feces consistency score according to the Waltham Feces Scoring Chart (28),

with score 1 indicating hard and dry feces and score 5 indicating watery diarrhea; additional particularities).

2.5. Chemical analyzes and calculations

Feces were pooled per day (including 24 h post first food consumption), resulting in maximum eight samples per cat per period/food. Feces were oven-dried at 60°C to reach a constant weight. Water loss after oven-drying was not used to calculate dry matter of the fresh feces as cat litter was still included, which renders such data inaccurate. After drying, all visible cat litter granules and cat hairs were manually removed and the cleaned feces were ground to pass a 1-mm sieve in an ultra-centrifugal mill (ZM100, Retsch B.V., Ochten, Netherlands). Fecal samples were analyzed by near-infrared reflectance spectroscopy (NIRS; Anadis Instruments Benelux BV & Nirvention BV, Almere, Netherlands), with the NIRS being calibrated by chemical analyzes of a subset of 50 feces samples obtained in this study. Samples of feces and foods were analyzed in duplicate for dry matter (30) (DM), nitrogen (31) (N), crude fat (32) (Cfat), and gross energy (33) (GE). Crude protein (CP) has been calculated as N × 6.25. Food samples were also analyzed for total dietary fiber (34). Ti concentrations in foods and all fecal samples were determined using inductively coupled plasma-optical emission spectrometry (ICP-OES, Iris intrepid II XSP, Thermo Fisher Scientific, Inc.) after destruction with H_2SO_4 using a microwave digestion system (MARS 6, CEM Corporation, Matthews NC, Unites States).

Apparent fecal nutrient digestibility was calculated as described elsewhere (5, 6):

$$Nutrient\ digestibility\,(\%) = 100 - \frac{[Nut_{feces}] \times [Ti_{food}]}{[Nut_{food}] \times [Ti_{feces}]} \times 100\%$$

where Nut_{feces}, $Nut_{food,}$ Ti_{feces} and Ti_{food} are the nutrient content (% DM) and Ti content (% DM) of feces and food, respectively.

2.6. Data processing and statistical analyzes

Negative digestibility values, which were predominantly observed during day 1, were omitted from the dataset (n = 49 out of 1,648 records). To model how nutrient digestibility values varied with the inclusion of a different number of fecal collection days, the digestibility values across subsequent fecal collection days were averaged. A new dataset was created including calculated pooled digestibility values per cat and food across 2 to 6 collection days.

All data were statistically analyzed using SAS (v. 9.4; SAS Institute, Cary, NC). The plateau (day) of time functions of fecal Ti concentrations as well as digestibility values were estimated with breakpoints in linear broken-line regressions (35) using the NLIN procedure. The broken-line function was as follows:

$$Y = a + b \times x - b \times 0.01 \times \log\left(1 + e^{(c-x)/0.01}\right)$$

where Y is the dependent variable, a is the starting value when $x = 0$, x is the day, b is the slope when $x < c$, and c is the breakpoint. Parameters of the broken-line function were estimated for the study cat population as well as per individual cat, and separately for each feeding period (1, 2) and for each food (A, B). These separate analyzes were considered more accurate and informative as feeding periods 1 and 2 differed in the food that cats received before the period started, with the owner's choice of food in period 1 (no Ti) vs. the experimental food in period 2, and this was expected to show in the starting values of fecal Ti concentrations and digestibility values. Due to missing values (e.g., because a cat did not defecate every day), the estimation of individual breakpoints in broken-line functions was not possible for every cat during each period and/or food and these cats were omitted from the dataset.

The required lengths of the adaptation and fecal collection periods were also determined from time-dependent variation in fecal Ti concentrations and digestibility values as assessed by a repeated measures ANOVA using the Proc MIXED procedure. Again, time effects were analyzed separately for each feeding period (1, 2) and for each food (A, B). Day was used as a REPEATED model statement (36) using a first-order autoregressive covariance structure [AR(1)] (37) and the model:

$$Y = \mu + D_i + \varepsilon_i$$

where Y is the dependent variable, μ is the average intercept, D_i is day i, and ε_i is the error term. Differences were considered significant at a probability <0.05, with posthoc pairwise comparisons were analyzed using the Tukey test. The consequences for the precision of digestibility estimates of the number of fecal collection days in combination with sample sizes were assessed using bootstrap sampling, including 10,000 replicates.

The effects of experimental factors (food and period) and cat characteristics on digestibility values were analyzed by repeated measures ANOVA in Proc MIXED, using an entire dataset across feeding periods and foods, but including only the study days after a constant marker excretion in the feces was reached (on cat population level). The statistical model was optimized for explaining variance in a dependent variable (DM, CP, Cfat, and GE digestibility) by using stepwise regression with the GLMSELECT procedure and the Schwarz Bayesian information criteria. The independent variables were the test food (A, B), period (1, 2), sex (female, male), neuter status (intact, neutered), age, weight and the two-way interactions between any two variables.

3. Results

3.1. Participants and owner compliance

This in-home digestibility study was started with 30 cats from 29 owners (20♀ 10♂, 5.9 ± 3.9 yr, 4.5 ± 1.3 kg; Supplementary Figure S1), of which 27 cats from 26 owners completed the study. Dropouts included cats that did not eat both foods ($n = 2$) and one owner who did not comply to the study protocol ($n = 1$). Four owners indicated that their cat had to get used to the provided cat litter and two of those switched back to their own cat litter during the study.

The owners reported that other items than the test food were consumed by 9 cats on 17 study days out of the total 432 records from 27 cats, including human food ($n = 4$ cats, on 5 days), cat food ($n = 5$

cats, on 6 days), cat treats ($n = 2$ cats, on 4 days) and dog food ($n = 1$ cat, on 2 days). Fecal samples were not available for 76 days, mainly because of no defecation ($n = 72$) and due to non-compliance ($n = 4$ days, by two owners).

Two fecal samples of one cat were too contaminated with cat litter and were excluded from chemical analyzes. Data for one cat were rejected for reasons of deviating fecal Ti concentrations (defined as >2× std. dev from mean) and suspected non-compliance of the cat owner, rendering data on 26 cats for statistical analyzes (16♀ 10♂, 5.5 ± 4.0 yr, 4.4 ± 1.2 kg; Supplementary Table S1).

3.2. Food transitions

The transitions between foods, from a cat's usual food to one of the two experimental foods and between experimental foods, caused minimal digestive discomfort in the cats. Vomiting was reported by owners on day 1 ($n = 1$; Food B), 5 ($n = 1$; Food B), 7 ($n = 1$; Food B), and 8 ($n = 1$; Food A). Extreme fecal consistency scores were rare (scores 1 or 5, $n = 6$), scores of 1.5 were recorded 109 out of 453 times, and scores 4.5 were not reported.

3.3. Variation in fecal Ti concentrations across days

Ti concentrations in the feces stabilized around the third day after first food consumption. The breakpoints in the fecal Ti concentration of all cats fed Food A were at day 3.17 ($n = 12$) for period 1 and 2.81 ($n = 14$) for period 2 (Figure 1). For all cats fed Food B, the breakpoints were estimated at day 2.28 ($n = 14$) for period 1 and 3.14 ($n = 12$) for period 2. The breakpoints for the individual cats ranged between 1.81–4.05 (Food A, period 1, $n = 10$), 2.07–3.42 (Food A, period 2, $n = 14$), 2.04–4.07 (Food B, period 1, $n = 12$) and 2.03–3.23 (Food B, period 2, $n = 9$) (Figure 1). The findings were supported by repeated measures analyzes of variance on the same data sets, which showed that fecal Ti concentrations in cats fed Food A (in both feeding periods) increased from day 1 to 2 ($p < 0.001$) and from day 2 to 3 ($p < 0.05$), with no differences from day 3 onwards (Figure 1). Fecal Ti concentrations in cats fed Food B differed from day 1 to 2 in both feeding period 1 ($p < 0.001$) and 2 ($p < 0.05$). For cats fed Food B there were no significant differences already from day 2 onwards, during both feeding periods, but in period 2 fecal Ti concentrations on day 2 tended to differ from those on days 3 ($p = 0.077$), 4 ($p = 0.052$), 7 ($p = 0.065$) and 8 ($p = 0.056$).

3.4. Variation in fecal apparent digestibility values across days

Similar patterns over time were present for apparent fecal digestibility values (Figure 2) to those of fecal Ti concentrations. The broken-line analyzes on the daily averages per food and period resulted in breakpoints ranging from 3.15 ± 0.36 for CP (Food A, period 1) to 3.63 ± 0.87 for Cfat (Food A, period 1; Table 2), except for Cfat digestibility values of cats fed Food B (breakpoint of 4.58 ± 0.92). The outcomes of the repeated ANOVA of the same datasets were in line with those of the linear broken-line regressions. Digestibility values for cats fed Food A in period 1 were constant from day 3 onwards for CP (Figure 2), DM and GE (Supplementary Figure S2), with no day effects for Cfat (Supplementary Figure S2). During period 2, digestibility values of cats fed Food A were constant from day 2 onwards for Cfat and from day 3 onwards for DM, CP and GE. Digestibility values for cats fed Food B were constant from day 2 onwards for period 1. In period 2, this was from day 1 onwards for CP, from day 2 for GE and from day 3 for DM and Cfat.

3.5. Number of fecal collection days and sample size

The use of multiple subsequent days, as opposed to fecal samples of day 3 only, did not decrease variation in the digestibility estimates. Bootstrap analyzes compared data from only day 3 to those for multiple days created by pooling (i.e., days 3–4 up to days 3–8), and the inclusion of multiple days did not reduce confidence interval width (Figure 3; Supplementary Figure S3). In the scenarios of increasing the number of cats, bootstrap analyzes showed decreasing variation and reductions in confidence interval width, both for Foods A and B (Figure 3; Supplementary Figure S3).

3.6. Experimental factors and cat characteristics

Digestibility values for all nutrients were higher in Food A compared to Food B (all nutrients $p < 0.001$, but Cfat $p < 0.05$), and unaffected by the test period and the cat characteristics sex, neuter status, and body weight ($p \geq 0.05$; Table 3). Significant interaction effects in Cfat digestibility showed that male cats had lower digestibility values compared to female cats, only when fed Food B (2-way interaction sex × food $p = 0.003$). Male cats showed a relatively steep increase in Cfat digestibility with increasing weight (sex × body weight $p = 0.013$) and a steeper decrease with increasing age (sex × age $p = 0.002$). Increases in Cfat digestibility with increasing body weight were most pronounced when Food B was fed (food × body weight $p = 0.022$). GE digestibility values in males decreased more strongly with age than those in females (sex × age $p = 0.019$). Main effects part of significant interactions are not addressed here as interactions provide the more accurate effects.

4. Discussion

In-home food digestibility trials with privately-owned cats have the potential to produce highly representative data for the target population, but currently there are no validated protocols. The present study is unique for determining cat food digestibility in-home and we analyzed variation in digestibility values to establish the minimal number of days for adaptation and fecal collection required for reliable apparent fecal digestibility estimates of dietary nutrients and energy. Also, we analyzed the impact of the study cat population, in terms of sample size and cat characteristics, on digestibility estimates in order to determine the minimal number of animals required for an *a priori* set acceptable margin of error.

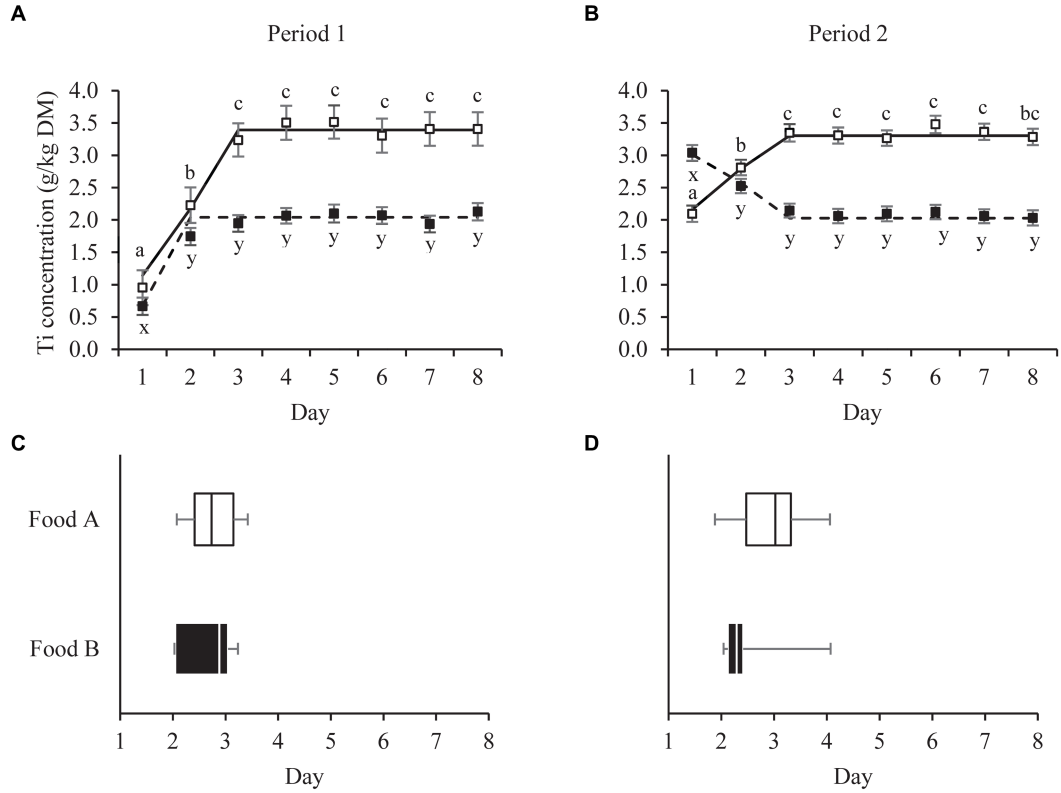

FIGURE 1
Mean daily fecal titanium (Ti) concentrations (panel **A,B**) of pet cats fed a relatively high (Food **A**; □) and low (Food B; ■) digestible food over two consecutive 8-day periods (P1, P2). Broken-line analysis showed fecal Ti concentrations to plateau at day (mean±SE) 3.17±0.24 (□, P1, *n*=12), 2.81±0.39 (□, P2, *n*=14), 2.28±0.17 (■, P1, *n*=14) and 3.14±0.26 (■, P1, *n*=12). Means within panel (**A,B**) and food (□, ■) with different superscripts (a,b,c or x,y,z) differ ($p<0.05$). Error bars are standard errors of the mean. Variation in breakpoint values for individual cats are shown in panel **C** and **D** (□, P1, *n*=10; □, P2, *n*=14; ■, P1, *n*=12; ■, P2, *n*=9). Box plots represent the lower quartile, median and upper quartile, the whiskers extend to the minimal and maximal values.

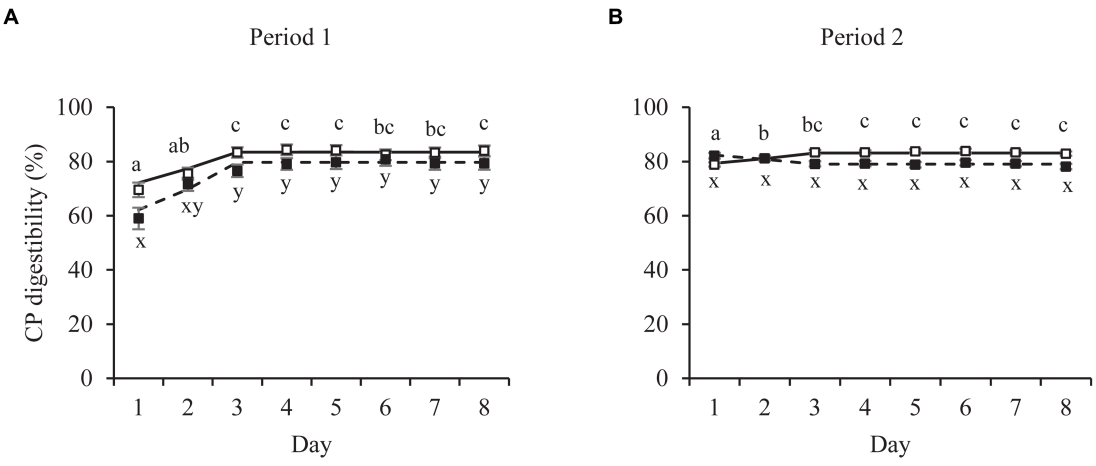

FIGURE 2
Mean daily crude protein (CP) fecal apparent digestibility values of pet cats fed a relatively high (□) and low (■) digestible food over two consecutive 8-day periods (P1, P2). Broken-line analysis showed CP digestibility values to plateau at day (mean±SE) 3.15±0.36 (□, P1, *n*=12), 3.21±0.58 (□, P2, n=14), 3.30±0.37 (■, P1, *n*=14) and 3.20±0.69 (■, P2, *n*=12). Means within panel **(A,B)** and food (□, ■) with different superscripts (a,b,c or x,y) differ ($p<0.05$). Error bars are standard errors of the mean.

4.1. Adaptation period

Food digestibility measurements with an indigestible marker require a sufficiently long adaptation period to ensure a constant rate of marker excretion in the feces, as well as to allow the adaptation of food-specific digestive processes (incl. gut motility, enzyme secretions, absorption, microbial fermentation, etc.).

4.2. Constant fecal marker excretion

This study shows that fecal samples from day 3 onwards can be used for the determination of apparent fecal digestibility values. Linear broken-line regressions revealed a plateau in the cats' fecal Ti concentrations from on average days 3.17 (period 1) and 2.82 (period 2) onwards for the more digestible food (Food A) and from respective days 2.28 and 3.14 onwards for the less digestible food (Food B). Repeated measures analyzes of variance confirmed that Ti concentration in fecal samples from cats fed the more digestible food (Food A) collected on day 3 (i.e., the interval of 48–72 h) did not differ ($p \geq 0.10$) from those in samples collected the following days, in both feeding periods. For cats fed the less digestible food this was true already ($p \geq 0.05$) on day 2 (i.e., the interval of 24–48 h).

TABLE 2 Pet cats (n=26) received 2 test foods for 2 subsequent 8-day periods. Digestibility values were calculated from daily fecal samples and analyzed for time effects by means of linear broken-line regressions. Presented are the estimated breakpoints in days ± standard error of fecal titanium (Ti) concentration and daily digestibility values of dry matter, crude protein, crude fat, and gross energy, separately for the first or second trial (period) and high digestible food (A) or low digestible food (B).

	Breakpoint	
	Food A	Food B
Period 1	$n = 12$	$n = 14$
Titanium	3.17 ± 0.24	2.28 ± 0.17
Dry matter	3.21 ± 0.32	3.35 ± 0.41
Crude protein	3.15 ± 0.36	3.30 ± 0.37
CfatCrude fat	3.63 ± 0.87	1.86 ± 0.16
Gross energy	3.17 ± 0.32	3.37 ± 0.43
Period 2	$n = 14$	$n = 12$
Titanium	2.81 ± 0.39	3.14 ± 0.26
Dry matter	2.53 ± 0.18	3.26 ± 0.33
Crude protein	3.21 ± 0.58	3.20 ± 0.69
Crude fat	3.09 ± 0.44	4.58 ± 0.92
Gross energy	2.67 ± 0.21	3.24 ± 0.34

Owners in the current study collected feces from the cats' litterbox on a daily basis. Although the time of feces collection was recorded, the precise defecation time was unknown. Feces collected during day 1 were produced between 0–24 h after the first morning meal, and therefore labelled as the average time point of 12 h post first food consumption. Subsequent days were labelled similarly. As such, day 2.28 represented 42.7 h (2.28 × 24 h – 12 h), whereas day 3.17 represented 64.1 h (3.17 × 24 h – 12 h) post first food consumption. Therefore, findings indicate that fecal samples from day 3 onwards can be used for the determination of apparent fecal digestibility values, which is 1 day later than was found in our similar in-home digestibility study with 53 privately-owned dogs (8).

In the in-home study with dogs, 1 day was sufficient to reach stable fecal marker (Ti) concentrations for both a relatively high and a low digestible dog food (8). In cats, peak marker (chromium oxide) concentrations in feces have been reported at (mean ± std) 26.5 ± 5.8 h post meal consumption in 3 year-old cats (n = 6) and 35.7 ± 14.1 h in 11 year-old cats (n = 6), as determined in dedicated feline research facilities (15). The latter study did not demonstrate significant differences between age groups, but the total transit times were highly variable across individuals, especially in the older cats. In the present study, breakpoints estimated for individual cats ranged between day 1.81 to 4.07, across foods and feeding periods, indicating the necessity to account for individual variation in transit time as a determinant of the time to reach stable fecal marker concentrations.

Gastrointestinal transit time can be influenced by several factors including dietary fiber content and source, the amount of food in the gastrointestinal tract, and the gastric emptying rate. A diet high in fiber generally decreases transit time and cats receiving a diet including 10% cellulose had a gastrointestinal transit time of 15.2 h

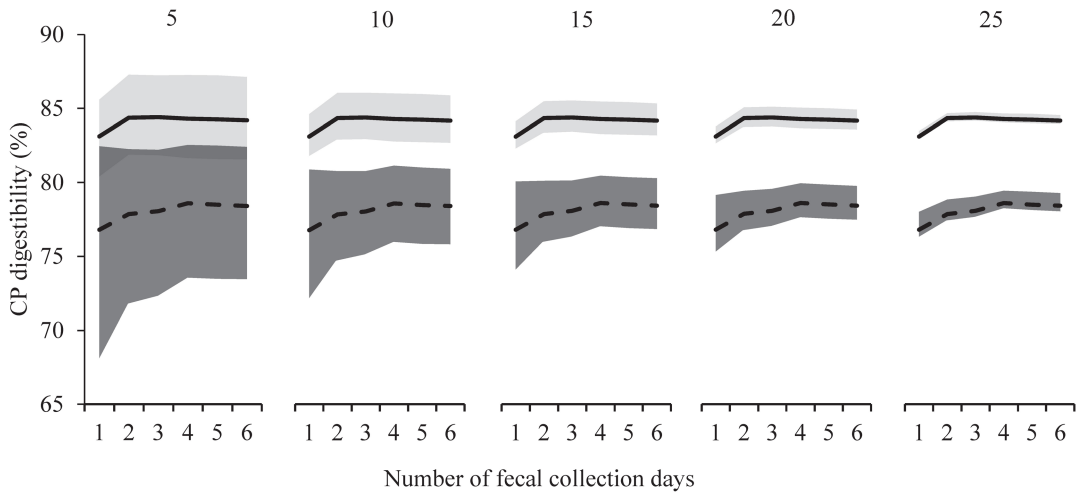

FIGURE 3
Bootstrapped estimates and confidence intervals of crude protein (CP) fecal apparent digestibility for the relatively high digestible food (Food A, upper solid line, light grey area) and relatively low digestible food (Food B, lower dashed line, dark grey area) with increasing number of fecal collection days (1 to 6, x-axis). Day one represents the first stable fecal collection day (i.e., trial day 3) with 3 to 6 days representing mean values from consecutive collection days (trial days 3 to 8, n=26 cats). Bootstrap sampling included 10,000 replicates and were ran for different sample sizes (5, 10, 25).

TABLE 3 Apparent fecal digestibility values (%) were determined in 26 pet cats that received 2 different foods (high digestible Food A and low digestible Food B) during 2 subsequent 8 day periods. The digestibility values for days 3–8, i.e., after values stabilized, were analyzed with repeated measures ANOVA for effects of test period, test food, and the cats' sex, neuter status, body weight and age. The latter two appeared to be non-significant and are not reported below. Presented are the least square means ± standard error of dry matter, crude protein, crude fat, and gross energy.

Factor	Dry matter	Crude protein	Crude fat	Gross energy
Period				
1 ($n=26$)	n.i.	n.i.	n.i.	n.i.
2 ($n=26$)				
Food				
A ($n=26$)	83.3 ± 0.4^{a}	83.0 ± 0.5^{a}	91.5 ± 0.6^{a}	85.5 ± 0.4^{a}
B ($n=26$)	74.6 ± 0.4^{b}	78.4 ± 0.5^{b}	87.8 ± 0.6^{b}	77.6 ± 0.4^{b}
Sex				
Female ($n=16$)	n.i.	81.4 ± 0.5	92.1 ± 0.6	82.8 ± 0.5
Male ($n=10$)		80.0 ± 0.6	87.3 ± 0.8	80.3 ± 0.6
Neuter status				
Intact ($n=3$)	n.i.	n.i.	n.i.	n.i.
Neutered ($n=23$)				
Food*Sex				
A -Female	n.i.	n.i.	92.4 ± 0.8^{a}	n.i.
A -Male			90.6 ± 1.0^{a}	
B -Female			91.7 ± 0.8^{a}	
B -Male			83.9 ± 1.1^{b}	

n.i., Not included in the analysis of variance after model selection using stepwise regression.
[a,b]Means differ significantly (within independent variable and nutrient) when they do not share any superscript letter ($p < 0.05$).

compared to 20.6 h for cats receiving a diet without cellulose (38). Similarly, cats receiving a diet with 16% fiber-rich beet pulp had a transit time of 15.2 h compared to 21.5 h for cats who did not receive beet pulp (39). In the present study, foods differed slightly in the time at which the fecal marker excretions were constant ($p < 0.05$), with 2 days for the more digestible food with 103 g/kg total dietary fiber and 1 day for the less digestible food with 191 g/kg total dietary fiber.

In addition to fiber content and source, the amount of food present in the gastrointestinal tract influences the gastrointestinal transit time. A large meal size reduces gastrointestinal transit by reducing gastric emptying (16). The gastrointestinal transit times of cats ($n=6$) prior and post feeding, determined by a capsule, were reported to be 28.9 h (range 18.4–90.9 h) and 46.6 h (range 15.0–109.4 h), respectively (14). The present study included cats with different consumption patterns, with 10 cats being normally fed meals and 17 *ad libitum*, but nevertheless shows 1–2 days to be sufficient to reach a stable fecal marker excretion, depending somewhat on the test food. Considering the importance of stable fecal marker excretions for accurate digestibility estimation, it is relevant to further study gastrointestinal transit times in cats for more extreme food contrasts such as wet versus dry food, food formulations, compositions, and feeding regimes.

4.3. Digestive adaptation

This study shows that the cats' digestive system appeared to adapt rapidly to novel foods and an adaptation period of 5 days as indicated by AAFCO (40) and FEDIAF (41) can be shortened to 2 days to yield stable digestibility values. An adaptation period in food digestibility studies should ensure that a cat's digestive system can adapt to the test food to achieve a steady state. Digestive adaptation, such as adjustments in digestive enzyme activity and gut microbiota composition, occur already within a few hours after changes in the amount of dietary protein, carbohydrate or lipid, in humans and several animal species (9, 11, 42). Digestive adaptation of the cats in the present study apparently required a maximum of 2 days as all nutrient apparent digestibility values were constant from a maximum of day 3 onwards. For the less digestible Food B, this time included 2 days for all nutrients in period 1 and 1 day for CP apparent digestibility values in period 2. The differences in time to reach stable digestibility values (Figure 2; Table 2; Supplementary Figure S2) between CP, DM, Cfat and GE indicate different adaptation processes/mechanisms. Compared to dogs (8), cats need 1 day longer to adapt, at least regarding Ti, CP, DM, Cfat and GE, which might be attributed to a less regular defecation pattern compared to dogs. The difference in ME requirements between dogs and cats (41), translates in higher amounts of food consumed by dogs compared to cats if foods with comparable ME contents are fed. A 4 to 7-y.o. dog with moderate activity consumes about 110 kcal ME/kg $BW^{0.75}$ whereas an adult cat (4 kg) consumed between 57 (neutered/indoor) to 88 (active) kcal ME/kg $BW^{0.75}$ (41). In case dogs and cats have largely similar digestive efficiencies (43–45), dogs would then also have a higher mass of undigested matter that is excreted via the feces than cats. In the present study, cats defecated on average 1.2 times/d whereas this was 2.3 times/d for dogs ($n=53$) fed dry foods in our previous study (8). The average fresh fecal weights also differed between dogs (4.1 g/kg $BW^{0.75}$) and cats (5.9 g/kg $BW^{0.75}$). Cats have profound lower feeding levels, defecated almost half as frequent as dogs and with larger fecal volumes per kg metabolic body weight, which underlies differences in adaptation periods as well as opportunities for fecal collection for in-home digestibility measurements.

4.4. Fecal collection period

Current guidelines recommend 5 fecal collection days for cat food digestibility studies with the marker method (41), although the scientific support for this period is unclear. In-home food digestibility trials are expected to have a relatively larger measurement variation, warranting more sample collection days, given the less controlled conditions and sources of variation such as consumption of other matter (e.g. treats, food) or contamination of feces (e.g. cat litter). Nevertheless, digestibility values in the present in-home study were stable from days 3 onwards, and 1 day of fecal collection seemed to suffice for a precise dietary digestibility determination of CP, DM, Cfat and GE. The pooling of fecal samples, which we modelled by combining results from 2 up to 6 fecal collection days, did not significantly decrease confidence interval width (Figure 3; Supplementary Figure S3). Additional fecal collection days do not increase precision substantially in food digestibility trials that make use of a marker, similar to what was found in the in-home study with

dogs (8). Practicing multiple fecal collection days, however, does have the advantage of being able to deal with infrequent defecations or small fecal volumes.

4.5. Study population

The appropriate number of cats for in-home digestibility studies relates strongly to variation in digestibility values and accepted margin of error of the digestibility assessment. The margin of error is described as half of the 95% confidence interval around the mean and was, on average 2.4% for both DM and CP, and 2.1% for GE, in apparent fecal digestibility trials with dry foods conducted following the standard AAFCO quantitative collection protocol at cat research facilities in the US ($n = 6$ cats per food, 129 foods from Hall et al. (46) (Hall personal communication)). Similar values of 2.9% for DM (range 0.8–4.7%), 2.5% for CP (1.0–4.0%) and 2.4% for GE (0.8–3.9%) were found at a research facility in Brazil (12 studies, 6 cats/food; Carciofi personal communication). The ranges in the latter studies indicate that the margin of error currently accepted for digestibility testing varies per test but also per nutrient of interest. The required number of cats for in-home digestibility testing can be based on these averages from Hall et al. (46) and maximal margin of errors from Carciofi. For the more digestible food this would result in a required sample size of <5 for DM, <5 to 6 for CP and < 5 for GE. For the less digestible food this would be <5 to 6 cats for DM, 8 to 12 for CP and < 5 to 7 for GE (Supplementary Figure S4). These two test foods were formulated to differ in composition and digestibility, but did not cover the full range as exists in commercial cat foods. For example, the crude protein content of test foods A and B were, respectively, 277 and 287 g/kg DM and apparent fecal crude protein digestibility values were 83 and 78%, whereas in complete dry cat foods the protein content can range from 234 to 489 g/kg DM (47) and the apparent fecal crude protein digestibility from 78 to 94% (20, 48–51). Future in-home digestibility trials with cat foods that range in composition and digestibility can further specify the required sample sizes.

Required sample sizes for in-home food digestibility trials depend on the test food used and the variation in digestibility values that originates from test subjects and study conditions (e.g., owner compliance and fecal sample contamination). The possible influence of cat characteristics on food digestibility values showed here as males having lower Cfat digestibility values when fed Food B, a steeper increase in Cfat digestibility values with increasing weight, and a steeper decrease in Cfat and GE digestibility values with increasing age, all compared to females (significant 2-way interactions with sex). In addition, the increase in Cfat digestibility values with increasing weight was seen across sexes, but only when fed Food B. The sex of a cat is a known influence on the digestion of food for example through effects on lipid metabolism. In a comparison between neutered lean and obese males ($n = 10$) and females ($n = 10$), the male cats were more sensitive to insulin for fatty acid uptake, especially when they were obese (52). Age too is well-known for influencing digestibility values in cats and aging decreases digestibility values for dry matter, protein, fat and energy (18, 20, 23, 53), potentially due to a reduced secretion and activity of digestive enzymes and a reduced capacity for the production, transport, and secretion of bile acids (54). Thus, a multitude of cat-related factors, separately or through interaction, potentially affect food digestibility measurements, causing variation and larger sample sizes required for heterogenous study populations. The advantage a heterogenous group of pet cats with varying characteristics is the good representativeness of study outcomes for a heterogenous target pet population.

4.6. Application for future in-home testing

In-home digestibility testing of cat food has the potential to produce data that are more representative for the pet cat population these foods are intended for than those currently derived from dedicated cat research facilities. Information on apparent nutrient digestibility is collected from cats with different characteristics (e.g. sex, neuter status, age, breed, food history) and living conditions (e.g. housing, eating pattern, activity), and additional information on perceived food quality characteristics can be obtained from the owner. For the realization of the in-home test potential, owner compliance is of crucial important. Non-compliance, like through the (accidental) provision of additional food or treats or fecal sample contamination (e.g. with cat litter), increases variation in digestibility values and can lead to misinterpretations. Owner compliance is influenced by the duration and complexity of the requested tasks (55) and designing studies of minimal length is of high relevance. Compliance may be further facilitated by short training programs to familiarize cat owners with the tasks and instruct them on study aspects such as fecal scoring (56). Future studies may assess the precise impact of non-compliance on digestibility values.

Indigestible markers are practical for pet food digestibility measurements in a home environment and TiO_2 has been validated as a digestibility marker for multiple animal species (3, 57–63). It has been used extensively in the past, but recently it was considered to be unsafe as a feed additive (64, 65), due to the potential of nanoparticles to be absorbed in the body. The TiO_2 used in the present study did not contain nanoparticles defined by a particle size <100 nm[29] and ingested concentrations (0.02 g/kg BW) were below the no-adverse effect level of 1 g/kg body weight. However, to address future concerns of owners regarding the safety of test foods, alternative markers should be investigated and made available for in-home digestibility studies.

The present digestibility values were not compared to those in cats at dedicated research facilities. We do not consider the latter as a benchmark for privately owned cats and presume that study outcomes are partly specific for the test conditions (research facilities vs. in-home). Further studies could focus on identifying and controlling sources of variation for in-home digestibility testing which will lead to improved repeatability, accuracy, and precision thereby making in-home testing more attractive for routine use in pet food testing.

5. Conclusion

In-home cat food digestibility trials require validated test protocols and this study sets key trial variables and also sheds new light on the digestibility test protocols currently used in cat research facilities. The findings support that 2 adaptation days and 1 fecal collection day suffice for accurate digestibility estimates. Three fecal

collection days are recommended though, as to account for the irregular defecation pattern of the cat. Assuming a margin of error currently accepted for digestibility testing, the required sample size for an in-home food test of digestibility would range from 5 to 12 cats depending on properties of food and nutrient of interest. Sources of variation in digestibility values are cat characteristics like age and sex, and owner compliance, which both warrant further investigation.

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. The animal study was reviewed and approved by Animal Welfare Body of Wageningen University (Wageningen, Netherlands). Written informed consent was obtained from the owners for the participation of their animals in this study.

Author contributions

EB, WH, BB, and GB contributed to the design of the study and interpretation of the findings. Execution of the study and chemical analyzes were done by EB. Data processing and analyzing were done by EB, GB, and BB. The manuscript has been written by EB and revised by GB, BB, and WH. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare 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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Supplementary material

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Effects of container type and size on thermal processing characteristics and B-vitamin retention of canned cat food

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Introduction: Rigid cans were the traditional container for canned cat foods, but semi-rigid trays/tubs and flexible pouches are popular options as well. Despite this, little is published on the effects of canned cat food container characteristics on thermal processing and retention of B-vitamins. Therefore, the objective was to evaluate the effects of container size and type on thermal processing and B-vitamin retention.

Materials and methods: Treatments were arranged in a factorial with two container sizes [small (85–99 g) and medium (156–198 g)] and three container types (flexible, semi-rigid, and rigid). A canned cat food formula was prepared, filled, and sealed into containers before retort processing to a heating cycle target lethality of 8 min. Internal retort and container temperatures were used to calculate accumulated lethality. Thiamin, riboflavin, niacin, pantothenic acid, pyridoxine, biotin, folic acid, cobalamin, and moisture contents were analyzed in pre- and post-retort samples by commercial laboratories. Thermal processing metrics were analyzed (SAS v. 9.4; SAS Institute, Cary, NC) with the fixed effects of container size, container type, and their interaction. Dry matter basis B-vitamin contents were analyzed with container size, container type, processing stage, and all two-way and three-way interactions as fixed effects. Means were separated using Fisher's LSD at a P-value <0.05.

Results and discussion: Total accumulated lethality was greater ($P < 0.05$) for semi-rigid and flexible containers (average 14.99 min) than for rigid containers (12.86 min). The greater processing of semi-rigid and flexible containers was likely influenced by required retort settings. Thiamin and riboflavin contents decreased ($P < 0.05$) by 30.4 and 18.3%, respectively, due to retort processing. Niacin, biotin, and cobalamin were not affected ($P > 0.05$) by processing. Processing increased ($P < 0.05$) pantothenic acid (9.1%), pyridoxine (22.6%), and folic acid (22.6%). This was likely caused by sampling or analytical variation. No interaction involving processing stage was significant for any B-vitamin ($P > 0.05$). B-vitamin retention was not influenced by differences in thermal processing caused by the packaging treatments. Thiamin and riboflavin were the only B-vitamins meaningfully impacted by processing and retention was not improved by any container characteristic.

KEYWORDS

B-vitamins, canned cat food, packaging, retention, riboflavin, thermal processing, thiamin, wet cat food

1. Introduction

Many pet cats are fed commercial foods designed to meet their nutritional requirements. These foods come in many formats, including canned foods. The term "canned" is defined as "… processed, commercially sterilized, and sealed according to 21 CFR part 113 in hermetically sealed containers such as but not limited to cans, pouches, tubs, and trays" (1). The Code of Federal Regulations (CFR) defines commercially sterilized as it applies to pet food as the absence of microorganisms and spores that pose a public health concern and microorganisms that reproduce at room temperature after a heat process (2). A retort, or a commercially-sized pressure cooker, is used to achieve commercial sterility of food products. The duration of time the retort is held at specific temperatures to achieve commercial sterility is termed the scheduled process (2). Foods processed with a retort must be contained within hermetically sealed containers, which are sealed in a way that prevents matter from moving into or out of the container (3).

As described in the Association of American Feed Control Officials (AAFCO) definition, canned pet food can be processed in different types of containers. The United States Department of Agriculture Food Safety and Inspection Service (USDA-FSIS) categorizes containers for thermal processing into three groups: flexible, semi-rigid, and rigid. These categories are defined by whether the food product significantly influences container shape and 0.7 kg/cm^3 of force under normal atmospheric conditions can change the container shape (3). As such, the internal food product only influences the shape of flexible containers and semi-rigid containers can deform with $<$0.7 kg/cm^3 of force, while rigid containers require forces $>$0.7 kg/cm^3 for deformation. While the rigid metal can is the most common container type, other options for canned cat foods include flexible pouches and semi-rigid trays (4). The popularity of these containers can be attributed to improved consumer convenience (3), but few peer-reviewed publications regarding canned cat food assess these container types.

Thiamin (vitamin B1) is not stable during retort processing and has been a focus in canned pet food research. Consumption of a diet deficient in thiamin by cats can cause severe neurological deficits such as decreased ability to learn in kittens (5) and seizures and ventroflexion in cats of all ages (6–8). This deficiency can become deadly quickly with reports of death within 2 or 3 days once movement is visibly difficult if treatment is not provided (9). It is the responsibility of pet food companies to ensure their products contain enough thiamin before they enter the marketplace. However, thiamin-deficient canned cat foods are still found on store shelves (10) even when pet food companies do their best to prevent this. At the same time, other B-vitamins (riboflavin, niacin, pantothenic acid, pyridoxine, folic acid, biotin, and cobalamin) also have recommended levels for inclusion in canned cat foods (1). Many of the B-vitamins interact with each other and deficiency of a single vitamin can have far-reaching consequences. For example, pyridoxine and folic acid are needed for ideal absorption of cobalamin and thiamin, respectively (11). Identification of a B-vitamin deficiency is challenging and often deficiencies of individual B-vitamins present similarly. As such, pet food companies need to ensure these vitamins also survive thermal processing.

The objective of this experiment was to determine the effects of packaging type and size on thermal processing characteristics and B-vitamin retention. The first hypothesis was that smaller containers made of flexible materials would achieve the target lethality faster than medium containers made of rigid materials. However, the second hypothesis was that retention of heat-labile B-vitamins would not be impacted by container type and size when processed to similar lethalities.

2. Materials and methods

2.1. Packaging types and sizes

Container types and sizes were chosen to encompass all categories of packaging materials and the container sizes most often used for commercial canned cat foods for a total of six containers. Container types were categorized as flexible, semi-rigid, and rigid based on USDA-FSIS guidelines (3) and container sizes were categorized as small and medium based on target fill weights. The small (85 g target fill weight; 95 mm width × 138 mm height × 25 mm gusset) and medium (170 g target fill weight; 140 mm wide × 180 mm high × 25 mm gusset) flexible containers used were made of multiple layers. Specifically, the small flexible container consisted of 12 μ PET, adhesive, Al Foil 8 μ, adhesive, and polypropylene 70 μ and the medium flexible container consisted of 12 μ PET, adhesive, Al Foil 7 μ, adhesive, Nylon 15 μ, adhesive, and polypropylene 60 μ. Small (99 g target fill weight) and medium (198 g target fill weight) semi-rigid containers were made of layers of polypropylene (1.2 mm for small and 1.4 mm for medium), EVOH 10%, and a polypropylene base sheet. Rigid containers were two-piece aluminum cans with modified vinyl coatings and target fill weights of 85 g (small; 209.5 × 107) and 156 g (medium; 307 × 109.3).

2.2. Canned cat food batter production

The canned cat food formula was designed to mimic a generic chicken-based commercial canned cat food (Table 1). This formula was intended to contain 78.0% moisture, 35.0% crude protein on a dry matter basis (DMB), 44.3% crude fat DMB, and 8.0% ash DMB with a pH of 6.5. Successful formulation was verified with analysis of pre- and post-retort moisture and pH and post-retort crude protein, crude fat, and ash (Supplementary Table 1). Vitamin premix level was ∼10× the typical production level to ensure high enough concentrations of B-vitamins for chemical analysis. The target levels of B-vitamins before retort processing on a DMB were 3,209.0 mg/kg thiamin, 90.9 mg/kg riboflavin, 909.1 mg/kg niacin, 136.4 mg/kg pantothenic acid, 272.7 mg/kg pyridoxine, 1.60 mg/kg biotin, 22.2 mg/kg folic acid, and 0.60 mg/kg cobalamin.

An independent batch of pre-retort batter (69 kg) was made for each replicate ($n = 2$) of each combination of package type ($n = 3$) and size ($n = 2$) for a total of 12 independent diet productions. Frozen blocks of mechanically separated chicken (Protein Inc/BHJ, St Joseph, MO) were ground through a pilot plant extructor and grinder with 9.5 mm openings and weighed into a horizontal

TABLE 1 Canned cat food formula super-fortified with B-vitamins.

Ingredient	Percentage, %
Water and steam	39.149
Mechanically separated chicken	55.333
Brown rice	3.000
Dried egg product	0.500
Potassium chloride	0.500
Soybean oil	0.500
Guar gum	0.350
Mineral premix	0.250
Vitamin premix[a]	0.200
Choline chloride	0.092
Kappa carrageenan	0.050
Taurine	0.041
Salt	0.035

[a]Target B-vitamin content in processed cat foods was 3,209.0 mg thiamin, 90.9 mg riboflavin, 909.1 mg niacin, 136.4 mg pantothenic acid, 272.7 mg pyridoxine, 1.60 mg biotin, 22.2 mg folic acid, and 0.60 mg cobalamin per kg of diet dry matter.

jacketed mixer (Rietz, Evansville, IN). Brown rice (Gulf Pacific Rice, Houston, TX), dried egg product (Rose Acre Farms, Seymour, IN), potassium chloride (Prince Agri Products Inc., Quincy, IL), soybean oil (preserved with mixed tocopherols; Columbus Foods, Des Plaines, IL), guar gum (Intercolloid, Wembley, Middlesex, UK), mineral premix (Prince Agri Products Inc., Quincy, IL), choline chloride (SEM Minerals, Quincy, IL), kappa carrageenan (Marcel Trading, Quezon City, Philippines), taurine (Prinova, Carol Stream, IL), and salt (Cargill, Hutchison, KS) were added to the mixer and blended for 5 min until uniform. Directly injected steam was used to bring the batter up to 43.3°C and water was added to bring the moisture content up to 78% (verified by rapid methods; CEM, Mathews, NC). At this time, a sample of the raw batter was taken for chemical analyses to capture the background vitamin content provided by the non-vitamin premix ingredients in the formula.

Next, the vitamin premix (DSM Nutritional Products, LLC, Parsippany, NJ) was added to the batter and mixed for 10 min prior to homogenization with 3 and 6 mm die plates (Karl Schnell Emulsifier, Winterbach, Germany). A sample was taken at this processing stage to capture the pre-retort moisture and B-vitamin contents.

2.3. Thermal processing of canned cat foods

Each replicate of each combination of packaging type and packaging size was thermally processed separately. Prior to container filling, a select number of packages (16 for small and medium rigid cans and 14 for small and medium flexible pouches and semi-rigid trays/tubs) were outfitted with type-T thermocouples (Ecklund-Harrison Technologies Inc., Ft. Meyers, FL). This number was chosen to allow for extra thermocouples beyond the minimum ten recommended by the Institute for Thermal Processing Specialists (12) in the event of thermocouple failure during retort processing. Additionally, two thermocouple leads were used to monitor temperature inside the retort during processing.

Fifty containers, including those with thermocouples, were filled with their respective target weight of batter and hermetically sealed with a sealer (small and medium cans: Ferrum, Schafesheim, Switzerland; small trays: Shinwa, Shinwa, Japan; medium trays: Raque Food Systems, Louisville, KP; small and medium pouches: PMP mini vacuum seamer, PakSource Global LLC., Sarasota, FL). Satisfactory closer was confirmed with pre- and post-retort processing burst tests for the flexible and semi-rigid containers and with post-retort vacuum measurements for the rigid containers (Supplementary Table 1).

Ballast containers comprised the two layers of containers above and below the experimental treatments to mimic a commercial production. Thermocouples and leads were attached to the data collection system (CALSoft v. 5.0.5.; TechniCAL LLC, Metairie, LA) at the start of retort processing. Separate retorts were used for the different packaging types due to their behavior during retort processing (cans: Reid Boiler Works Inc. Bellingham, WA; trays and pouches: JBT, Madera, CA). Temperatures inside the retort (Figure 1) and inside containers (Figure 2) were recorded every 30 s and used to calculate lethality (Equation 1) by the data collection system using the trapezoidal method (Equation 2). In these equations, $T_C(t)$ represented the temperature recorded by a container's thermocouple at time t and Δt represented the time interval between measurements in minutes (0.5). The retort automatically entered the water cooling phase once the last thermocouple reached the minimum lethality value of 8 min. Temperatures were recorded during the cooling cycle to allow for calculation of accumulated lethality during the heating cycle, cooling cycle, and throughout the entire scheduled process. The units of lethality are equivalent minutes of processing at 121.11°C. As such, greater lethality values indicate more intense processing.

$$Lethality = \int 10^{\frac{T_C(t)-121.11^\circ C}{10^\circ C}} \Delta t \quad (1)$$

$$Lethality = \sum_{0}^{t} 10^{\frac{T_C(t)-121.11^\circ C}{10}} \Delta t \quad (2)$$

Once cooled, packages were removed from the retort and stored at ambient temperature overnight. The following day, four containers from each replicate were combined to create a composite sample of the post-retort diet for chemical analysis.

2.4. Chemical analyses

Pre-retort batters (before and after the addition of vitamin premix) and post-retort diets were analyzed in duplicate for moisture (AOAC 934.01), and thiamin [EN 14122:2003; (13)] at a commercial laboratory (Nestle Purina Analytical Laboratory, St. Louis, MO). Riboflavin (AOAC 944.33), niacin (AOAC 944.13), and pyridoxine (AOAC 961.15) contents were measured in duplicate in pre-retort batters and post-retort diets by the vitamin premix supplier (DSM Nutritional Products, LLC; Parsippany, NJ). Pre-retort batters and post-retort diets were analyzed once for

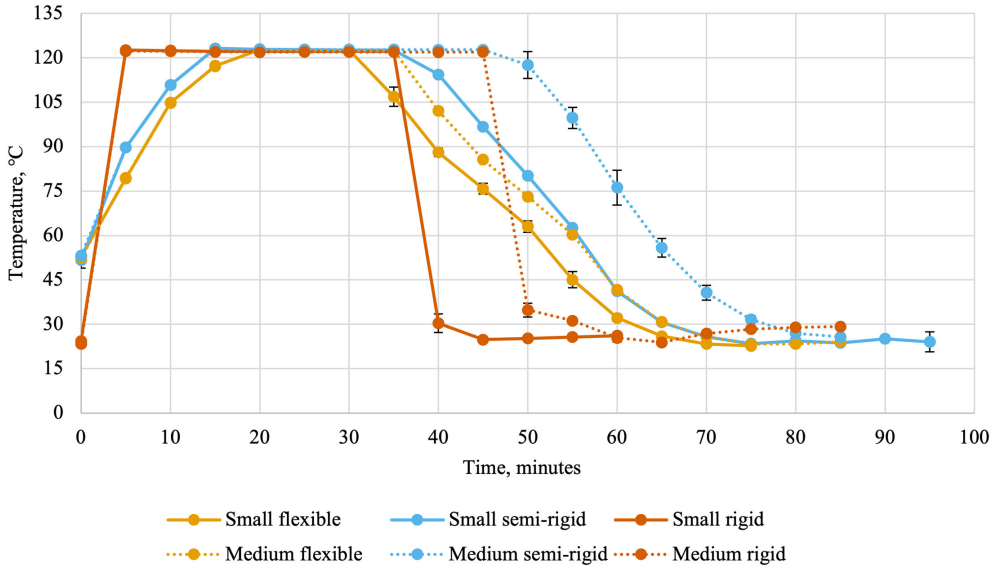

FIGURE 1
Retort temperatures (average ± standard deviation) every 5 min during thermal processing of canned cat food processed in containers of two different sizes and three different types.

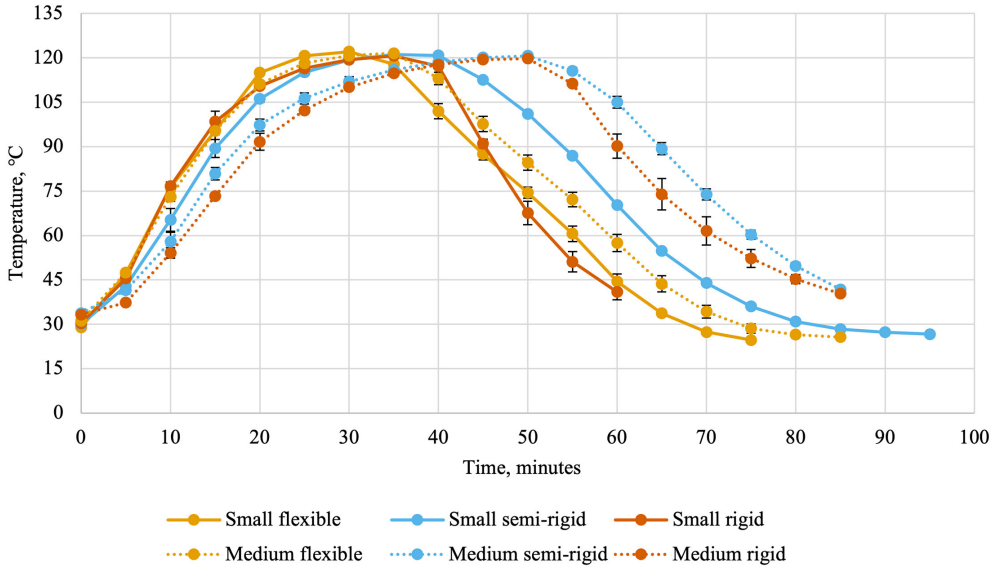

FIGURE 2
Internal can temperatures (average ± standard deviation) every 5 min during thermal processing of canned cat food processed in containers of two different sizes and three different types.

pantothenic acid (AOAC 945.74), biotin (14), folic acid (AOAC 992.05), and cobalamin (AOAC 952.20) by a commercial laboratory (Covance Laboratory, Madison, WI). Analysis of crude protein (AOAC 990.03), crude fat (AOAC 920.39), and ash (AOAC 942.05) in post- retort diets was conducted in duplicate by a commercial laboratory (Nestle Purina Analytical Laboratory, St. Louis, MO). pH readings were taken of pre-retort batters during production and of post-retort diets after diet production and sampling.

2.5. Statistical analysis

Moisture content, crude protein content, crude fat content, ash content, and pH were presented as average values ± standard deviation. Initial can temperature, time to reach target lethality and accumulated lethality values were analyzed as a 2 × 3 factorial with the fixed effects of container type and container size (GLIMMIX procedure, SAS v. 9.4; SAS Institute, Cary, NC). B-vitamin contents were analyzed as a 2 × 3 × 2 factorial with the

main effects of container size (small and medium), container type (flexible, semi-rigid, and rigid), and processing stage (pre- and post-retort processing), and all two-way and three-way interactions. An analysis of variance was employed to determine the significance of the models' main effects and interactions. Fisher's least significant difference was used to separate means at an α of 0.05.

3. Results

3.1. Thermal processing metrics

Thermocouple failure occurred during the processing of small flexible pouch replicates 1 and 2 (four and two failures, respectively), small semi-rigid tray/tub replicates 1 and 2 (two failures for both), small rigid can replicate 1 (one failure), medium flexible pouch replicates 1 and 2 (two and three failures, respectively), and medium rigid can replicate 1 (one failure). No thermocouple failures occurred during processing of medium semi-rigid trays/tubs.

On average, medium size containers were initially warmer ($P < 0.05$) than small size containers (32.21 vs. 29.73°C; Table 2). However, there was no difference ($P > 0.05$) in initial container temperatures between container types or the interaction of container size and type. Medium size containers (41.6 min) required 23.4% more time to achieve the target lethality of 8 min than small size containers (33.7 min). The amount of time required for rigid and semi-rigid containers (40.9 min) was similar ($P > 0.05$) but greater than for flexible containers (31.2 min). These relationships were observed for the interaction of container size and type, wherein medium rigid and semi-rigid containers (average 42.0 min) needed 17.3% more time ($P < 0.05$) than small rigid and semi-rigid containers (average 35.8 min). Medium flexible containers (32.8 min) required less time ($P < 0.05$) and small flexible containers (29.5 min) required the least ($P < 0.05$) amount of time overall.

Lethality accumulated during the heating cycle was not affected ($P > 0.05$) by container size, type, or their interaction (average 9.74 min; Table 2). However, lethality during the cooling cycle was 9.0% greater ($P < 0.05$) for small containers than for medium containers (4.70 vs. 4.31 min). Semi-rigid containers (5.87 min) accumulated more ($P < 0.05$) lethality during the cooling cycle than rigid containers (3.25 min) with cooling cycle lethality for flexible containers (4.40 min) intermediate. Differences in the total lethality accumulated during retort processing were observed between container types, with flexible and semi-rigid containers (average 14.99 min) accumulating 16.6% more ($P < 0.05$) lethality than rigid containers (12.86 min).

3.2. B-vitamin content of pre- and post-retort samples

Riboflavin, niacin, and pyridoxine levels in pre-retort samples before the addition of vitamin premix fell below detectable limits. Pantothenic acid (21.8 mg/kg DMB), biotin (0.319 mg/kg DMB), folic acid (0.509 mg/kg DMB), and cobalamin (0.0588 mg/kg DMB) were lower prior to inclusion of the vitamin premix. Thiamin was only detectable in one sample of pre-retort batter without the vitamin premix, resulting in an average thiamin content of 2.57 mg/kg DMB for one replicate of medium pouches prior to vitamin premix addition.

Thiamin content was affected ($P < 0.05$) by the main effects of container size and processing stage. On average, small containers (2,996.1 mg/kg DMB) contained 4.8% more thiamin than medium containers (2,858.0 mg/kg DMB) separate from container size and/or processing stage (Figure 3A). Not considering container size or type, thiamin content decreased by 30.4% due to retort processing (Figure 3B). However, the main effect of container type and all interactions were not significant ($P > 0.05$; Table 3). Similar relationships were observed for riboflavin content, where small containers contained 5.2% more ($P < 0.05$; Figure 4A) riboflavin than medium containers and degradation due to retort processing was 18.3% ($P < 0.05$; Figure 4B).

Niacin content was 5.75% greater ($P < 0.05$; Figure 5) in small containers than in medium containers but was not affected ($P > 0.05$; Table 3) by container type, processing stage, or any interactions. Pantothenic acid and pyridoxine contents were higher ($P < 0.05$) in post-retort samples than in pre-retort samples by 9.1% (Figure 6) and 11.6% (Figure 7), respectively. These B-vitamins were not affected ($P > 0.05$; Table 3) by container size, container type, or any interactions. Rigid containers (13.672 mg/kg DMB) contained higher ($P < 0.05$; Figure 8A) levels of folic acid than semi-rigid and flexible containers (average 12.502 mg/kg DMB), which were not different ($P > 0.05$) from each other. Folic acid content was also greater ($P < 0.05$; Figure 8B) in post-retort samples than in pre-retort samples by 22.6%. The interaction between container size and processing stage was also significant ($P < 0.05$; Figure 8C); small containers post-retort processing (14.385 mg/kg DMB) contained the highest level of folic acid, followed by medium containers post-retort (13.565 mg/kg DMB) with pre-retort samples in small and medium containers (average 11.583 mg/kg DMB) containing the lowest level. The main effect of container size and all unmentioned interactions did not affect ($P > 0.05$; Table 3) folic acid content. Finally, biotin (average 1.528 mg/kg DMB; Supplementary Table 2) and cobalamin (average 0.4173 mg/kg DMB; Supplementary Table 2) were not affected ($P > 0.05$; Table 3) by container size, container type, processing stage, and any interactions.

4. Discussion

The goal of this experiment was to determine if container size and type influenced retort processing and retention of B-vitamins in canned cat food. Minimum recommended levels should be met for a pet food to be deemed "complete and balanced" in the United States (1). As such, it is important for pet food companies to ensure their products contain enough of each B-vitamin to support animal health. Other research on vitamin degradation due to retort processing has utilized rigid cans as the container type (15–17). However, flexible pouches and semi-rigid trays/tubs have become popular offerings for canned cat foods and research with human food products suggests there are differences in their processing.

TABLE 2 Thermal processing metrics for a canned cat food processed in two different container sizes and three different container types.

Measurement	Small			Medium			Size × Type SEM[a]	*P*-values		
	Flexible	Semi-rigid	Rigid	Flexible	Semi-rigid	Rigid		Size	Type	Size × type
Initial can temperature, °C	28.91	30.02	30.26	31.15	33.77	32.01	0.874	0.0111	0.1809	0.5292
Time to target lethality, min	29.5	36.1	35.5	32.8	46.4	45.6	0.63	<0.0001	<0.0001	0.0023
Total lethality, min	15.02	15.14	12.67	14.43	15.35	13.05	0.510	0.9939	0.0077	0.6178
Heating lethality, min	10.23	9.28	9.23	10.42	9.49	9.76	0.435	0.4160	0.1365	0.9096
Cooling lethality, min	4.79	5.87	3.45	4.01	5.88	3.06	0.150	0.0194	<0.0001	0.0970

[a]SEM, standard error of the mean.

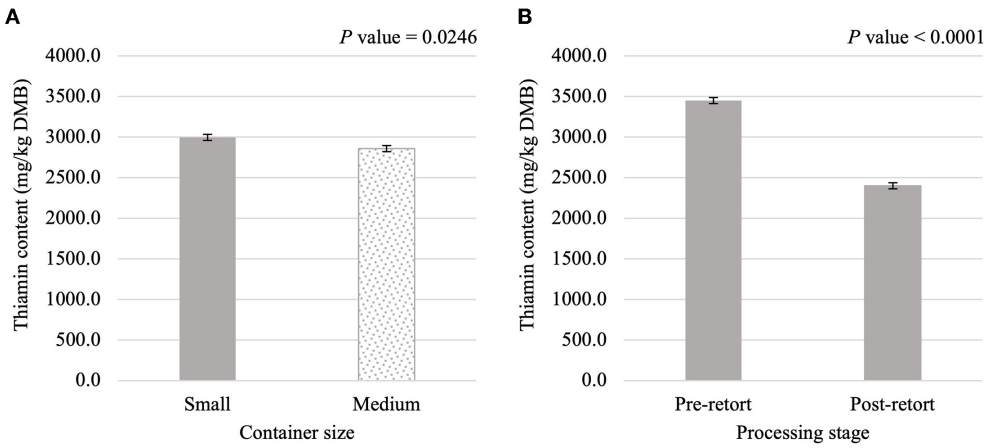

FIGURE 3
Main effects of container size **(A)** and processing stage **(B)** on dry matter basis (DMB) thiamin content (average ± pooled standard error of the mean) of canned cat food processed in containers of two different sizes and three different types.

4.1. Thermal processing metrics

Thermocouple failure was expected simply due to the large number employed in the present experiment and has been documented in other experiments (15, 18). However, the minimum number of 10 thermocouples recommended by the Institute for Thermal Processing Specialists (12) was achieved in the processing of each replicate.

Flexible and semi-rigid containers required slightly different scheduled processes than rigid containers as they are more susceptible to damage during thermal processing (19). This is evidenced by the differences in retort temperatures and internal can temperatures. The small and medium rigid cans were sturdier and could withstand faster retort come-up times than the small and medium semi-rigid and flexible containers. Other packaging-related factors of heating rates include container wall thickness, thermal conductivity of the container material, and the container's ratio of surface area to volume (4). In the present experiment, the effects of container wall thickness and thermal conductivity are included in the container type factor and the container surface area to volume ratio is captured within both factors.

Small containers were cooler at the beginning of retort processing than medium containers. The intention was to keep initial can temperatures similar across all treatments because the initial can temperature can influence the heating rate and the intensity of processing (4). In general, food products with lower initial temperatures will accumulate less lethality than food products with higher initial temperatures processed under the same scheduled process (20). The scheduled process in the present experiment was dependent on when the final containers achieved the target lethality of 8 min and may have mitigated some effects of differences in initial temperature. In future experiments, initial can temperatures could be controlled better by placing filled and sealed containers in a warm water bath during the filling and seaming processes.

The time to achieve the target lethality was affected by container size, container type, and their interaction. Thermocouples were placed in containers to measure temperature in the coldest spot, or the location that heats and cools the slowest. Therefore, the

TABLE 3 *P*-values for a factorial arrangement of treatments evaluating the effects of two container sizes, three container types, and two processing stages on the content of B-vitamins in a canned cat food.

Vitamin	Size	Type	Processing stage	Size × type	Size × processing stage	Type × processing stage	Size × type × processing stage
Thiamin	0.0246	0.6825	<0.0001	0.1824	0.4238	0.2173	0.8322
Riboflavin	0.0375	0.3506	<0.0001	0.6230	0.2428	0.3796	0.7100
Niacin	0.0339	0.5214	0.0891	0.6519	0.9151	0.1252	0.7032
Pantothenic acid	0.0529	0.2256	0.0012	0.6115	0.8647	0.6115	0.0927
Pyridoxine	0.5003	0.0686	0.0009	0.2299	0.2442	0.7504	0.7747
Biotin	0.4984	0.4316	0.9533	0.4504	0.5045	0.1137	0.2669
Folic acid	0.2368	0.0208	<0.0001	0.4251	0.0320	0.3061	0.6058
Cobalamin	0.5797	0.2461	0.3723	0.1850	0.9521	0.8663	0.8208

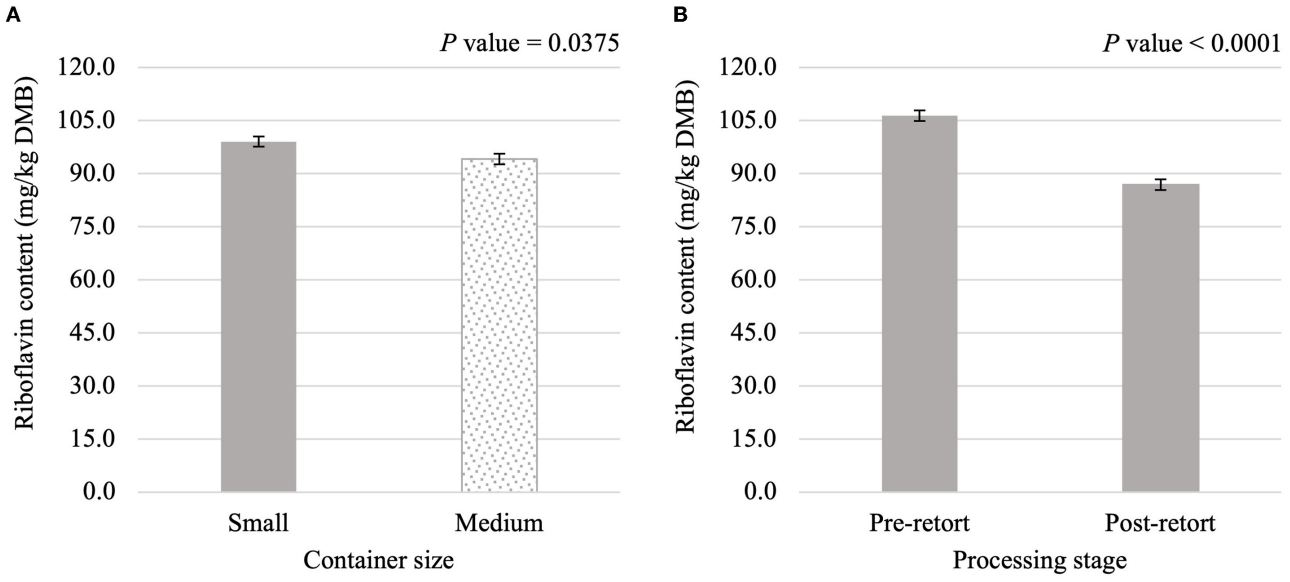

FIGURE 4
Main effects of container size **(A)** and processing stage **(B)** on dry matter basis (DMB) riboflavin content (average ± pooled standard error of the mean) of canned cat food processed in containers of two different sizes and three different types.

decreased time required for small containers compared to medium containers was not unexpected. Flexible containers also required less time than semi-rigid and rigid containers to achieve the target lethality. A reduction in calculated process time with the use of flexible pouches instead of rigid cans has been noted in the retort processing of shrimp in a sauce (21) and trout, pollock, and shrimp alone (22). These researchers suggested the differences in the surface area to volume ratio between the two container types contributed to their findings. As in their research, the flexible pouches in the present experiment were flatter than the semi-rigid and rigid containers, which can reduce the time required for thermal processing (23). This is echoed when the interaction between container size and container type is analyzed. Flexible containers required the shortest amount of time and the difference between medium and small flexible containers was smaller than between medium rigid or semi-rigid containers and small rigid or semi-rigid containers. Other researchers have also found a greater effect of container size on calculated process time when food was processed in rigid cans than in flexible pouches to achieve a target lethality (24). Process developers should take these differences in heat penetration into account when designing scheduled processes to minimize over-processing.

All container types and sizes were processed to average heating lethalities greater than the target of 8 min. This was not unexpected, as other researchers have also struggled to achieve target lethalities when processing canned cat foods (18, 25). Initiating the cooling cycle when the final thermocouple-containing container reached the target lethality meant all other containers would be "over-processed", thus resulting in a higher than intended average heating lethality. However, the lack of difference in heating lethalities suggested a successful retort processing.

Internal container temperature may continue to rise during early portions of the cooling phase and contribute to accumulated lethality (26). This was observed in the present experiment,

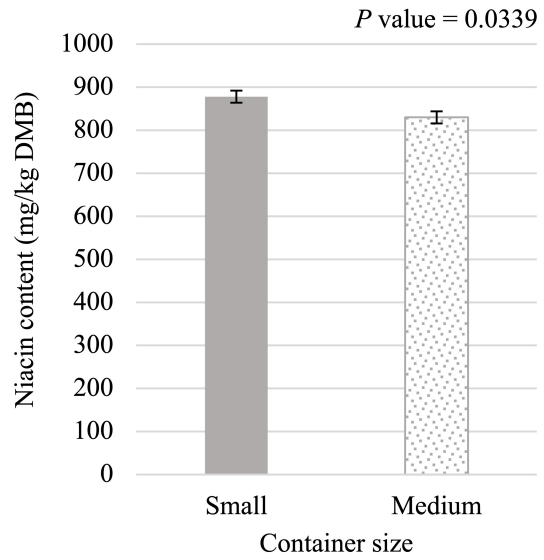

FIGURE 5
Main effect of container size on dry matter basis (DMB) niacin content (average ± pooled standard error of the mean) of canned cat food processed in containers of two different sizes and three different types.

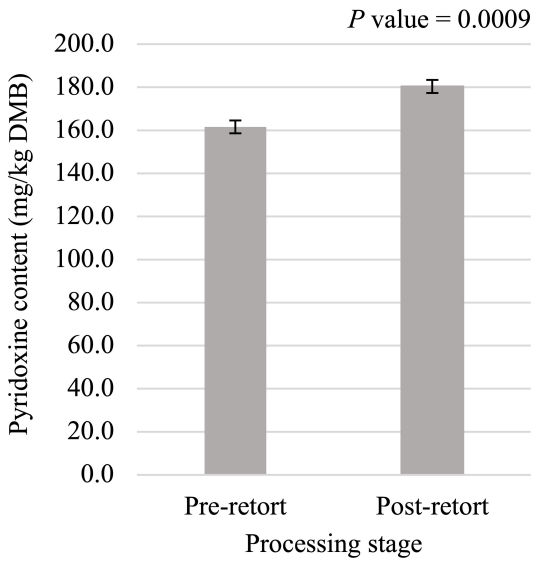

FIGURE 7
Main effect of processing stage on dry matter basis (DMB) pyridoxine content (average ± pooled standard error of the mean) of canned cat food processed in containers of two different sizes and three different types.

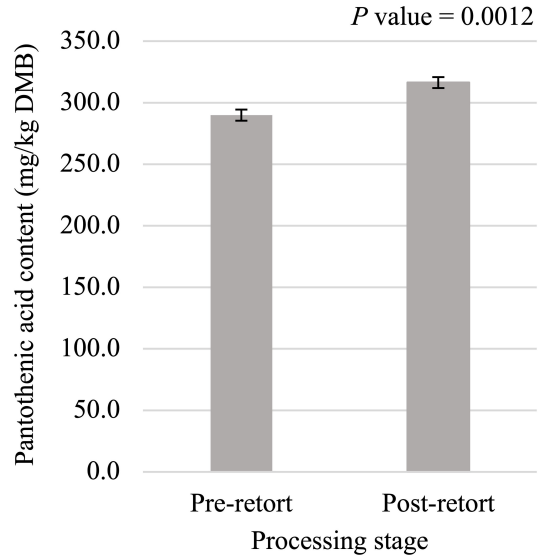

FIGURE 6
Main effect of processing stage on dry matter basis (DMB) pantothenic acid content (average ± pooled standard error of the mean) of canned cat food processed in containers of two different sizes and three different types.

wherein the main effects of container size and container type were significant. The greater cooling cycle lethality for small containers compared to medium containers was unexpected. This finding suggested that the cooling of small containers was slower than for medium containers (20). It is important to note than only a 9% difference was observed between container sizes and this relationship was not observed for total accumulated lethality. Greater differences were observed between container types, where the greatest cooling cycle lethality was accumulated in semi-rigid containers, followed by flexible containers, and rigid containers accumulated the lowest lethality. The scheduled process for rigid containers utilized a faster retort cool-down that was not possible with the semi-rigid and flexible containers. This was required to prevent deformation of semi-rigid and flexible containers by cooling them too quickly. However, this may have led to greater cooling cycle lethalities in those container types. The fact that flexible containers accumulated less lethality during the cooling cycle than semi-rigid containers suggested a faster cooling rate similar to the differences observed in the time to achieve target lethality between container types.

Regardless of these outcomes, total accumulated lethality was only affected by container type. The grouping of semi-rigid and flexible containers accumulating more lethality than rigid containers suggested that differences in the scheduled process for these container types, particularly during the cooling cycle, played a role in the overall processing intensity. Nevertheless, total accumulated lethality for all six treatments was similar to the reported typical lethality of 12–14 min for commercial canned cat foods (25).

4.2. B-vitamin content of pre- and post-retort samples

The addition of vitamin premix was necessary to meet the intended thiamin level target prior to retort processing as well as the minimum recommended level for cats in adult maintenance or growth and reproduction [5.6 mg/kg DMB (1)]. This agrees with

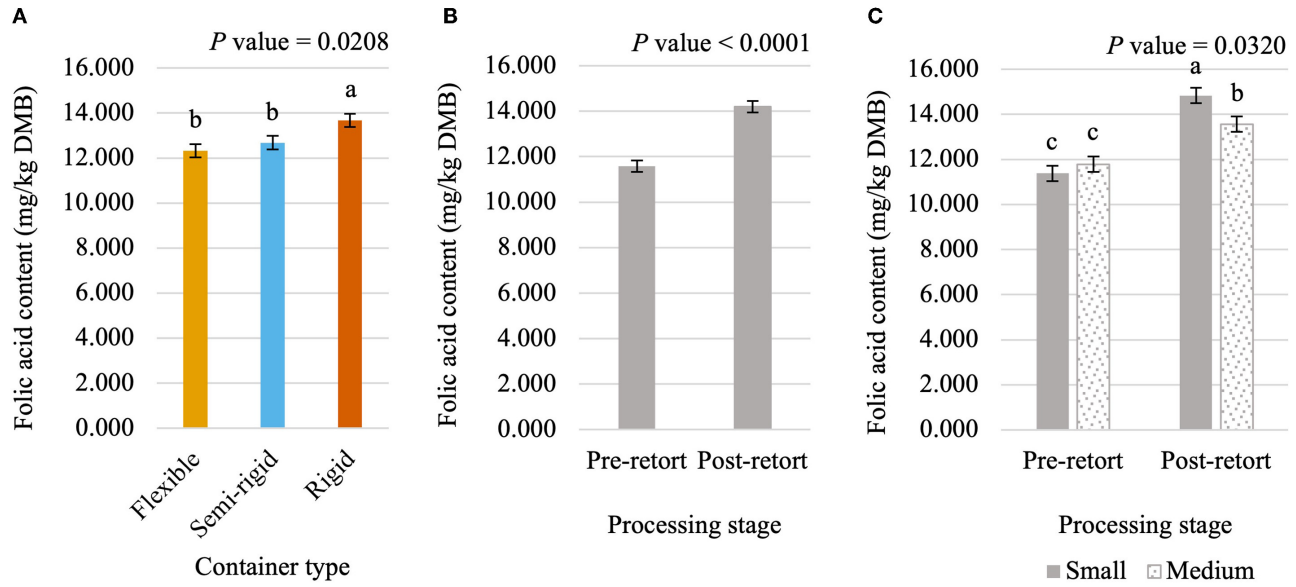

FIGURE 8
Main effects of container type **(A)** and processing stage **(B)** and the interaction of container size and processing stage **(C)** on dry matter basis (DMB) pyridoxine content (average ± pooled standard error of the mean) of canned cat food processed in containers of two different sizes and three different types. [abc]Means within a main effect or interaction without a common superscript are different ($P < 0.05$).

previous research that found low levels of thiamin in ingredients used to manufacture chicken-based canned cat foods (15). Levels of other B-vitamins were also low in pre-retort samples without vitamin premix in the present experiment. Undetectable levels of riboflavin, niacin, and pyridoxine indicated that other ingredients present in the canned cat food did not provide meaningful contributions. As such, supplementation of these vitamins through the inclusion of a vitamin premix is necessary to meet the minimum recommended levels of cats [4.0 mg/kg DMB; 60 mg/kg DMB, and 4.0 mg/kg DMB, respectively (1)]. It is likely that the mechanically separated chicken, brown rice, and dried egg product provided minimal levels of the other B-vitamins. All three ingredients have been documented with measurable levels of pantothenic acid and folic acid while only dried whole egg contained measurable levels of cobalamin (27). Regardless, the minimum recommended levels of pantothenic acid, biotin, and cobalamin for adult maintenance and growth and reproduction for cats [5.75 mg/kg DMB, 0.07 mg/kg DMB, and 0.020 mg/kg DMB, respectively (1)] were met without the addition of vitamin premix prior to retort processing. The level of folic acid did not meet the recommended level [0.8 mg/kg DMB (1)] without the vitamin premix, indicating that inclusion of a vitamin premix is also necessary for this vitamin.

The main effect of container size was significant for thiamin, riboflavin, and niacin contents and the main effect of container type was significant for folic acid content. This indicated low levels of variability in the addition of the vitamin premix to the formula and/or in the vitamin analysis. Analysis of these main effects did not differentiate between pre-retort and post-retort samples; these main effects do not suggest improved vitamin retention with a specific container size or type.

Thiamin degradation due to thermal processing is well-documented in canned foods. Experimentally, thiamin degradation in canned cat food ranged from 45.8 to 82.9% when different thiamin sources were included in the diet (15). On the other hand, industry recommendations describe thiamin degradation due to processing as high as 90% (1). These reports are much higher than the average 30.4% thiamin loss observed in the present experiment. None of the interactions between processing stage and container size and/or container type were significant in the present experiment. This does not agree with research that documented greater thiamin retention in rainbow trout, pollock, and shrimp processed in pouches instead of cans (22). However, statistical analysis was not used to make this conclusion. Other researchers identified a trend for lower thiamin retention when cat food was processed in 156 g cans vs. 349 g cans (16). That research group's experimental design included processing time as a factor, which may have led to smaller cans accumulating greater lethality during the heating cycle. If this were the case, it is reasonable to assume the cooling cycle exacerbated this difference and confounds the data. Heating lethality was not different among the treatments in the present experiment and total lethality for semi-rigid and flexible containers by 2.13 min. This suggests there may be a range of total lethalities before differences in thiamin degradation are observed, as the interaction between container type and processing stage was not significant. Others have found nearly twice the thiamin degradation when a canned cat food containing thiamin mononitrate was processed to a total lethality around 80 min (15). Future research should process one canned cat food formulation to increasing total lethalities and measure thiamin before and after processing to determine how much overprocessing is acceptable.

This is the first experiment to document decreased riboflavin content in canned cat food due to thermal processing. The 81.7%

retention observed was not unexpected. Retention of riboflavin in beef or veal home-canned in different sizes of cans and jars ranged from 83 to 112% and were not considered significant (28). Cut potatoes and cow peas also retained 83.7 and 85.2%, riboflavin, respectively, after the retorting process (29). However, riboflavin retention was lower for retorted tuna [49.8%; (30)] and for immature seeds of varieties of the common bean [44.3–59.1%; (31)]. It is challenging to compare riboflavin retention across experiments with different processing conditions without a common measure of processing intensity, such as total lethality. Future research could investigate the effect of total lethalities on riboflavin retention in a canned cat food. However, the higher riboflavin retention compared to thiamin retention in the present experiment suggested that thiamin may be more important when optimizing formulation and the scheduled process.

The B-vitamins niacin, biotin, and cobalamin were not affected by retort processing in this experiment. Niacin is considered stable during food processing, but retention is affected when retorting temperature and time are altered (32, 33). These factors were kept as consistent as possible in the present experiment but could not be exact due to the constraints of the flexible and semi-rigid container types. It appeared the slightly higher total lethality accumulated by semi-rigid and flexible containers was not great enough to influence niacin retention. Very little is published about the retention of cobalamin during retort processing. Preliminary findings saw no difference in cobalamin content when canned pet food was processed for 45, 60, or 90 min in a retort (17). This indicated that cobalamin is not heat-labile and agreed with the findings in the present experiment. Even less is published regarding the effect of retort processing on biotin content; this may be due to minimal instances when biotin supplementation in pet food is necessary (1). It appeared that niacin, cobalamin, and biotin were stable during the retort process when minimum heating lethality values were targeted in the present experiment. This may not be the case if the canned cat foods were processed to higher lethality values and could be addressed in future experiments.

Post-retort samples were higher in pantothenic acid, pyridoxine, and folic acid compared to pre-retort samples. This finding is counter-intuitive and was more than likely influenced by sampling variation and analytical variation, leading to the conclusion that pantothenic acid, pyridoxine, and folic acid are minimally affected by retort processing. Reports of pantothenic losses due to retort processing range from 19.7% [water-blanched spinach (34)] to around 30.0% [home-canned beef and veal (28)]. However, other researchers found that pantothenic acid is not heat-labile and suggested the vitamin may be more protected in food systems than in model systems (35). Similarly, preliminary research with canned cat food identified minimal differences in pantothenic acid content when retort processing time was increased (17). There is greater consensus for pyridoxine and folic acid. Losses of pyridoxine in strained lima beans, stained beef, and tomato juice were 10% or lower and were similar between conventional retorting and high temperature-short time aseptic processing (36). This led researchers to conclude that pyridoxine is minimally impacted by heat processing. Pyridoxine and folic acid were also stable during retort processing of pinto beans (37) soybeans (38) when variability and leaching of vitamins into cooking water were considered. These vitamins are important in supporting pet cat health, but degradation is not a major concern when canned cat foods are processed to minimum intensities.

5. Conclusions

Processing of flexible and semi-rigid containers required different retort processing settings than rigid containers. This was evident in the greater accumulation of lethality during the cooling cycle for these containers and was likely due to the slower cooling of containers to prevent deformation. Flexible containers required less time to achieve target lethality than semi-rigid and rigid containers and small containers required less time than medium containers. Pet food processors should take these findings into consideration when designing the scheduled processes for flexible and semi-rigid containers and create processes that balance food product changes due to over-processing and the risk of not meeting regulations due to under-processing.

Vitamin premix supplementation was necessary to meet minimum recommended levels of thiamin, riboflavin, niacin, pyridoxine, and folic acid. Retort processing decreased levels of thiamin and riboflavin, but container size and container type did not influence degradation differently even though containers were processed to slightly different total lethalities. The other B-vitamins (niacin, pantothenic acid, pyridoxine, folic acid, biotin, and cobalamin) were stable during retort processing. However, it is important to remember that foods were processed to low lethalities and these vitamins could be more sensitive if processing was more intensive. Future research in this area should process the same formula in the same container to increasing lethalities to evaluate this. The same experiment could also identify the accumulated lethality a canned cat food must be processed to for a difference in thiamin and/or riboflavin degradation to be observed. Based on the data in this experiment, pet food formulators should formulate canned cat foods with higher than necessary levels of thiamin and riboflavin to account for processing losses. They should also consider over-formulating the other B-vitamins if the processing facility cooks to higher lethalities than done in this experiment.

Data availability statement

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

Author contributions

CA designed the experiment. LM performed the experiment. AD performed the statistical analysis and wrote the manuscript. All authors revised and provided intellectual input on this manuscript. All authors contributed to the article and approved the submitted version.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships

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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.2023.1175819/full#supplementary-material

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Comparison of two software programs used to determine the relative supersaturation of urine ions

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Introduction: Relative supersaturation (RSS) values for urine crystals are a measure of the risk of urinary stone formation and have been shown to be lowered in foods shown to aid in the management of urolithiasis. In order to calculate RSS in pets, computer programs have been developed to calculate RSS and aid in the understanding of stone formation in veterinary medicine. However, some older programs have not been updated for use in animals, and the specific coefficients used are not publically available. One of the first RSS programs was developed in BASIC computer language and published in 1985 which was called EQUIL2. The EQUIL2 program was updated to a compiled version compatible with a PC platform. However, the formulas could not be read or altered.

Methods: This study evaluates a new program with known coefficients to the original EQUIL2 program. The RSS values of the two programs were compared through a *t*-test, calculating the r^2 from correlation analysis, Lin's concordance correlation coefficient, and by a Bland–Altman analysis of outputs from the two programs using urine samples from healthy dogs and cats.

Results and Discussion: Our results show that for both magnesium ammonium phosphate (struvite) and calcium oxalate, the RSS values of the original program could be calculated from the new programs RSS values. Although the actual RSS values were different (as might be expected through the use of the updated coefficients and different thermodynamic stability constants in the calculations) the results were highly correlated, finding elevations and reductions in RSS proportionally in the same urine samples. The current work creates a foundation for using the modernized program to calculate RSS and provides a shared method for understanding the risk of struvite and calcium oxalate stone formation.

KEYWORDS

cats, dogs, EQUIL software, calcium oxalate, struvite, uroliths, stone risk

1. Introduction

Uroliths are abnormal mineral (stony) deposits occurring in the urinary tract. Stones form by precipitation when precursor molecules exceed their saturation level in the urine and inhibitors fail to prevent agglomeration into substantial calculi. The saturation level is the maximum amount of solute that can be dissolved in a solvent at a specific temperature and is influenced by the

solvents pH, its milieu of ions and factors influencing precipitation. Within the urinary system of a living mammal, there is an absence of large temperature fluctuations, so the saturation level is mainly dependent on the pH of the solution (urine) and the concentration of the specific molecules. A relative supersaturated solution occurs when more than the usual amount of solute required to form crystals is dissolved in the solution at a stable temperature; this phenomenon occurs when pH changes increase the solubility of the solute as well as the interaction of the molecules in the system serving to stabilize the solution. Therefore, relative supersaturation is defined as the ratio of the actual concentration of a dissolved solute in urine to its theoretical solubility in water (1). The RSS ratio is a unitless number reflecting urine pH and specific ion concentration, taking into account the thermodynamic stability constants and their interaction.

Urine becomes supersaturated when it contains more material than can be dissolved by water under normal circumstances. Supersaturation is required for stone formation; however, a relative supersaturation above 1 in companion animals' canine and feline urine may or may not result in stone formation (2–4).

Urolithiasis is common in humans, affecting about 9% of the population in the United States (5), while nephroliths are over 5% (6) and urolithiasis is a significant disease globally (7). Urolithiasis is also prevalent in dogs and cats, causing morbidity, and in some cases, mortality. These stones commonly cause lower urinary tract disease (LUTD) in dogs and cats. Urolithiasis accounts for 18% of LUTDs in dogs (8) and about 7–22% of cases in cats (9–11). Struvite and calcium oxalate (CaOx) comprise most stones in dogs and cats (12).

Relative supersaturation (RSS) is a method for measuring the risk of specific crystal formation in urine and may help predict the risk of urolith formation. The RSS method uses mathematical determinations to predict the predisposition to form stones by analyzing urine pH and the concentrations of different minerals and compounds known to influence the formation of crystals. Risk factors for stone formation include breed, age and genetic makeup. However the controllable food choice and feeding practices also play significant roles (13–17). There are specific changes in food that can influence stone risk including fatty acids (18), hydroxyproline and starch consumption (19), salt consumption (15), and water intake (20). Also, it has been reported that feeding a specific food with the attribute of a reduced urine RSS is beneficial for the management of uroliths (14, 21, 22). Understanding the contribution that food makes in either resolving or avoiding stone formation has been an area of particular interest to pet food manufacturers for decades (1).

There are several methods proposed for calculating urine supersaturation. An ion-activity product index has been developed (23) for estimating urinary CaOx saturation. Marshall and Robertson (24) developed the nomogram for estimation of saturation of urine for different solutes such as CaOx, calcium phosphate, magnesium ammonium phosphate ($MgNHPO_4 \cdot H_2O$; struvite), uric acid, and others. The SUPERSAT program was developed in 1969 (25). An empirical activity product ratio (APR) was also developed and refined (26, 27). The EQUIL2 program (Control for this study) was developed as the first computer program for calculating urinary saturation (28) in order to provide a computerized analysis of supersaturation indices for ions in urine for routine clinical applications. The EQUIL2 program which was previously described for estimating urinary saturation (28, 29) has also been applied to evaluate the RSS in pet urine (13, 30–32).

The EQUIL2 method of computation uses urine pH and the total concentrations of 13 major urinary ions, Na, K, Ca, Mg, NH_4, PO_4, SO_4, oxalate, citrate, urate, pyrophosphate, CO_2, and Cl, together with their thermodynamic stability constants, to calculate the free ion activity products of these urinary ions (28). The ratio of free ion activity products to the thermodynamic solubility products of their salts is then used to calculate the relative supersaturation (RSS) of the different salts. There have been previous enhancements (29) to the EQUIL software, updating the thermodynamic constants, and taking into account CaOx monohydrate's complex chemistry. The EQUIL2 program has been utilized in various applications, including clinical evaluation of human patients suspected of stones and evaluating *in vitro* assays of lithogenic materials (33–36).

In the present study, we document the RSS values of the two common uroliths in the urine of dogs and cats, namely CaOx and magnesium ammonium phosphate (struvite) computed using the new coefficients and the previously established EQUIL2 program for RSS calculations. The goal of the study was to evaluate the relationship between the reported RSS of the two programs thus allowing a comparison of the RSS estimates from EQUIL2 which has previously been used in nutritional research. Also, to provide an understanding of the program we are now making available for general use.

2. Materials and methods

2.1. Origin of urine samples

The study included urine ion analysis from 380 healthy adult beagle dogs and 494 healthy adult shorthair cats. The health status of the dogs and cats was ascertained by daily observations from animal care technicians and annual evaluations, which included a physical examination, blood work (complete hematology and serum biochemistry panels), urinalysis, and diagnostic imaging (abdominal radiographs and ultrasound). All pets were under the care of a veterinarian (an individual for the canine colony and another for the feline colony) who was consulted on and oversaw any health related concerns. Urinary tract health was under frequent evaluation as the pets urine was frequently evaluated for crystals and signs of infection. Any dog or cat with a urinary tract health-related problem or other systemic disease was not eligible for participation. The dogs and cats were maintained separately in a temperature-controlled facility with natural daylight and opportunities for environmental enrichment and exercise. All dogs and cats were fed to maintain their optimal body weight for at least 2 weeks prior to sampling. Quantities of food offered and refused were weighed and recorded daily. Drinking water was offered *ad libitum*. During the adaptation phase the dogs and cats were housed collectively by species. The dogs and cats were also housed individually in lodges, to which they had been acclimated via a habituation program, for the 24 h of urine collection. During the collection period all naturally voided urine was collected in bottles containing thymol. A minimum of 30 mL of urine was saved for analysis. Urine was held at body temperature during collection and pH was measured. Aliquots were frozen at-80°C for all other analysis. The protocols were approved by appropriate Animal Care and Use Committees and adhered to the sponsor's Animal Welfare Policy.

2.2. Urine ion analysis

Urine was analyzed for Ca^{+2}, Cl^{-}, citrate, Mg^{+2}, oxalate, PO_4^{-3}, K^{+}, Na^{+}, SO_4^{-2}, NH_4^{+}, and pH. The specific analyte methods were validated in the laboratory doing the analysis following the FDA Guidelines for Biochemical Analyses. The mineral content of the urine, specifically, Ca, Mg, K, Na and P, are measured using ICP-OES (AOAC Official Method 968.08, Minerals in Animal Feeds and Pet Food, Atomic Absorption Spectrophotometric Method). The minerals (Ca, Mg, K, Na, and P) are measured using an Agilent 5,100 Inductively Coupled Plasma—Optical Emission Spectrometer. The urine is prepared by acidifying 500 µL of the urine with dilute hydrochloric acid. The instrument is calibrated using traceable solutions containing each of these elements. The concentrations of chloride, sulfate and citrate are measured simultaneously using hydroxide ion exchange chromatography. Each sample of whole urine is diluted 400:1 with deionized Water (AOAC Method 993.3, Inorganic Anions in Water by Ion Chromatographic Method). The instrument is calibrated using traceable standards of each ion of interest. The concentration of oxalate in each urine sample is measured using ion exclusion chromatography. The raw urine is acidified using dilute phosphoric Acid prior to freezing to ensure that the oxalate stays in solution. The method used is similar to the previously published method (37). The instrument is calibrated using gravimetrically prepared solutions of sodium oxalate. The urine pH was measured by glass electrode (VWR SympHony B30PCI). The meter was calibrated using phosphate buffer standards at pH 4, 7, and 10. Sulfate, Chloride, and Citrate concentrations are measured using a Thermo-Dionex Ion Chromatograph using conductivity for detection and Chromeleon 7 Chromatographic software. The ammonium content is measured on whole undiluted urine using a method based on the previously published method (38). Basically, alkaline hypochlorite and phenol react with ammonia using sodium nitroprusside as a catalyst to form indophenol. The absorbance of indophenol at 595 nm is directly proportional to the ammonia concentration. The method is standardized with known solutions of ammonium chloride. Ammonium is measured using a Varian Varioskan microtiter plate reader using their SkanIt software. Oxalate is measured using an Agilent 1200 HPLC with a Diode Array Detector watching absorbance at 210 nm for response and OpenLab Chemstation version C.01.07 chromatographic software.

2.3. Pet health

Dogs and cats were assigned to the study if they were above 1 year of age and known to be healthy. Health was established initially by veterinary evaluation and monitored daily through observation by animal care technicians. Urinary tract health was monitored on average more than once a month through evaluation of urine for microbes, crystals or any abnormality (such as an unusual change in pH, urine specific gravity or microscopic abnormalities).

2.4. Statistical analysis

The relationship between values of RSS for CaOx and struvite calculated with the control and new software programs using the measured ion concentrations in urine from dogs and cats was examined using four assessments: a paired *t*-test; Lin's concordance correlation coefficient (CCC) (39); calculation of the r^2 statistic; and scatterplots with Bland–Altman plots (40). The residuals from the paired *t*-test were evaluated by inspection to assure they were normally distributed. Regarding the relationship between the two RSS estimates the scatterplots and the Bland–Altman plots are a visual depiction of the relationship between the different measurements. The Lin's CCC is an assessment of agreement corrected for bias. These and other statistical tests to examine the repeatability of a measurement or whether two methods of measurement produced similar results have been discussed in detail (41). The graphical method, called a Bland–Altman plot, is a plot of the difference versus the average of the two different measures. On the y-axis reference lines are drawn at ±2 standard deviations (SD) and ± 3 SD of the difference. A Bland–Altman plot allows for assessment of the magnitude of disagreement, for error and bias. Linear regression was used to determine if the new program values can be used to predict old program values. A 95% confidence interval (CI) was calculated for the slope estimate. If the 95% CI contained 1, this was further confirmation of agreement between the two programs. However, even if the slope was significantly different from 1, the new program values may still be useful for predicting old program values. The strength of the prediction equations was ascertained using the r^2 statistic. This statistic is a measure of the proportion of variation in old program values explained by the new program values. All statistical analyses were performed using SAS®, version 9.4 program (SAS Institute, Cary, NC, United States).

3. Results

3.1. Relative supersaturation in dogs

3.1.1. CaOx

Table 1 gives the summary statistics of RSS values for CaOx in dog urine samples as determined by the EQUIL2 (Control) and the EQUIL-HL21 software with the new coefficients (New). Mean RSS

TABLE 1 Calculated canine RSS for calcium oxalate using the control and new programs.

	Canine RSS calcium oxalate	
Statistic	Control	New
N[1]	378	378
Mean	15.44	13.15
SD	11.73	9.85
CV	76.0	74.9
Minimum	1.03	0.95
Median	12.675	10.764
Maximum	74.69	68.40
Difference (SE)	2.29 (0.12)	
Prob > |t|	<0.0001	

[1]Two values were removed because they exceeded 3 standard deviations from the population mean.

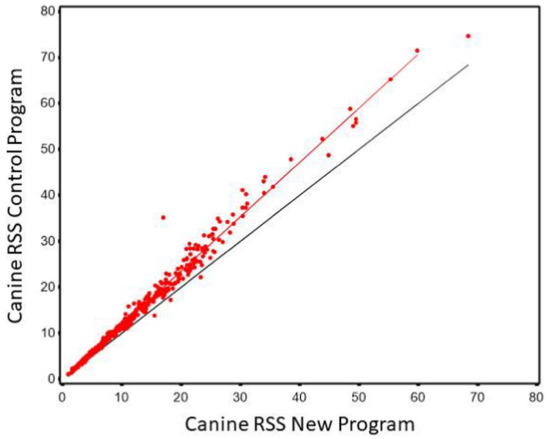

FIGURE 1
The relationship between control and new RSS calculations for calcium oxalate in canine urine. The red line indicates the best fit line from the scatter plots. The black line represents a 1:1 relationship.

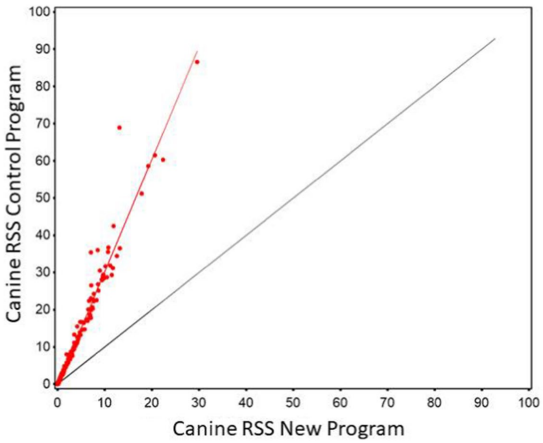

FIGURE 3
The relationship between control and new RSS calculations for struvite in canine urine. The red line indicates the best fit line from the scatter plots. The black line represents a 1:1 relationship.

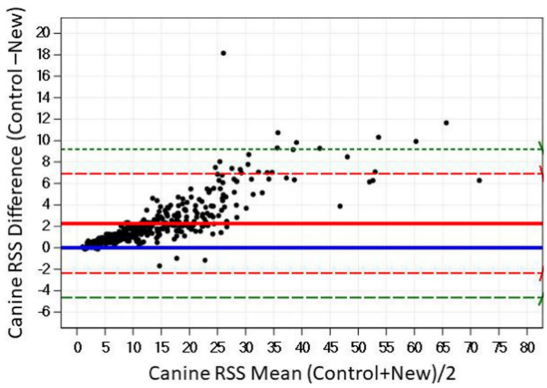

FIGURE 2
The Bland–Altman relationship between control and new RSS calculations for calcium oxalate in canine urine. The solid line indicates the mean of the difference between the values; the dashed red lines indicate 2 standard deviation limits, and the green indicate the limits at ±3 standard deviations, providing an estimate for 99% of the differences.

TABLE 2 Calculated canine struvite RSS using the control and new programs.

	Canine RSS struvite	
Statistic	Control	New
N[1]	375	375
Mean	6.06	2.27
SD	11.30	5.95
CV	187	263
Minimum	0	0
Median	1.010	0.337
Maximum	86.56	92.78
Difference (SE)	4.05 (0.40)	
Prob > |t|	<0.0001	

CaOx values were significantly lower (difference = 2.29, SE = 0.12, p = <0.01) using the new coefficients.

Regression of CaOx values calculated using the new software program could be used to predict CaOx RSS values calculated using the control program with high precision (r^2 of 0.985). The Lin's CCC was 0.956 showing the agreement between the two values is high. However, the slope was significantly different from 1 (slope = 1.181, 95% CI 1.166, 1.196). This results in the average difference between the two values as shown in Figure 1. This indicates that it is at the higher RSS estimates for CaOx where the two software programs have the largest discrepancy and that they are not interchangeable.

The Bland–Altman plots confirm the results from the regression analysis in that as RSS values for calcium oxalate increased, the difference in calculated values between the two software programs also increased (Figure 2).

3.1.2. Struvite

Summary statistics of RSS values for struvite in dog urine are given in Table 2. Mean RSS values for struvite were significantly lower (difference = 4.05, SE = 0.4, p < 0.001) using the new software program.

Regression of struvite values calculated using the new software program could be used to predict struvite values calculated using the old software program with high precision. The strength of the prediction equation was high (r^2 = 0.967). However, the slope significantly different from 1 (slope = 3.028, 95% CI 2.97, 3.09) as shown in Figure 3. The Lin's CCC was 0.461 showing a modest agreement.

The Bland–Altman plots confirm the results from the regression analysis in that as RSS values for struvite increased, the difference in output between the two software programs also increased (Figure 4).

3.2. RSS in cats

3.2.1. CaOx

Summary statistics of RSS values for CaOx values in cat urine samples as determined by the two software programs are given in

Table 3. There was a significant difference in mean RSS values for CaOx (difference = 0.199, SE = 0.014, $p < 0.0001$) between the two software programs.

As in the case with the canine the relationship of the two software programs was such that the old RSS could be predicted from the results of the new (r^2 of 0.987) as shown in Figure 5. However, the slope was significantly different from 1 (slope = 1.048, 95% CI 1.037, 1.059). Thus, it is at the higher RSS estimates for CaOx where the two software programs have the most significant discrepancy. As in the canine CaOx RSS the Lin's CCC showed there was a high agreement between the two values (CCC = 0.996).

The Bland–Altman plots confirm the results from the regression analysis in that as RSS values were closely aligned (Figure 6).

3.2.2. Struvite

Summary statistics of RSS values for struvite in cat urine are given in Table 4. Mean RSS values for struvite were significantly greater (difference = 7.1, SE = 0.61, $p < 0.0001$) from the New software as compared to the Control.

Regression of MAP values calculated using the new software program could be used to predict MAP values calculated using the old software program with high precision (r^2 of 0.971, Figure 7). However, as in the in the case with canine the slope was significantly greater than 1 (3.314, 95% CI 3.26, 3.37). The Lin's CCC showed only modest agreement (CCC = 0.476).

The Bland–Altman plots confirms the results from the regression analysis that as RSS values for struvite increased, the difference in the output between the two software programs also increased (Figure 8).

4. Discussion

This study compared the two computer programs - the previously published EQUIL2 (Control) and the EQUIL-HL21 program with new coefficients (New) for calculating the relative supersaturation (RSS) of dog and cat urine with respect to CaOx and struvite. The original programs take up to 15 and 23 user-inputted ion

TABLE 3 Calculated feline calcium oxalate RSS for calcium oxalate using the control and new programs.

	Feline RSS calcium oxalate	
Statistic	Control	New
N[1]	488	486
Mean	3.42	3.22
SD	2.47	2.35
CV	72.4	73.0
Minimum	0.48	0.53
Median	2.775	2.67
Maximum	31.96	30.29
Difference (SE)	0.199 (0.014)	
Prob > |t|	<0.0001	

[1]Six values were removed because they exceeded 3 standard deviations from the population mean.

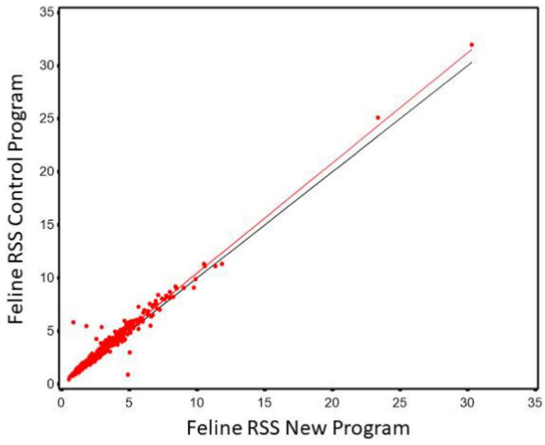

FIGURE 5
The relationship between control and new RSS calculations for calcium oxalate in feline urine. The red line indicates the best fit line from the scatter plots. The black line represents a 1:1 relationship.

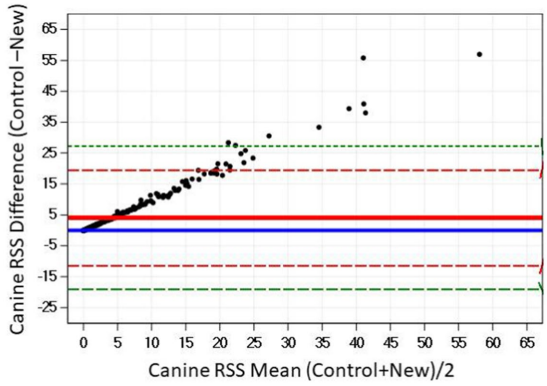

FIGURE 4
The relationship between control and new RSS calculations for struvite in canine urine. The solid red line indicates the mean of the difference between the values; the dashed red lines indicate 2 standard deviation limits, and the green indicate the limits at±3 standard deviations, providing an estimate for 99% of the differences.

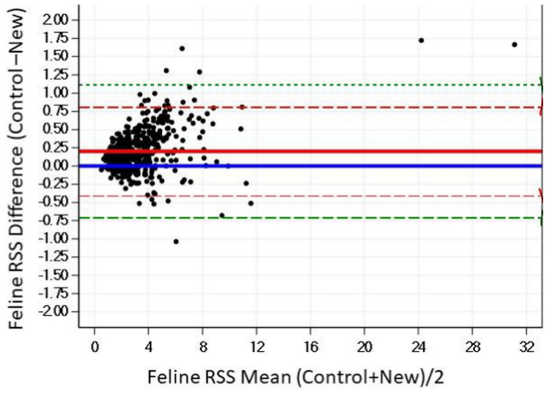

FIGURE 6
The Bland–Altman relationship between control and New RSS calculations for calcium oxalate in feline urine. The solid line indicates the mean of the difference between the values; the dashed red lines indicate 2 standard deviation limits, and the green indicate the limits at±3 standard deviations, providing an estimate for 99% of the differences.

TABLE 4 Calculated feline struvite RSS using the control and new programs.

	Feline RSS struvite	
Statistic	Control	New
N	494	492
Mean	10.34	3.14
SD	19.09	5.66
CV	184.66	180.26
Minimum	0.01	0.003
Median	5.79	1.90
Maximum	296.39	88.51
Difference (SE)	7.11 (0.61)	
Prob > |t|	<0.0001	

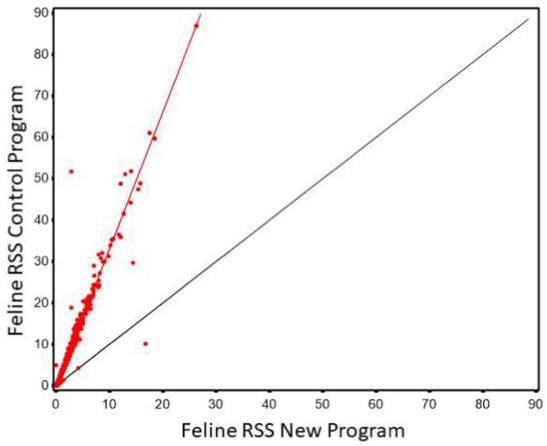

FIGURE 7
The relationship between control and new RSS calculations for struvite in feline urine. The red line indicates the best fit line from the scatter plots. The black line represents a 1:1 relationship.

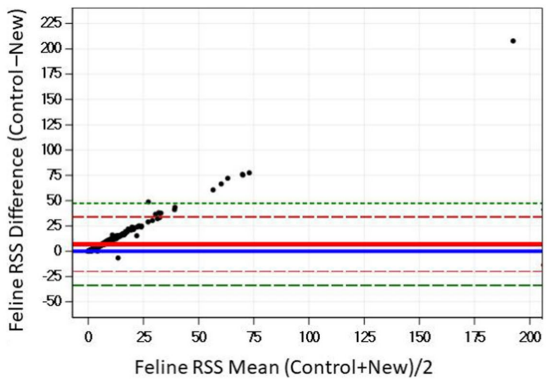

FIGURE 8
The Bland–Altman relationship between control and new RSS calculations for struvite in feline urine. The solid red line indicates the mean of the difference between the values; the dashed red lines indicate 2 standard deviation limits, and the green indicate the limits at±3 standard deviations, providing an estimate for 99% of the differences.

concentrations, respectively. However, these programs are robustly designed to calculate RSS in other sample types (i.e., blood) and for multiple stone types. As such not all input variables have meaning in the urine samples or stone types considered here. This study used the analytes which are present in urine and expected to have an influence on struvite or CaOx supersaturation. The data from this work show that the two programs are highly related in that knowing the RSS from the New calculation allows one to calculate the RSS from the Control program. However, they do not produce identical results. The difference is due to the different thermodynamic stability constants used in the computations by the two programs.

The RSS values for CaOx in dogs were significantly higher using the Control program compared with the New software. This relationship generated the slope of 1.181 so that the increase was found consistently with the higher values showing the greatest differences. For the RSS of CaOx in cat urine, the agreement between the programs was good with a slope of 1.048 (Control/New). For RSS of struvite in dog urine, the slope was 3.08 with higher values in the Control program as compared to the New. Similarly, for RSS of struvite in cat urine the slope was 3.314 showing clear differences and higher values of RSS using the Control program.

We hypothesize the discrepancies in the data obtained using the two programs can be explained by the slightly different coefficients and thermodynamic stability constants used for computations between the two programs. The greater divergence in RSS at higher values indicates that a constant factor in the algorithms is responsible, although the contribution of super-anion binding sites may play a role for CaOx (42). Also, it has been shown that the thermodynamic solubility product of magnesium ammonium phosphate hexahydrate, which is used by the programs to calculate RSS for struvite, is higher than previously reported (43). This may also explain the difference in the values of RSS for struvite for the same sample computed by the two programs.

The study was designed to document the RSS values generated by the two software programs and determine the level of alignment between the values generated by both. The study therefore, does not give information on what program generates values that best represent clinical manifestations or laboratory investigations. Moreover, a limitation in this work is that urine from healthy dogs and cats, which were not known stone formers, was used to determine the range of RSS. This limits the evaluation of the algorithms. The study does provide evidence that when comparing RSS values the comparisons should be made using values calculated by the same program or through understanding the relationship between the two programs used. We also freely offer the program to researchers (Supplementary Table 1), so that well-designed, randomized, controlled clinical studies and cohort or case–control studies can be performed as desired to determine the relative effectiveness of interventions on urinary stone risk.

Comparisons between the Control program and other methods regarding their ability to predict urine supersaturation are available in the scientific literature (24, 26, 29, 44, 45). The predictive values generated by the Control program (using 10 input variables) and by the proprietary SUPERSAT program (Arup Laboratories, aruplab.com, 500 Chipeta Way, Salt Lake City, UT) or a program offered by Royal Canin, using human, dog and cat urine have been reported (39). Values for both programs were similar for CaOx but differed for struvite with the Control program calculating significantly higher struvite RSS (30). The SUPERSAT program used the 10 input variables of EQUIL2 plus uric acid. It is unlikely that the inclusion of

uric acid accounts for a difference in the RSS for struvite or CaOx as uric acid only has influence of supersaturation for RSS of urate stones. Unfortunately, the SUPERSAT program and its coefficients have not been made publically available to laboratories outside the corporation that commissioned the work. LITHORISK, a new predictive software, was developed by Marangella's group (44, 46) for calculations and graphic visualization of risk profiles for stone formation using urine samples. Data obtained from LITHORISK showed good correspondence with those obtained with the Control program in one study (46). However, another study comparing Control and LITHORISK values showed significant differences between the two programs (47). These studies collectively show that these programs can be used to predict urine supersaturation, and no one method has been established as being superior to the others. However, when comparisons are made between diagnoses, treatments, outcomes, or foods, it is essential to use RSS values generated by the same program.

This study has a limitation in that it did not use all possible analytes for predicting RSS and did not have any pets with urolithiasis as data points to evaluate what RSS prediction value is associated with stone formation. Furthermore, there is the general limitation of all RSS values as actual stone formation is dependent on nucleation and the surface energy of the solid phases of stone-salts (48, 49).

In conclusion, this study shows that the relative supersaturation of dog and cat urine with respect to CaOx or struvite can be calculated using either of these programs. Although the programs are highly related they are not interchangeable. The advantage of the new program is that it is compatible with modern operating systems and can serve as an open-source platform for use by the veterinary research community.

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 of Hill's Pet Nutrition, Inc. Written informed consent was obtained from the owner for the participation of the animals in this study.

Author contributions

JB was responsible for statistical analysis. PW was responsible for the coefficients used and the new program to calculate relative super saturation. All authors contributed to the writing and editing of the manuscript and agreed to be accountable for the content of the work.

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Conflict of interest

RA, SD, JM, PW, and DJ were employed by Hill's Pet Nutrition, Inc. JB was employed by Alpha Statistical Consulting.

Publisher's note

Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2023.1146945/full#supplementary-material

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Effect of diet on postprandial glycemic and insulin responses in healthy dogs

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Introduction: Dog owners have gradually changed their approach, paying more attention to the nutrition and health of their animals. Various pet foods with different ingredients and nutritional characteristics are available on the market. The present study aimed to evaluate the administration of three diets, namely, two grain-free (GF1 and GF2) and one grain-based (CB), with different sources of carbohydrates that can influence the glycemic and insulin postprandial responses in healthy dogs.

Materials: Fifteen healthy dogs were dived in three groups and alternatively fed each diet for 50 days. Blood samples were collected at beginning of each feeding period. Glycemia and insulin were measured before and after 120, 240 and 360 minutes diet administration to evaluate postprandial responses.

Results: GF2 diet showed the highest level of albumin and mean insulin concentration ($p < 0.001$). Furthermore, the GF1 diet caused the smallest ($p < 0.001$) glucose and insulin area under the curve (AUC) and the lowest ($p < 0.05$) glucose nadir. Otherwise, GF1 showed the highest ($p < 0.01$) insulin time to peak. The GF2 diet showed the highest level of albumin while reporting the lowest amount of fructosamine ($p < 0.05$). The diet GF2 registered the highest ($p < 0.001$) level of insulin zenith. The cereal-based (CB) diet reported the highest amount of fructosamine ($p < 0.05$). The CB diet had the highest levels of glucose and the highest ($p < 0.001$) glucose and insulin mean concentrations. Diet CB reported the lowest ($p < 0.001$) insulin nadir.

Discussion: Diets with different carbohydrate sources and chemical compositions could modulate the glycemic response in healthy dogs. Bearing in mind that glycemic/insulin postprandial responses influence energy availability and that different dogs have specific lifestyles, it may be preferable to also consider these aspects when choosing a maintenance diet for animals

KEYWORDS

starch, protein, glucose, fructosamine, insulin, energy

1. Introduction

The role of companion animals in society has undergone great changes over time, especially in large population centers, where they have become indispensable (1). In 2022, the annual report of the Federation of European Pet Food Industries (FEDIAF) reported that 90 million European households own at least one pet. Most pet owners pay special attention to their animals, including their diet. Before the advent of industrial feeds, dogs were often fed kitchen and/or butcher shop

scraps. Owners' approach has gradually changed as new knowledge about companion animal nutrition has developed (2, 3). As a result, a huge amount of pet foods characterized by different ingredients and nutrient concentrations have entered the market over the past two decades. In this regard, the content of carbohydrates (e.g., soluble sugars, starch, and dietary fiber) varies greatly among commercial pet food brands. Over the years, the domestication of dogs has improved their ability to digest and metabolize carbohydrates (4). As indicated by Carciofi et al. (5), starch is known as a palatable and digestible source of energy. In addition, carbohydrates allow dogs to store essential nutrients, such as amino acids or fatty acids, especially during specific stages of life. However, no specific carbohydrate requirements have been indicated for companion animals (2, 3, 6). Intrinsic carbohydrate availability may change owing to variations in protein and fat content and the technological processes used. Furthermore, it has been shown that starch digestibility is highly variable, and it is influenced by several factors, such as sources, particle size, amylose:amylopectin ratio, processing methods (7), and starch:protein ratio (8). In addition, all these factors can affect postprandial glycemic levels in healthy dogs (5, 9, 10). The postprandial glycemic response can be assessed on both single and mixed foods. However, the presence of protein and fat may affect the responses and vary the differences between foods. This study aimed to evaluate whether the administration of three diets, namely, two grain-free diets (GF1 and GF2) versus one cereal-based diet (CB), formulated with different sources and amounts of carbohydrates, can influence the postprandial glycemic response in healthy dogs.

2. Materials and methods

2.1. Animal and diets

All the procedures used in the study were 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).

In all, 15 neutered healthy adult dogs (mean age 5.00 ± 1.30 years, body weight 21.1 ± 5.36 kg, and BCS 4.20 ± 0.86 on 5 points scale) were recruited in a private kennel located in the province of Naples (Italy) and homogeneously divided into three groups, which were alternatively fed with three commercial kibble diets (Figure 1). At the time of recruitment, no clinical signs, clinicopathological changes, or the presence of mainly canine vector-borne diseases were observed.

The diets were formulated using the same main protein source (chicken), but different carbohydrate sources and were named GF1, GF2, and CB. The ingredients, chemical compositions, and essential amino acids of each diet are reported in Tables 1–3, respectively.

Dogs were fed to meet maintenance requirements (ME, kcal = $132 \times BW^{0.75}$ kg; 3). Each diet was alternatively administered to all dogs for 50 days (15 of feeding adaptation and 35 of administration). In addition, during the experimental trial, the diets were adjusted according to the weight of the animals.

2.2. Clinical examination and blood sampling

Blood samples were collected (±10 mL) at recruitment and at the end of each nutritional phase in two tubes: one with EDTA, for blood count, and one with separator gel to obtain the serum for the biochemical profile. 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 approximately 15 min until the clot formed and then 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, United States): globulin, total protein (TP), albumin (Alb), alkaline phosphatase (AP), glutamic pyruvic transaminase (GPT), alanine transaminase (ALT), γ-glutamyl transferase (GGT), aspartate

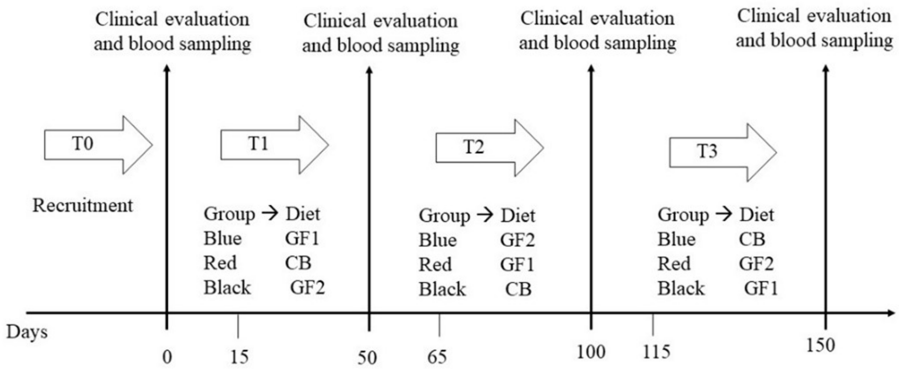

Figure 1. Study timeline.

FIGURE 1
Study timeline.

TABLE 1 Ingredients of three tested diets.

Diet	Ingredients
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.
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.
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

GF1, grain-free diet 1; GF2, grain free diet 2; CB, cereal-based diet.

TABLE 2 Means and standard deviation of the chemical composition of tested diets (% as it is).

	GF1	GF2	CB
Crude protein	36.3 ± 0.21	31.6 ± 0.06	31.7 ± 0.25
Total fat	18.5 ± 0.30	19.3 ± 0.23	19.4 ± 0.81
Crude fiber	2.30 ± 0.10	2.37 ± 0.06	2.47 ± 0.12
TDF	7.68 ± 0.08	8.37 ± 0.05	9.43 ± 0.10
IDF	3.02 ± 0.02	3.76 ± 0.07	3.48 ± 0.08
SDF	4.66 ± 0.01	4.91 ± 0.03	5.95 ± 0.04
Ash	6.50 ± 0.30	6.00 ± 0.06	6.03 ± 0.17
Starch	25.0 ± 0.06	28.0 ± 0.02	27.9 ± 0.58
ME*	3990 ± 2.65	3990 ± 1.82	3997 ± 2.25

GF1, grain-free diet 1; GF2, grain-free diet 2; CB, cereal-based diet; IDF, Insoluble dietary fiber; SDF, soluble dietary fiber. *ME, Metabolizable energy (kcal/kg), calculated according to the predictive equation indicated by NRC (3).

transferase (AST), glutamate dehydrogenase (GLDH), fructosamine (Fr), insulin, α-amylase, lipase (LP)l cholesterol (Col), triglycerides (Tri), creatinine (Crea), BUN, and creatine kinase (CK). Physical examination was conducted and the weight and the body condition score (BCS) of tested dogs were evaluated at the beginning of each experimental period. The blood count and biochemical profile at recruiment are reported in Tables 4, 5.

2.3. Postprandial glucose and insulin response tests

Blood samples were collected at 8:00 a.m. when dogs had been fasting for 12 h (baseline sample, time 0) to determine all the hematological parameters. In addition, blood samples were collected at 120, 240, and 360 min after the meal to measure dogs' postprandial glycemic and insulin responses. The dogs received 50% of the ration after the first sampling (time 0) and the rest of the meal (50%) after the last sampling (360 min). Blood was collected at the beginning of each sampling (3 mL) in a Na-heparin tube, centrifuged (378 × *g* for 5 min), and the plasma was separated into two Eppendorf tubes. One drop of blood from the same sample was immediately used to measure glycemia using a portable digital glucometer (Sinocare Safe-Accu, Safecare Bio-tech, Yuhang, China). All the blood samples from the studied groups were obtained by an expert veterinarian to avoid possible mistakes during blood collection and measurement using the glucometer. Plasma samples were kept under refrigeration (4°C) for a maximum of 2 h before analysis. Insulin plasma samples were frozen (−80°C) for a maximum of 2 months before they were analyzed (11). Insulin was assessed by Chemiluminescence Enzyme Immunoassay (CLIA).

2.4. Calculations

The integrated area under postprandial glucose and insulin response curves for each dog was calculated using the trapezoidal method (JMP 14, SAS Institute, NC, United States). Subsequently, the area of each dog was averaged to determine the AUC of each diet. In addition, based on the blood samples collected from each dog, the average concentration (mean concentration), maximum (zenith) and minimum (nadir) peaks, and the time to reach the maximum increase (time to peak) of glucose and insulin for each diet were determined.

2.5. Statistical analysis

The effect of diet was observed using a mixed model, in which time and animals were the random factors and the diet was the fixed factor. Tukey's HSD test was used when significant differences were observed. All statistical analyses were performed using the software JMP 14 (SAS Institute, NC, United States).

3. Results

3.1. Biochemical profile

Table 6 shows the biochemical profile of tested dogs. During the trial, all parameters fell in the range indicated as physiological for the species (3). The highest level of albumin was registered when dogs were fed the GF2 diet, whereas the lowest levels were observed when the dogs were fed the GF1 diet. CB and GF2 diets resulted in the highest and lowest amounts of fructosamine, respectively ($p < 0.05$). Similarly, when the dogs were fed the cereal-based diet, they had the highest levels of glucose, whereas the GF1 group reported the lowest amount.

3.2. Glucose and insulin postprandial responses

Table 7 shows the variation of serum glucose recorded during the trial. The GF1 diet showed the lowest ($p < 0.001$) values of AUC and the lowest ($p < 0.05$) nadir peak compared to the other diets. The mean glucose concentration was significantly lower ($p < 0.01$) in GF2 diet. The use of the CB diet showed the highest ($p < 0.001$) AUC values related to glycemia.

Table 8 shows the trend of insulin response in the function of the administered diet. CB diet reported the highest insulin response in terms of AUC and mean concentration ($p < 0.01$). The cereal-based (CB) diet had the lowest ($p < 0.01$) zenith and nadir insulin levels ($p < 0.01$), and

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TABLE 3 Essential amino acids profile of the tested diets (means and standard deviation, % as it is).

Amino acid	GF1	GF2	CB
Arginine	2.11 ± 0.30	1.88 ± 0.26	1.80 ± 0.25
Histidine	0.76 ± 0.11	0.72 ± 0.10	0.68 ± 0.10
Isoleucine	1.13 ± 0.16	1.12 ± 0.16	0.97 ± 0.14
Leucine	2.32 ± 0.32	2.20 ± 0.31	2.07 ± 0.29
Lysine	2.08 ± 0.29	2.11 ± 0.30	1.65 ± 0.23
Phenylalanine	1.30 ± 0.18	1.22 ± 0.17	1.18 ± 0.17
Proline	2.20 ± 0.31	1.77 ± 0.25	2.21 ± 0.31
Threonine	1.27 ± 0.18	1.22 ± 0.17	1.08 ± 0.15
Tyrosine	0.87 ± 0.12	0.84 ± 0.12	0.77 ± 0.11
Valine	1.54 ± 0.22	1.48 ± 0.21	1.35 ± 0.19
Cysteine + Cistin	0.42 ± 0.06	0.37 ± 0.05	0.43 ± 0.06
Methionine	1.10 ± 0.15	1.00 ± 0.14	0.93 ± 0.13
Tryptophane	0.33 ± 0.03	0.33 ± 0.03	0.32 ± 0.03

GF1, grain-free diet 1; GF2, grain free diet 2; CB, cereal-based diet.

TABLE 4 Blood count of the tested dogs at recruitment.

Items	Units	Mean value	Reference value
RBC	M/μL	6.80 ± 0.64	5.50–7.90
WBC	K/μL	14.0 ± 3.02	6.00–16.0
Hgb	g/dL	16.2 ± 1.59	12.0–18.0
Hct	%	47.1 ± 4.53	37.5–55.0
MCV	fL	69.3 ± 2.53	60.0–76.0
MCH	Pg	23.9 ± 1.07	20.0–27.0
MCHC	g/dL	34.5 ± 0.44	32.0–38.0
Plt	K/μL	315 ± 87.7	240–400

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.

TABLE 5 Biochemical profile of the tested dogs at recruitment.

Items	Units	Mean value	Reference value
BUN	mmol/L	6.33 ± 2.06	3.2–10.3
Crea	μmol/L	79.8 ± 23.7	44–133
Tri	mmol/L	1.26 ± 0.77	0.3–5.3
Chol	mmol/L	4.14 ± 1.27	3.6–10.3
TP	g/L	63.0 ± 17.9	54–76
ALT	U/L	38.6 ± 14.7	25–122
Bil	μmol/L	2.52 ± 0.86	0–6.8
AP	U/L	43.8 ± 21.3	14–147
GGT	U/L	2.73 ± 1.46	2–13

Crea, Creatinine; Tri, Triglyceride; Chol, Cholesterol; TP, Total protein; ALT, Alanine Transaminase; Bil, Bilirubin; AP, Alkaline phosphatase; GGT, Gamma-glutamyl transferase.

time at peak append earlier in comparison to the other diets ($p < 0.05$). Compared to other diets, the GF2 diet presented the highest ($p < 0.001$) zenith insulin level.

TABLE 6 Biochemical profile of the tested dogs.

Items	Units	GF1	GF2	CB	RMSE	Reference values
Gl	g/L	36.0	36.4	36.7	3.40	24–43
TP	g/L	66.4	67.1	66.0	3.65	54–76
Alb	g/L	28.6^{b}	30.2^{a}	29.3^{ab}	1.14	28–43
AP	U/L	32.6	33.7	35.7	9.02	14–147
Crea	μmol/L	76.1	79.9	80.3	9.01	44–133
BUN	mmol/L	6.19	6.48	6.54	1.40	3.2–10.3
CK	U/L	126	112	114	50.2	41–378
ALT	U/L	45.6	43.3	44.9	12.9	25–122
GGT	U/L	3.12	2.96	3.34	0.95	2–13
AST	U/L	32.3	32.5	34.2	4.84	14–59
Fr	μmol/L	196^{ab}	193^{b}	206^{a}	11.6	177–314
Glu	mmol/L	4.63^{b}	4.86^{ab}	4.97^{a}	0.42	3.2–7.0
α-amylase	U/L	781	783	779	101	333–1264
LP	U/L	86.4	87.4	90.2	18.7	0.1–250
Chol	mmol/L	4.85	4.72	5.10	0.77	3.6–10.3
Tri	mmol/L	0.62	0.71	0.71	0.32	0.3–5.3
Bil	μmol/L	3.33	3.05	3.25	0.56	0.0–6.8

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; Fr, Fructosamine; Glu, Glucose; LP, Lipase; Chol, Cholesterol; Tri, Triglyceride; Bil, Bilirubin. Along the row, lowercase letters indicate differences for $p < 0.05$. RMSE, root means square error.

Figures 2A–C describe the glucose and insulin postprandial curves obtained when dogs were fed the GF1, GF2, and CB diets, respectively. With diet GF1, the level of glucose was always higher than the insulin concentration. Nevertheless, after 360 min both concentrations seemed to be overlapping. Insulin concentration with the GF2 diet was greater than glucose concentration after 120 min. However, at 240 min glucose level increased compared to insulin concentration. With the CB diet, the glucose concentration was always greater than the insulin level, except at 240 min.

4. Discussion

Considering the nutritional characteristics, all the tested diets satisfied the nutritional requirements of adult dogs placed in a kennel (3). No refusals were observed during the experimental period, meaning that the diets were palatable. Furthermore, the amount of feed administered was correctly calculated during the trial, considering that no significant differences were observed regarding live weight and body condition scores.

4.1. Blood metabolic profile

All biochemical parameters fell into the physiological range for canine species (12). In our study, the highest levels of albumin and lowest concentration of fructosamine were reported in the dogs that were fed the GF2 diet. Furthermore, the dogs registered the lowest level of glucose

TABLE 7 Postprandial glucose response in the tested dogs.

Diet	GF1	GF2	CB	RMSE
Glucose (mg/dL)				
AUC 0–360 min	34236^{B}	36371^{A}	36406^{A}	909
Mean concentration	87.3^{AB}	82.8^{B}	89.3^{A}	4.26
Zenith	91.2	92.4	92.5	4.13
Nadir	76.7^{b}	86.1^{a}	82.0^{ab}	5.52
Time to peak (min)	221	221	220	158

GF1, grain-free diet 1; GF2, grain-free diet 2; CB, cereal-based diet; AUC, area under the curve; Mean Concentration, medium level of serum glucose; Zenith, maximum level of glucose; Nadir, lowest level of glucose. Along the row, capital letters indicate $p < 0.01$, and lowercase letters indicate $p < 0.05$. RMSE, root mean square error.

TABLE 8 Postprandial insulin response in the tested dogs.

Diet	GF1	GF2	CB	RMSE
Insulin (mIU/L)				
AUC 0–360 min	3550^{B}	3674^{B}	3897^{A}	186
Mean concentration	9.73^{B}	9.44^{B}	11.9^{A}	1.83
Zenith	14.7^{B}	18.1^{A}	13.1^{B}	2.52
Nadir	9.80^{A}	9.22^{A}	6.45^{B}	1.68
Time to peak (min)	316^{a}	251^{ab}	168^{b}	109

GF1, grain-free diet 1; GF2, grain-free diet 2; CB, cereal-based diet; AUC, area under the curve; Mean Concentration, medium level of serum insulin; Zenith, maximum level of insulin; Nadir, lowest level of insulin. Along the row, capital letters indicate $p < 0.01$, and lowercase letters indicate $p < 0.05$. RMSE, root means square error.

and fructosamine when GF1 and GF2 diets were administered, respectively. These results were unexpected and could be related to the relatively high variability recorded for these parameters (13).

Serum proteins, such as albumin, act as important carrier substances and contribute to the regulation of acid–base balance. Moreover, the body's immune system depends on protein substances (14). Serum fructosamine and plasma glucose are frequently used to assist in the diagnosis and monitoring of diabetes mellitus (15). The term fructosamine is a result of a nonenzymatic chemical reaction between a molecule of glucose and a free amino group (16). Furthermore, serum fructosamine reflects the degree of glycation of serum proteins and the mean serum glucose concentration from the previous 1–3 weeks in dogs, so it could be considered a longer-term marker of glycemic control in comparison with serum glucose measurement, which is a short-term marker (17). Moreover, the same authors observed that the serum concentration of fructosamine is not affected by acute increases in blood glucose concentration, which occur with glucose during stress or excitation.

4.2. Glycemic and insulin response

The postprandial glycemic response shows changes in blood glucose concerning different carbohydrate-containing foods (18). The interpretation of postprandial glycemic responses depends on several factors, such as ingested amount, processing, and diet composition (9). The amount of starch consumed and digested is one of the major factors that affects glucose response to the meal. In our study, the amount of starch intake was quite similar between the diets (75, 80, and 78 g/d for the GF1, GF2, and CB diets, respectively), suggesting a role of starch source on the glycemic and insulin responses. Carciofi et al. (5) investigated the effects of different starch sources, observing that extruded diets composed of similar ingredients but different starch sources can reveal important differences in postprandial glycemic response. Similarly, in the present study, we tested the use of three diets with similar nutritional characteristics but formulated with different carbohydrate sources (cereals grain vs. sweet potatoes vs. pea starch). However, it is difficult to compare our data with the literature due to some limitations in the experimental design (feed administration and sampling). The obtained results suggest that specific characteristics of these ingredients and their level of inclusion affected glycemic response (19, 20). The GF1 diet always reported the lowest values of glucose and insulin AUC. This result could be ascribed both to the lower starch amount of this diet (25 vs. 28% in the GF1 and the other diets, respectively) and to the digestibility of sweet potatoes, which were the main source of starch in the GF1 diet.

Carbohydrate sources, such as potatoes, sweet potatoes, peas, chickpeas, or lentils, are often used in pet foods. These ingredients also provide plant-based protein (21). *In vitro* studies have shown that sweet potatoes can result in a lower glycemic index (GI) due to their higher fibrous fraction and the higher proportion of amylose and resistant starch (RS), which may slow gastric emptying and reduce glucose absorption rate (22, 23). The term resistant starch (RS) indicated the starch residue left after hydrolyzing starch first with sulfuric acid (2 M) and then by incubating the residue with α-amylase and pullulanase (4, 18). Furthermore, the high amount of amylose appears to lower the rate of glucose delivery to blood, promoting a lower glycemic index (24).

Dogs that received the GF2 diet showed the highest glucose and lowest insulin AUC. In the GF2 diet, the main source of starch was pea starch. Pea starch is mainly available as a by-product of protein extraction. In our study, pea starch derived from wrinkled peas, which is more susceptible to be attacked by α-amylase. Furthermore, starch purification process often leads to changes in the starch structure and improves digestibility (25). In addition, thermal processing significantly increases the rapidly digestible starch and decreases the resistant starch fractions in pea starch (26). In this regard, legume starches are more digestible than potato starches, which are rich in amylose but less digestible than starches of several cereal grains (26). As reported by Yang et al. (27), amylopectin is more easily digested than amylose because amylopectin polymers have more intramolecular hydrogen bonds and less surface area. These characteristics could explain the glycemic and insulin responses of GF2 diet. Similarly, GF2 and CB diets showed higher glycemic responses. Whereas GF1 and GF2 diets registered similar insulin responses between the groups.

The observed digestion pattern could be related to the ingredients used in the formulation and the raw material processing method. As reported by Ottoboni et al. (7) and Giuberti et al. (28), technological treatments could cause starch gelatinization (not measured in the present work), which affects glucose release. In our case, some raw materials were heat-treated prior to the extrusion process and then subjected to double heat treatment.

The CB diet showed the highest glucose and insulin AUC and the lowest nadir and zenith values. These results could be due to the high proportion of whole spelt and oats (20%) in the diet, which allows faster energy availability compared to sweet potatoes and pea starch, as suggested by the lower time to peak. The results obtained when the dogs were fed the CB diet suggested a rapid digestibility of that diet. As suggested by Monti et al. (29), as faster and more complete the digestion

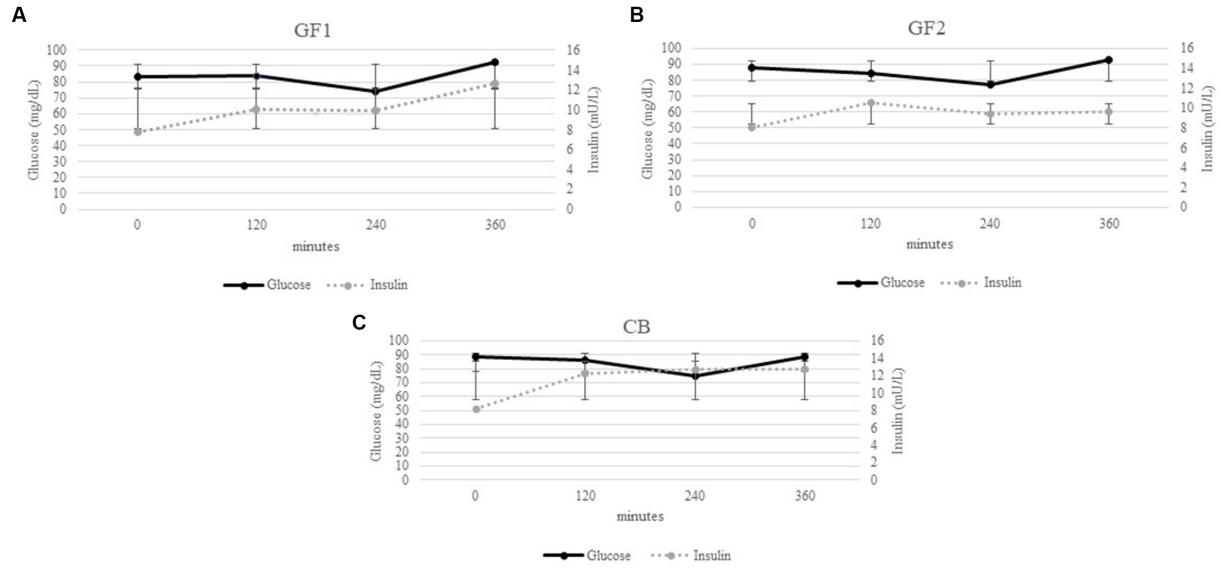

FIGURE 2
Glucose (mg/dL), Insulin (mL/UL).

and absorption of starch are, the greater the postprandial responses. However, compared to GF1 and GF2 diets, the CB diet is richer in total and soluble dietary fiber being composed by whole oat and spelt (30). Brennan and Clearly (31) reported the beneficial effects of soluble fibers on health. Cereal-based diets play a role in modulating the glucose absorption period and in lessening the variation in glucose and insulin concentrations (32). All these considerations were confirmed by the glycemic curve of three diets (Figures 1, 2A,B). In particular, a slower insulin response was observed in the GF1 curves when compared to the GF2 and CB ones. Moreover, the differences registered among the diets could be ascribed to differences in crude protein, total dietary fiber, ether extract, and starch. Indeed, all these nutrients could contribute to the plasma glucose and insulin response (33).

5. Conclusion

In recent years, owners of companion animals have been paying ever more attention to the nutrition and health of their animals. In this respect, the choice of the right diet is crucial regarding several factors, such as age and body weight. Despite some limitations in the experimental design, the obtained results show how different starch sources can lead to a different glycemic response. The grain-free diets (GF1 and GF2), even reported the lack of cereals, showed a different glycemic and insulin response due to the different starch digestibility. Whereas the CB diet showed an increase in glycemic response probably due to the rapid absorption of starch. Further studies are needed on the starch characteristics of tested diets and the potential benefits of these carbohydrates to dog health.

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 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

MG, MC, and SC contributed to the conception and design of the study. AV performed the statistical analysis. AV, AR, MA, and MF performed the analysis. AV, MG, and AR wrote the first draft of the manuscript. AR and MA wrote sections of the manuscript. All authors contributed to the manuscript revision and read and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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Oligosaccharides and diet-related dilated cardiomyopathy in beagles

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Introduction: In 2018 the US Food and Drug Administration reported a potential link between grain-free, legume-containing dog foods and the development of canine dilated cardiomyopathy in atypical breeds. One hypothesis was that high oligosaccharide content in legumes reduced bioavailability of taurine, an amino acid with some previous links to canine dilated cardiomyopathy.

Methods: To address this hypothesis, in the present study, 8 Beagle dogs consumed four diets: a husbandry commercial dental diet, and three test diets formulated with either 30% rice (control), 30% pea (grain-free) or 30% rice with the addition of 1% raffinose (the predominant oligosaccharide found in peas). The study was conducted in a randomized, crossover design with 5 week feeding periods. Measurement of basic health parameters (weight, body condition score, complete blood cell count, chemistry panel), plasma amino acids, cardiac biomarkers (plasma N-terminal pro-brain natriuretic peptide (NT-proBNP) and cardiac-specific troponin I), fecal bile acids and echocardiographic exams were completed pre-study after feeding the husbandry diet as well as after each test feeding period.

Results: Echocardiography showed 9–11% reduction in ejection fraction and 17–20% greater left ventricular end systolic volume with the husbandry diet compared to both grain-containing test diets. Concentrations of plasma NT-proBNP were 1.3–2 times greater after the husbandry diet compared to the grain-based diet, with the oligosaccharide and pea-based diets showing intermediate levels. Plasma taurine levels were unchanged across diets, while plasma methionine levels were highest and cysteine/cystine levels were lowest after dogs ate the husbandry diet.

Discussion: Results indicate that raffinose in the diet is sufficient, but not required to see an increase NT-proBNP, but did not induce any changes in cardiac function after 5 weeks of feeding. Whether this could progress to reduction in cardiac function with longer term feeding is uncertain. A reduced cardiac function along with the greatest increase in NT-proBNP was observed after feeding the husbandry diet that contained the highest amount of insoluble fiber but did not contain legumes or oligosaccharide. Further research into the impact of insoluble fiber in the dental diet is needed to support these novel observations.

KEYWORDS

dilated cardiomyopathy, grain-free dog food, oligosaccharides, dietary fiber, taurine

1. Introduction

In 2018, the US Food and Drug Administration (FDA) reported they were investigating the increased incidence of canine dilated cardiomyopathy (DCM) in breeds not predisposed to the disease who were being fed grain-free dog food with high legume or potato content (1).

The FDA provided updates on the investigation as new information was available, but in none of the updates have they been able to provide an explanation for the observed link (2–5). Since the initial report, research into possible mechanisms has been ongoing with many causes proposed. The causal link however, is yet to be established, and in December 2022 the FDA announced they would no longer be providing updates until critical scientific discoveries are made and urged more research (6).

Dilated cardiomyopathy is a serious chronic myocardial condition characterized by impaired systolic function, left ventricle chamber dilation and ventricular wall thinning that can be fatal (7). Diagnosis of DCM is ideally done through echocardiography and heart failure from DCM progression is defined as an ejection fraction (EF) < 40% (7). Additional screening for DCM includes monitoring for elevations in either a marker of cardiac stretch, blood levels of N-terminal pro-brain natriuretic peptide (NT-proBNP), or a marker of cardiomyocyte damage, cardiac-specific troponin I (8–10). Previously, genetic DCM was known to occur in certain breeds with a predisposition including Deerhounds, Newfoundlands, Irish Wolfhounds, Doberman Pinschers and Cocker Spaniels (11). However, with the FDA report, a nutritionally-mediated DCM has become apparent with peas being the most common ingredient among the reported DCM cases (12). Since then, DCM has been reported to be nutritionally inducible in Golden Retrievers or a mixed breed population after feeding diets with high pea content (13–15), while another study failed to find any echocardiographic changes despite increased blood markers of cardiac disease (16). Moreover, switching dogs to a grain-inclusive or pulse-exclusive diet has been reported to improve cardiac function in dogs with diagnosed DCM (8, 17–19).

Initial research surrounding diet-related DCM centered on the sulfur containing amino acid taurine, whose deficiency has an established link to some forms of canine DCM (20–22). Taurine is found exclusively in animal protein and is completely absent from plant sourced proteins. However, taurine is not considered an essential amino acid for dogs because they can synthesize it from the other sulfur-containing amino acids, methionine, and cysteine (23). Taurine has many roles in the body and is found in particularly high concentrations in heart muscle, where it is thought to be important for cellular energy utilization and membrane integrity (24). Another important function is in the formation of the bile acid taurocholate by conjugating taurine with cholesterol-derived bile acids (25). Taurocholic acid (TCA) is the only conjugated bile acid synthesized by dogs that is then released into the small intestine to assist with the absorption of dietary fat. Most bile acids are reabsorbed and recycled by the body, while some is lost in the feces, which must then be replaced with *de novo* synthesis of TCA (26). There is also the potential for TCA to be transformed by intestinal bacteria through deconjugation of taurine (27). This results in the loss of taurine through consumption by the microbiome, and the formation of secondary bile acids, the majority of which are excreted in the feces (28). Compared to grains, pulses have relatively high concentrations of soluble fiber and oligosaccharides, which have the potential to bind with bile acids in the intestine and facilitate their excretion in stool (28–31). Moreover, oligosaccharides are a direct source of nutrition to the microbiome, and it may be that a higher oligosaccharide intake supports a larger microbial population, leading to reduced bile acid reabsorption and an increase loss in taurine to microbial metabolism (32, 33).

Thus, the connection between grain-free diets and canine DCM may be related to the high oligosaccharide content of legumes, which reduces taurine availability through microbial catabolism and increased fecal bile acid loss. Therefore, it was hypothesized that the consumption of oligosaccharide-containing diets may cause DCM-like cardiac changes and increase fecal bile acid losses, irrespective of the presence or absence of legumes or grains. To address this hypothesis, eight adult beagles were fed three high protein test diets in a randomized, crossover design of 5 week feeding periods. The test diets included a control rice-based diet (GB), an oligosaccharide-enriched rice-based diet (Oligo), and a grain-free, pea-based diet (GF). At the start of the study and at the end of each feeding period, cardiovascular function was assessed using echocardiography as well as plasma NT-proBNP and troponin measured. At the end of each feeding period, measurements included standard blood chemistry and cell counts, plasma levels of sulfur-containing amino acids (taurine, methionine and cysteine/cystine), fecal total bile acid and fecal TCA.

2. Materials and methods

All procedures were conducted following a protocol approved by the University of Saskatchewan's Animal Research Ethics Board and adhered to the Canadian Council on Animal Care guidelines for humane animal use (protocol #20190055). Chemicals and ingredients were obtained from Sigma Aldrich (Markam, ON, Canada) unless specified otherwise.

2.1. Animals

Eight adult colony Beagle dogs (4 spayed females, 4 neutered males, ages 3–5 years old) were obtained from certified scientific breeders (Marshal Bioresources, North Rose, NY, USA and King Fisher International, Stouffville, ON, Canada). The dogs were housed at the Western College of Veterinary Medicine's Animal Care Unit (ACU) at the University of Saskatchewan. During the day, the animals were group housed in a large enclosure with toy enrichment, but individually kenneled overnight and while feeding. Except in inclement weather, the dogs had 24-h access to outdoor runs. Food was provided individually twice daily, with water available *ad libitum*. Dogs received daily walks and socialization. Health was monitored daily, with body weights collected weekly and teeth brushing using only water daily. A veterinarian examined the dogs prior to the start and after the end of the study to ensure good health, and all vaccinations and deworming kept up to date.

2.2. Diets

Diets were formulated using Concept 5 software (Creative Formulation Concepts, Pierz, MN, USA) to meet the Association of American Feed Control Officials (AAFCO) (34) nutrient requirements for adult dogs, with 30% inclusion of either rice or pea flour (Table 1). Three test diets were formulated for the feeding trials, including a control, grain-based rice diet (GB), the rice diet with a 1% inclusion of raffinose, the primary oligosaccharide found in peas (Oligo), and a grain-free, pea-based diet (GF) with a 30%

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TABLE 1 Formulation of test diets.

Ingredients (% inclusion as fed)	GB	Oligo	GF
Rice flour, white	30	30	–
Pea flour, CDC inca	–	–	30
Pork and bone meal	30	30	20.6
Salmon meal	10	10	10
Potato flour	18.6	17.6	28
Canola oil	6	6	6
Salmon oil	3	3	3
Raffinose	-	1	-
Celite	1	1	1
Vitamin/mineral premix	1	1	1
Salt	0.3	0.3	0.3
Choline chloride	0.1	0.1	0.1

Diets were formulated to have 30% inclusion rate of either rice or pea flours, while remaining closely isonitrogenous. GB, grain-based (rice) diet; Oligo, grain-based (rice) diet with 1% added raffinose, an oligosaccharide; GF, grain-free, pea-based diet.

inclusion of whole smooth pea flour (CDC Inca) (35). A previous study found that oligosaccharide content of peas ranged from 2 to 6% (dry matter or DM), which guided the decision to add 1% raffinose to approximate the naturally occurring oligosaccharide content of the GF diet with 30% inclusion of pea flour (12). Celite®, a non-digestible marker made of diatomite, was included at 1% for the purpose of measuring total tract apparent digestibility. In order for the test diets to maintain similar nitrogen content, pork meal content was reduced in the GF diet due to the higher protein content of the pea flour compared to the rice flour. Moreover, potato flour was used to balance diets, with potato flour replacing a portion of the pork and bone meal in the GF diet compared to the GB diet.

2.3. Feeding trial

For 5–6 weeks prior to the start of the feeding trial, all dogs were fed a legume-free, veterinary-grade dental kibble as a husbandry diet (see Supplementary Table S1 for a list of ingredients of this diet). The dogs were then divided into three treatment groups and fed test diets twice daily over 5-week feeding periods in a blinded, randomized, crossover design. Body weights and body condition scores (BCS) using a 9-point scale were measured weekly. For several months prior to this feeding trial, individual daily portions were adjusted as needed to maintain BCS within ideal range (BCS 4–5), any uneaten food weighed to calculate actual food consumption and individual calorie requirement per day calculated from energy density (metabolizable energy or ME) of the husbandry diet according to the guaranteed analysis on the label. During the cross-over periods with our test diets, individual isoenergetic meal portions were calculated based on ME of test diet proximate analyses. All meal portions were kept to a consistent energy for each dog throughout the cross-over study, aside from one dog who required an 8% increase in portion size on the 3rd week of the first feeding period while on the Oligo diet due to unexplained weight loss and a drop in BCS below ideal. This dog regained her weight after 3 weeks and was able to maintain a stable weight within the ideal BCS at this increased portion size.

2.4. Nutrient and fiber analysis

Random 10–25 g subsamples from the test and husbandry diets were ground in the lab, then sent to Central Testing Laboratory Ltd. (Winnipeg, MB, Canada) for proximate and amino acid analysis according to AOAC standards as listed in Tables 2, 3. Dietary fiber content of all diets was assessed by Eurofins Scientific (Toronto ON, Canada) according to method AOAC 2011.25-M. Dietary fiber was differentiated into the categories of low molecular weight dietary fiber (LMWDF), insoluble high molecular weight dietary fiber (IHMWDF), and soluble high molecular weight dietary fiber (SHMWDF). LMWDF includes oligosaccharides, as well as other soluble and prebiotic fibers such as inulin, polydextrose and others. IHMWDF includes dietary fibers that do not dissolve in water, including cellulose and lignin, as well as resistant starch. SHMWDF consists of dietary fibers that dissolve in water, including hydrocolloids, gums, soluble pectin, and cereal ß-glucan. Individual amounts of the three main oligosaccharides found in legumes (raffinose, stachyose and verbascose) was also performed by Eurofins Scientific (29, 36).

2.5. Total tract apparent digestibility

Feces were collected during the final 2 days of each feeding period from each dog immediately after defecation (taken before feces fell to the ground or within a few seconds of landing on the ground) and stored at −20°C until used in further analyses. Samples were then pooled for a given individual, mixed and dried for 72 h at 65°C before being ground to a powdered consistency. Portions were sent to an external lab for proximate nutrient and amino acid analysis (Central Testing Laboratory Ltd., Winnipeg, MB, Canada). The Celite® present in the feces was measured as acid insoluble ash (AIA) in the proximate analysis. This value was used to calculate total tract apparent digestibility using the formula (37):

$$\textit{Nutrient Digestibility}\,(\%) = \left[1 - \left(\frac{\%\ \textit{nutrient in feces}\ x\ \%\ \textit{AIA in food}}{\%\ \textit{nutrient in food}\ x\ \%\ \textit{AIA in feces}}\right)\right] x100$$

2.6. Fecal bile acid analysis

A portion of the powdered, dried feces was used to measure total fecal bile acid content using a commercial assay kit (Total Bile Acid Assay Kit, Cell Biolabs Inc. Sand Diego, CA, USA). The assay measured bile acid content through a colorimetric enzyme driven reaction with 3α-hydroxysteroid dehydrogenase, NADH and thio-NAD^+. A second portion of feces was used to measure TCA content using a commercial assay kit (Taurocholic Acid (TCA) ELISA Kit, Abbexa LTD, Cambridge, UK). This ELISA used a competitive inhibition assay with a colorimetric detection.

TABLE 2 Proximate analysis of commercial husbandry diet and lab-formulated test diets.

Nutrient %DM (g/1000 kcal)	Husbandry		GB		Oligo		GF	
Metabolizable energy (kcal/kg)[1]	4,023		3,640		3,698		3,694	
Moisture[2]	8.1	(20)	10.7	(29)	12.9	(35)	10.6	(29)
Crude protein[3]	28.7	(71)	30.4	(84)	31.2	(84)	30.2	(82)
Crude fiber[4]	2.82	(7.0)	0.33	(0.9)	0.63	(1.7)	1.89	(5.1)
Fat[5]	13.2	(33)	10.6	(29)	11.2	(30)	10.9	(30)
Acid insoluble ash[6]	0.18	(0.5)	1.10	(3.0)	1.10	(3.0)	1.07	(3.0)
Non-fiber carbohydrates[7]	47.3	(118)	46.7	(128)	45.4	(123)	46.2	(125)
Total digestible nutrients[8]	81.2	(202)	79.7	(219)	79.8	(216)	79.3	(215)

% DM, percent dry matter; GB, grain-based (rice) diet; Oligo, grain-based (rice) diet with 1% added raffinose, an oligosaccharide; GF, grain-free, pea-based diet.
[1]Determined by Central Testing Laboratory Ltd. (Winnipeg, MB, Canada) using method AOAC 930.15.
[2]Determined by Central Testing Laboratory Ltd. (Winnipeg, MB, Canada) using method AOAC 930.03.
[3]Determined by Central Testing Laboratory Ltd. (Winnipeg, MB, Canada) using method by Ankom Technologies (2017) based on AOCS Ba 6a-05.
[4]Determined by Central Testing Laboratory Ltd. (Winnipeg, MB, Canada) using method AOAC A 5–04.
[5]Determined by Central Testing Laboratory Ltd. (Winnipeg, MB, Canada) using method AOAC 990.03.
[6]Determined by Central Testing Laboratory Ltd. (Winnipeg, MB, Canada) using method by J. Van Keulen and B.A. Young, AOAC 942.05.
[7]Determined by Central Testing Laboratory Ltd. (Winnipeg, MB, Canada) using equation Non-Fiber Carbohydrates (DM): 100 – [(% Dry Matter/100) + % Protein + %Fat + %Ash + %Crude Fiber].
[8]Determined by Central Testing Laboratory Ltd. (Winnipeg, MB, Canada) using equation Total Digestible Nutrients (DM): 100 – [%Dry Matter/100) + %Ash + %Crude Fiber] – 8.

TABLE 3 Sulfur-containing amino acids cystine, methionine and taurine content of commercial husbandry diet and lab-formulated test diets.

Amino acid %DM (g/1000 kcal)	Husbandry		GB		Oligo		GF		AAFCO min requirements	
Cystine	0.46	(1.14)	0.30	(0.82)	0.38	(1.03)	0.26	(0.70)	N/A	
Methionine	0.54	(1.34)	0.33	(0.91)	0.43	(1.16)	0.24	(0.65)	0.33	(0.83)
Cystine + Methionine	1.00	(2.49)	0.63	(1.73)	0.81	(2.20)	0.50	(1.35)	0.65	(1.63)
Taurine	0.07	(0.17)	0.14	(0.38)	0.19	(0.51)	0.13	(0.35)	N/A	

% DM, percent dry matter; GB, grain-based (rice) diet; Oligo, grain-based (rice) diet with 1% added raffinose, an oligosaccharide; GF, grain-free, pea-based diet.
Determined by Central Testing (Winnipeg, MB, Canada) using method AOAC 994.1.

2.7. Blood plasma analysis

Blood was collected in the morning on the final day of each test period after an overnight fast through jugular venipuncture. Samples of whole blood were collected into a heparinized and serum tubes, then sent to an external laboratory (Prairie Diagnostic Services, Saskatoon, SK, CA) to perform complete blood counts (CBC) and basic chemistry panels. Another portion of blood was collected in an EDTA tube and centrifuged at 2200 RPM for 10 min. Plasma was aliquoted and stored at-80°C until needed for further analyses. One set of plasma aliquots were sent to an external lab for measurement of the sulfur-containing amino acids cysteine/cystine, methionine and taurine (University of Victoria Proteomics Centre, Victoria, BC, CA) using Ultra-high Pressure Liquid Chromatography-Multiple Reaction Monitoring-Mass Spectrometry methodology. Other plasma aliquots were used in commercial kits for plasma canine NT-ProBNP and canine high-sensitivity cardiac specific troponin I (troponin) following manufacturer's directions (Nordic BioSite Life Sciences, Täby, Sweden). Both of these latter kits were colourimetric-based immunoassays validated by the manufacturer for canine samples.

2.8. Echocardiographic assessment

All dogs were acclimated to the echocardiographic and blood pressure measurement procedures prior to beginning the feeding trial. On the 35th day of each feeding trial, echocardiograms were completed on the dogs while in fasted states according to previously described methods from this lab (13, 38). All measurements and imaging were performed by the primary researcher (EB). Training on ultrasound technique had been provided by an experienced veterinarian and greater than 200 h of experience performing ultrasonography was completed prior to the study. Blood pressure (BP) and heart rate were determined by the mean of 3 measurements taken from the base of the tail using a high-definition veterinary oscillometer (VET HDO® High Definition Oscillometry, Babenhausen, Germany). A Sonosite Edge II ultrasound machine (FUJIFILM Sonosite Inc., Toronto, ON, CA) was used to measure left ventricle chamber size and function during diastole and systole using Simpson's rule for 2D ultrasonography in the left parasternal apical two-and four-chamber views. End-point measurements include ejection fraction (EF), cardiac output (CO), stroke volume (SV), left ventricle end diastolic volume (EDV), left ventricle end systolic

volume (ESV) and peak mitral valve velocity during early diastole (Vmax E). Volumes were calculated using Simpson's rule of disks. M-mode imaging was done through the right parasternal short-axis view at the level of the papillary muscles to assess left ventricular internal diameter during diastole (LVIDd) and systole (LVIDs). End-point measurements were taken as an average from at least two values per individual collected in separate cine loops during the exam. Values collected from echocardiographic assessment were normalized to the dog body weight for Simpson's rule volumes, while M-mode obtained values for LVIDd and LVIDs measurements were standardized to different exponents of body weight according to guidelines suggested by Visser et al. (39).

2.9. Data handling and statistics

Data was analyzed using IBM SPSS 28 Statistics Software (IBM SPSS Statistics Software, Armonk, NY, USA). Data was tested for normality using the Kolomogorov–Smirnov (KS) test and homogeneity of variance using Levene's test. When appropriate, data was log transformed to meet normality. If parametric assumptions were met, a repeated-measures, one-way ANOVA was performed, followed by Fisher's least significant difference *post-hoc* test if significance was found ($p \leq 0.05$). If normality was not met, analysis was done using Friedman's test for ranked data.

3. Results

3.1. Diet proximate analysis

The three lab-formulated test diets contained similar amounts of metabolizable energy, crude protein, and crude fat per (Table 2). Calorie density fell between 3,640 kcal/kg to 3,698 kcal/kg among the test diets, while the husbandry diet contained higher energy content at 4,023 kcal/kg. Crude protein content ranged from 28.7–31.2%DM (71.3–84.4 g/1000 kcal) among all diets, all much higher than minimum protein requirements for adult dog maintenance of 18%DM (45 g/1000 kcal) as established by AAFCO (40). Crude fat content was highest in the husbandry diet at 13.2%DM (32.8 g/1000 kcal), while the formulated test diets contained similar crude fat content ranging from 10.6–11.2%DM (29.1–30.0 g/1000 kcal). Crude fiber ranged from 0.33–2.82 %DM (0.91–7.01 g/1000 kcal) among the diets, with the following rank order from lowest to highest: GB < Oligo < GF < husbandry diet.

3.1.1. Fiber and amino acid content in diets

Total dietary fiber was found to be highest in the husbandry diet, and lowest in the GB and GF diet (Table 4). Quantity of low molecular weight dietary fiber (LMWDF) was highest in the GF diet, followed by the Oligo diet, while neither the husbandry nor GB diets had detectable levels of LMWDF (Table 4). While none of the test diets or the husbandry diets had detectable levels of the oligosaccharides, stachyose or verbascose, the 1% inclusion of raffinose in the Oligo diet resulted in similar raffinose content to the GF diet. In contrast, the GB diet and the husbandry diet contained no detectable levels of raffinose. Insoluble fiber was considerably higher in the husbandry diet compared to all test diets, while the GF diet had lower levels compared to both grain-containing test diets. The rank order of SHMWDF from lowest to highest was: GF = Oligo < husbandry diet < GB diet.

The husbandry diet had a higher content of cysteine and methionine compared to all test diets, while containing lower taurine (Table 3). Among the test diets, the Oligo diet had the highest amount of all 3 sulfur-containing amino acids, followed by the GB diet. Analysis of amino acid content found that the methionine and methionine + cystine content of the GF diet fell below the AAFCO minimum requirements for adult dogs of 0.33 %DM (0.83 g/1000 kcal) methionine, and 0.65 %DM (1.63 g/1000 kcal) methionine + cystine. Methionine + cystine content of the GB diet also fell below minimum requirements when measured as %DM, but not if adjusted to g/1000 kcal.

3.2. Weight and condition

No significant differences in weight or body condition score were observed in dogs after being fed different test diets for 5 weeks (Table 5). Daily calorie provisions were similar among test diets, but greater in the husbandry diet. This was unintentional and due to a combination of overestimation of calorie amount in the formulations of the test diets, and the manufacturer's reported calorie content being lower than what we later determined in proximate analyses after the feeding study was complete.

3.3. Digestibility

Apparent total tract digestibility of crude protein, fat and non-fiber carbohydrate (NFC) was statistically similar across the test diets (Table 6). Digestibility could not be measured in the commercial dental husbandry diet since it lacked a non-digestible marker. Among the test diets, there was also no difference in digestibility of cysteine. However, methionine digestibility was lower in the GF diet compared to both GB and the Oligo diet. Taurine digestibility was also lower in the GF diet compared to the Oligo diet, but not the GB diet.

3.4. Plasma amino acids

Plasma taurine levels were unchanged across all diets at the end of each 5-week feeding period (Table 7). In contrast, plasma methionine levels were significantly higher after dogs were fed the husbandry diet compared to either the Oligo diet or the GF diet, but intermediate after the GB diet. Cysteine/Cystine levels differed in dogs after feeding the different diets, with the significantly highest level observed in dogs after feeding the GB diet compared to after all other diets, while the husbandry diet had half this value that was significantly the lowest.

3.5. Fecal bile acid and taurocholate analysis

Total fecal bile acid was significantly lower after dogs were fed the husbandry diet for 5-weeks compared to after the GB and GF diets, but

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TABLE 4 Fiber content of the commercial husbandry diet and lab-formulated test diets.

Fiber parameter % w/w (g/1000 kcal)[1]	Husbandry	GB			Oligo		GF	
LMWDF	<0.6	(<1.6)	<0.6	(<1.8)	0.8	(2.5)	0.9	(2.7)
IHMWDF	6.6	(17.8)	2.9	(8.9)	3.2	(9.9)	2.6	(7.9)
SHMWDF	1.6	(4.3)	1.8	(5.5)	1.3	(4.0)	1.3	(3.9)
Total DF	8.2	(22.2)	4.7	(14.5)	5.3	(16.5)	4.8	(14.5)
Raffinose	<0.2	(<0.5)	<0.2	(<0.6)	0.8	(2.5)	0.8	(2.4)
Stachyose	<0.2	(<0.5)	<0.2	(<0.6)	<0.2	(<0.6)	<0.2	(<0.6)
Verbascose	<0.2	(<0.5)	<0.2	(<0.6)	<0.2	(<0.6)	<0.2	(<0.6)

% w/w, percent weight by weight; GB, grain-based (rice) diet; Oligo, grain-based (rice) diet with 1% added raffinose, an oligosaccharide; GF, grain-free, pea-based diet; DFLMWDF, low molecular weight dietary fiber; IHMWDF, insoluble high molecular weight dietary fiber; SHMWDF, soluble high molecular weight dietary fiber; DF, dietary fiber.
[1]Determined by Eurofins (Toronto ON, Canada) using method AOAC 2011.25-M.

TABLE 5 Body weight, body condition score, daily portions, and daily calorie allotment (kcal/d) of dogs fed a commercial husbandry diet, grain-containing rice diets without (GB) or with (Oligo) the addition of the oligosaccharide raffinose, or a grain-free pea-based diet (GF).

	Husbandry	GB	Oligo	GF	*P*-value
Weight (kg)	9.26 ± 0.911	9.13 ± 0.858	9.16 ± 0.914	9.11 ± 0.874	0.476
BCS	4.7 ± 0.28	4.8 ± 0.164	4.5 ± 0.21	4.6 ± 0.18	0.613
Daily portion (g)	175 ± 19.8	180 ± 18.4	176 ± 18.7	179 ± 18.4	0.401
Kcal/d (kcal)	707 ± 79.6[a]	655 ± 66.9[b]	652 ± 69.3[b]	662 ± 68.0[b]	0.001

BCS, body condition score.
$N = 8$ Data is shown as Mean ± SEM. Statistics with one-way repeated measures ANOVA.
Different letters indicate significant differences in Fisher's LSD *post hoc* analysis ($p < 0.05$).

TABLE 6 Percent total tract apparent digestibility of test diets, including grain-containing rice diet without (GB) or with the addition of the oligosaccharide raffinose (Oligo), or a grain-free pea-based diet (GF).

	GB	Oligo	GF	*P*-value
Protein[1]	85.6 ± 2.54	84.4 ± 1.11	84.3 ± 0.45	0.849
Fat[1]	98.8 ± 0.15	98.4 ± 0.33	98.5 ± 0.24	0.537
Carbohydrate[1]	94.9 ± 1.14	95.0 ± 0.52	92.7 ± 0.60	0.142
Cysteine[2]	84.2 ± 3.32	85.7 ± 2.03	81.4 ± 1.69	0.404
Methionine[2]	93.5 ± 1.35[a]	94.5 ± 0.96[a]	89.6 ± 1.65[b]	0.006
Taurine[2]	84.9 ± 3.16[ab]	87.6 ± 2.14[a]	80.2 ± 3.16[b]	0.050
Total amino acids[2]	84.8 ± 2.82	86.6 ± 1.43	81.4 ± 1.33	0.203

$N = 8$ Data is shown as Mean ± SEM. Statistics with one-way repeated measures ANOVA.
Different letters indicate significant differences in Fisher's LSD *post hoc* analysis ($p < 0.05$).
[1]Determined by Central Testing (Winnipeg, MB, Canada) according to the methods listed in Table 2.
[2]Determined by Central Testing (Winnipeg, MB, Canada) using method AOAC 994.12.

there was no statistical difference among the test diets (Table 8). No statistically significant difference in TCA excretion or the ratio of this parameter to total bile acids across the any of the diets was observed.

3.6. Blood and plasma analysis

The CBC showed no difference in white blood count among any of the diets after feeding them for 5-weeks (Table 9). Red blood cell count fell below the lower limit of the reference range after feeding the husbandry diet, which was significantly lower than what was seen with all other diets. Both hemoglobin and hematocrit were also found to be statistically lower after feeding dogs the husbandry diet compared to after any of the test diets. Mean corpuscular volume was higher while mean corpuscular hemoglobin and mean corpuscular hemoglobin concentration were both significantly lower after feeding the husbandry diet compared to after feeding the test diets. Red cell distribution width was not statistically different among any of the diets, and except for red blood cells, all values remained within the reference ranges.

Blood indicators of renal function, digestive enzymes and fasting blood glucose showed no statistical differences after dogs were fed diets and all values remained within the reference ranges (Supplementary Table S2). Indicators of hepatic function fell within the reference range aside from total protein, which ranged from 51 to 52 g/L, which is below the reference range, but with no statistical differences among the diets (Supplementary Table S3). Cholesterol levels remained within reference range, but were statistically higher in dogs after they were fed the husbandry diet compared to after the test diets. Total bilirubin significantly decreased, while alkaline phosphate increased in dogs after being fed the husbandry diet compared to after all test diets. Globulin levels in dogs after being fed the husbandry diet were significantly higher than after being fed the GF diet, while the GB and Oligo diet were intermediate and did not differ from any other. Albumin to globulin ratio was significantly lower in the dogs after being fed the husbandry diet compared to after the Oligo and GF diets.

In general, blood electrolyte levels all remained within the reference range across all diets, and no statistical differences were seen between in dogs after any of the diets (Supplementary Table S4). Exceptions were a significant reduction in bicarbonate and an

TABLE 7 Plasma levels of sulfur-containing amino acids of dogs fed grain-containing diets without (GB) or with the addition of the oligosaccharide raffinose (Oligo), or a grain-free pea-based diet (GF) over 5 week feeding periods.

Amino acid (μM)	Husbandry	GB	Oligo	GF	*P*-value
Taurine	74.3 ± 10.65	81.03 ± 9.70	74.29 ± 8.79	74.16 ± 7.31	0.521
Methionine	60.03 ± 3.12^{a}	57.34 ± 4.05^{ab}	54.38 ± 7.31^{b}	53.24 ± 3.99^{b}	0.023
Cysteine/Cystine	18.65 ± 1.35^{a}	32.87 ± 3.23^{b}	25.39 ± 9.70^{c}	26.08 ± 2.53^{c}	0.002

$N = 8$. Statistics with one-way repeated measures ANOVA. Different letters indicate significant differences in Fisher's LSD *post hoc* analysis ($p < 0.05$).

increased anion gap in dogs after being fed the husbandry diet compared to other test diets, but both values remained well within normal reference range.

3.7. Plasma N-terminal pro-brain natriuretic peptide and cardiac-specific troponin I

We found significant differences in plasma NT-proBNP after feeding dogs the different diets (Figure 1). Specifically, after being fed the husbandry diet for 5 weeks, dogs had significantly higher NT-proBNP levels than after all other diets. In addition, NT-proBNP levels in dogs after being fed the Oligo and GF diets were intermediate to this, but statistically greater than after the GB diet. Although a similar trend was observed for mean plasma troponin levels, owing to significant variability after feeding each diet, no statistically significant differences were detected.

3.8. Echocardiography and cardiovascular health

Blood pressure and heart rate did not differ in dogs after being fed the different diets for 5-weeks, with all values falling within the reference range for a healthy adult dog (Table 10). Ejection fraction (EF) in dogs after feeding the husbandry diet was significantly lower than after both grain-containing test diets but did not differ from after the GF diet (Table 10). After the GF diet, dogs also had a significantly lower EF compared to after the Oligo diet, but not after the GB diet. End systolic volume (ESV) in dogs after feeding the husbandry diet was significantly larger than after both grain containing diets. No other significant differences in the other echocardiogram measurements were detected in dogs after feeding any of the diets.

4. Discussion

4.1. Important outcomes

The outcomes of this study support there being a role of fiber in the link between atypical DCM and diet, but not in the way we hypothesized. Notably, we saw adverse changes in the markers of cardiac health and function among the diets after only 5-weeks of feeding the diets, which reversed with diet change. We saw an increase in NT-proBNP after feeding both the Oligo and the GF diets compared to after feeding the GB diet, but we found no corresponding differences in cardiac function as measured by echocardiography. However, the results after feeding the commercial, grain-containing husbandry diet were most striking. The husbandry diet (a commercial dental diet) led to the greatest increase in NT-proBNP and a coincident lower EF secondary to increased ESV compared to the grain containing diets, all consistent with early subclinical DCM. These changes were independent of plasma taurine status, as it did not differ among any of the diets and stayed within the reference range of 41–97 μM (21).

4.2. Diets

We succeeded in developing test diets with similar nutrient profiles while incorporating equal amounts of either rice or pea flour. Pea flour was chosen because it is the most common legume among the diets reported by the FDA and has recently been identified as the primary ingredient of concern in a foodomic analysis of the original DCM cases (6, 41). Our inclusion of 1% w/w raffinose in the Oligo diet correctly approximated the naturally occurring oligosaccharide of our GF diet. We also maintained similar protein levels near 30% for all the test diets. This not only meets the AAFCO minimum protein requirements of 18%, but is comparable to high protein content of many premium pet foods on the market (34, 42). Levels of methionine and combined cystine/methionine fell below the required minimums established by AAFCO in both our GF and GB diet when measured as %DM, although the GB diet did meet the requirements when presented as g/1000 kcal (34). These levels of methionine and combined cystine/methionine are comparable to previous studies with pea-based diets from our lab where diets were intentionally low in sulfur-containing amino acids to push dogs to more readily develop DCM-like changes (13, 43). However, the high overall crude protein and higher taurine content of the diets in the current study could lessen concerns about the low levels of precursor amino acids and mimic current premium diets where purified taurine is now commonly included.

A legume-free, grain-containing dental diet was chosen as a husbandry diet to support dog oral health and fed for 5–6 weeks pre-study. Crude protein and non-fiber carbohydrates content in the husbandry diet was similar to the test diets, but calorie density and fat content was slightly greater. Dental diets often include higher amount of certain dietary fibers to facilitate the removal of dental plaque through abrasion (44, 45). This is consistent with what we found in our husbandry diet, which listed both dried beet pulp and cellulose in the ingredient list, leading to considerably higher in IHMWDF compared to the test diets (Supplementary Table S1). The subclinical DCM-like cardiac changes observed after feeding the husbandry diet, which contained no measurable oligosaccharides, does not support our hypothesis that oligosaccharides are responsible for the development of nutrition related DCM.

TABLE 8 Bile acid content per gram feces, including both total bile acid (total BA) and taurocholic acid (TCA) quantity in dogs fed grain-containing diets without (GB) or with the addition of the oligosaccharide raffinose (Oligo), or a grain-free pea-based diet (GF) over 5 week feeding periods.

	Husbandry	GB	Oligo	GF	*P*-value
Total BA (μM/g)	45.6 ± 2.1[a]	53.1 ± 2.2[b]	49.7 ± 0.9[ab]	52.4 ± 0.7[b]	0.033[1]
TCA (nM/g)	80.8 ± 45.1	41.4 ± 27.9	10.6 ± 2.6	12.4 ± 3.6	0.075[2]
TCA to Total BA (%)	0.218 ± 0.140	0.073 ± 0.048	0.022 ± 0.007	0.024 ± 0.007	0.051[1]

$N = 8$. Data is shown as mean ± SEM. Analysis was done with [1]one-way repeated measures ANOVA and [2]Friedmans nonparametric test. Different letters indicate statistical differences using Fisher's least significant difference *post-hoc* analysis ($p < 0.05$).

TABLE 9 Complete blood count (CBC) and serum cholesterol of dogs fed grain-containing diets without (GB) or with (Oligo) the addition of the oligosaccharide raffinose, or a grain-free pea-based diet (GF) over 5 week feeding periods.

	Reference range	Husbandry	GB	Oligo	GF	*P*-value
WBC (10^9/L)	4.9–15.4	6.3 ± 0.43	6.0 ± 0.50	5.7 ± 0.38	5.9 ± 0.49	0.567
RBC (10^{12}/L)	5.80–8.50	5.67 ± 0.17[a]	6.21 ± 0.145[b]	6.13 ± 0.15[b]	6.15 ± 0.15[b]	<0.001
HGB (g/L)	133–197	135 ± 3.0[a]	150 ± 2.7[b]	148 ± 3.0[b]	149 ± 1.9[b]	<0.001
HCT (L/L)	0.390–0.560	0.405 ± 0.008[a]	0.434 ± 0.007[b]	0.429 ± 0.008[b]	0.429 ± 0.007[b]	0.019
MCV (fL)	62.0–72.0	71.6 ± 1.26[a]	70.1 ± 0.80[b]	70.0 ± 0.92[b]	70.0 ± 0.79[b]	0.012
MCH (pg)	21.0–25.0	23.9 ± 0.33[a]	24.1 ± 0.32[b]	24.3 ± 0.32[b]	24.2 ± 0.21[b]	0.026
MCHC (g/L)	330–360	333 ± 2.0[a]	344 ± 2.4[b]	347 ± 1.3[b]	346 ± 2.2[b]	<0.001
RDW (%)	11–14	12.7 ± 0.62	13.1 ± 0.26	12.8 ± 0.50	12.8 ± 0.36	0.080
Cholesterol (mmol/L)	2.70—5.94	5.04 ± 0.406[a]	4.27 ± 0.241[b]	4.14 ± 0.211[b]	3.96 ± 0.186[b]	<0.001

$N = 8$. Data is shown as mean ± SEM. Analysis was done with one-way repeated measures ANOVA. Different letters indicate statistical differences using Fisher's least significant difference *post-hoc* analysis ($p < 0.05$).
Reference range provided to show if values fall outside of normal limits and were for qualitative comparison only to experimental results.
WBC, white blood cell; RBC, red blood cell count; HGB, hemoglobin; HCT, hematocrit; MCV, mean corpuscular volume; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; RDW, red cell distribution width.

4.3. Amino acid digestibility and plasma concentration

Due to the known link between taurine deficiency and some cases of DCM, we compared the effects of the diets on taurine and its precursor amino acids methionine and cysteine. The digestibility of the amino acids methionine and taurine were lower in the GF diet compared to the Oligo diet, agreeing with previous studies from this lab (43). This suggests that raffinose does not negatively impact the digestibility of sulfur containing amino acids, keeping in mind that results of apparent fecal digestibility can be misleading when the microbiome can act upon that nutrient (46). Reduced sulfur amino acid digestibility after feeding pea-based GF diets was attributed to increased total dietary fiber and amylose content in a previous study (43). We did not see this same relationship since the husbandry, GB and Oligo diets contained higher amounts of IHMWDF, and the GB contained higher SHMWDF compared to the GF. However, amylose content was not specifically assessed in this study so we cannot dismiss this as also being a factor.

There was no difference in dog plasma taurine levels among the diets, despite differences in digestibility, and lower taurine content in the husbandry diet. This aligns with what our lab has seen previously in both 7 day and 28 day feeding periods of pulse-based diets compared to grain-based diets in Beagles (13, 38, 43, 47). Effects of diet on plasma taurine in other studies were less consistent. With observational studies reporting both higher and lower taurine levels after feeding grain-free diets (16, 48). Breed differences appear to be at play, with one study finding that Beagle dogs are less susceptible to taurine depletion compared to larger mixed breed dogs (33). This study also suggested that plasma taurine is a less sensitive indicator of taurine status compared to skeletal muscle as a proxy for cardiac muscle, which may offer some explanation for conflicting results.

While all values remained within the reference range in the current study, plasma methionine levels were highest in the dogs after feeding the husbandry diet (49). The higher plasma methionine may be due to the higher dietary methionine in the husbandry diet. However, this explanation does not address the lower plasma cystine/cysteine levels in dogs after feeding the husbandry diet, which is unexpected given that this diet contained higher amounts of cysteine compared with the test diets. The lower plasma cysteine/cystine may be related to taurine availability since taurine stores are maintained in part by increased taurine synthesis through cysteine degradation in the liver (50, 51). The need for increased taurine synthesis may also be behind the lower plasma cystine/cysteine in dogs after feeding the Oligo diet compared to after the GB despite greater amount of dietary cystine in the Oligo diet. This would suggest that the additional oligosaccharide did lead to increased taurine loss.

4.4. Fecal bile acid

When examining whether oligosaccharides may cause taurine depletion through increased fecal bile acid losses, we found no

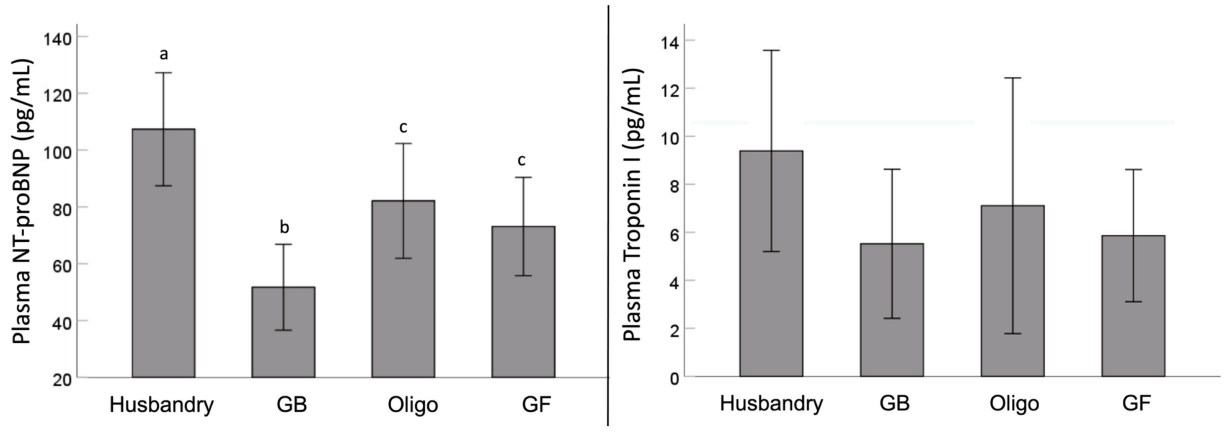

FIGURE 1
Plasma levels of N-terminal pro-brain natriuretic peptide (NT-proBNP) and high sensitivity cardiac specific troponin I (Troponin I) levels of dogs fed grain-containing diets without (GB) or with the addition of the oligosaccharide raffinose (Oligo), or a grain-free pea-based diet (GF) over 5 week feeding periods. Different letters indicate statistical differences using Fisher's least significant differenes *post hoc* analysis.

TABLE 10 Parameters of cardiac function determined with echocardiography in dogs fed grain-containing diets without (GB) or with the addition of the oligosaccharide raffinose (Oligo), or a grain-free pea-based diet (GF) over 5 week feeding periods.

	Husbandry	GB	Oligo	GF	*P*-value
DBP (mmHg)	66 ± 2.5	69 ± 2.6	68 ± 3.2	70 ± 1.8	0.685
SBP (mmHg)	120 ± 5.3	135 ± 4.2	129 ± 5.4	127 ± 3.4	0.142
Heart rate (BPM)	75 ± 5.7	79 ± 4.2	74 ± 4.6	79 ± 2.9	0.750
EF (%)	62.0 ± 1.77^{a}	67.4 ± 1.96^{bc}	68.8 ± 2.15^{c}	63.9 ± 2.65^{ab}	0.044
CO (L/min/kg)	0.10 ± 0.008	0.11 ± 0.007	0.11 ± 0.008	0.11 ± 0.007	0.803
SV (ml/kg)	1.4 ± 0.12	1.4 ± 0.12	1.5 ± 0.12	1.4 ± 0.08	0.611
EDV (ml/kg)	2.24 ± 0.132	2.09 ± 0.142	2.22 ± 0.147	2.19 ± 0.077	0.447
ESV (ml/kg)	0.84 ± 0.036^{a}	0.68 ± 0.045^{b}	0.70 ± 0.067^{b}	0.80 ± 0.054^{ab}	0.047
LVIDd (cm/kg$^{0.299}$)	1.30 ± 0.053	1.27 ± 0.051	1.26 ± 0.068	1.33 ± 0.055	0.533
LVIDs (cm/kg$^{0.387}$)	0.75 ± 0.042	0.71 ± 0.033	0.71 ± 0.040	0.76 ± 0.036	0.148
Vmax E (cm/s)	66.3 ± 5.14	65.9 ± 2.64	62.0 ± 3.47	63.4 ± 3.00	0.633

$N = 8$. Data is shown as mean ± SEM. Analysis was done with one-way repeated measures ANOVA. Different letters indicate statistical differences using Fisher's least significant difference *post hoc* analysis ($p < 0.05$).
DBP, diastolic blood pressure; SBP, systolic blood pressure; BPM, beats per minute; EF, ejection fraction; CO, cardiac output; SV, stroke volume; EDV, end diastolic volume; ESV, end systolic volume; LVIDd, left ventricular internal diameter – diastolic; LVIDs, left ventricular internal diameter – systolic; Vmax E, early-wave maximum velocity through the mitral valve.
EDV, ESV, SV, and CO utilized volume measurements taken from 2/4-chamber views and Simpson's method of disks, while LVIDs and LVIDd values were taken from *M*-mode echocardiogram.

differences in total bile acid losses after feeding our test diets regardless of oligosaccharide content. However, we did see a reduction in total bile acid excretion with the husbandry diet, which contained no detectable oligosaccharides but higher IHMWDF. These results agree with what we have previously seen in our lab, where increased amounts of IHMWDF led to reduced fecal bile acid excretion (13, 43). The diets associated with decreased bile acid excretion in these previous studies also contained higher levels of oligosaccharides, but the results of the current study do not support a role for oligosaccharides, or at the very least not raffinose, in mediating decreased total fecal bile acid excretion.

Although there were large relative differences in the excretion of the primary bile acid TCA in the feces, the values did not meet the criteria for statistical significance due to a high variability. The proportion of TCA to total bile acids (BA) also presented large differences in variability, and only slightly missed the threshold of statistical significance (Friedman's test $p = 0.051$). The TCA/BA proportion was nearly 10x greater in the husbandry diet compared to the Oligo and GF diets. Since oligosaccharides are known to promote overgrowth of intestinal microbes, the lower TCA excretion in the Oligo and GF could be explained by greater deconjugation of bile acids by the microbiome (52, 53). Pezzali et al. (54) looked at bile acid excretion in dogs fed grain-based and grain-free diets, and similarly found no difference in total bile acid excretion but did observe a greater excretion of primary bile acid and percent primary bile acid in the higher-oligosaccharide, grain-free diet. The differences seen in bile acid composition in both studies supports the premise that the gut microbiome is acting upon bile acids differently with different dietary treatments. These shifts in the microbiome may be impacting taurine availability, which could explain the discrepancy in plasma cysteine/cystine levels seen between

the Oligo and GB diet. Although it is established that the gut microbiota can impact taurine levels, the role of the microbiome in the development of nutrition-related DCM remains largely unexplored, and there is a growing need for more thorough research in this area (22, 33, 55).

4.5. Basic health and blood analysis

Throughout this study all the dogs remained healthy, without any progression to clinically significant DCM or heart failure despite changes consistent with early DCM after dogs were fed the husbandry dental diet. Although the husbandry diet provided greater calories daily than the test diets, weight and body condition score remained consistent throughout the study. The reduced red blood cells and other changes in hemoglobin parameters after dogs were fed the husbandry diet were consistent with a mild anemia, agreeing with a previous similar study by Bakke et al. that involved a feeding trial of a high legume diet and a retrospective analysis of health records of dogs diagnosed with DCM (56). Our lab has also seen lowered red blood cells after both 7 and 28-day feeding periods of feeding diets high in insoluble fiber (13, 47). Although IHMWDF was not measured directly in the 7-day feeding trial, crude fiber provides a relative approximation of insoluble fiber content, since it does not capture any soluble fiber or oligosaccharide present.

In the current study, we observed an increase in total cholesterol levels in dogs after feeding the husbandry diet compared to our test diets. This agrees with a previous study reporting dogs diagnosed with DCM compared to healthy controls have coincident elevations in cholesterol (10). This observed increase is likely related to bile acid excretion, since bile acids are produced by the conjugation of taurine with cholesterol, and we saw lower fecal bile acids after dogs were fed the husbandry diet. This is consistent with the understanding that insoluble fiber does not bind to bile acids as readily as soluble fiber, so is less likely to facilitate their excretion in stool (57). Lower fecal bile acid excretion suggests a higher reuptake of bile acids, and less cholesterol lost in the feces (28, 31, 58).

4.6. Cardiac biomarkers and echocardiography

One of the most noteworthy results in our study is the diet-related differences observed with plasma NT-proBNP which is stimulated by cardiac stretch and can be indicative of the severity of DCM (59, 60). The increases we saw in plasma NT-proBNP after feeding the husbandry diet were coincident with impaired systolic function (reduced EF and increased ESV), consistent with early changes associated with DCM. In contrast, NT-proBNP was also significant higher in dogs after feeding the GF and Oligo diets compared to the GB control diet, albeit to a lesser extent than after the husbandry diet and with no corresponding cardiac function changes. Circulating NT-proBNP is recognized as being one of the earliest indicators of cardiac dysfunction, and its levels may rise before any physical changes can be observed (10). However, any increase in a short feeding period of only 5-weeks in a relatively resistant breed such as the Beagle indicates the potential to develop into overt DCM if fed longer term or to more susceptible breeds (9, 10, 60).

These results align with previous studies where DCM-related changes in cardiac function have been observed with short-term diet change (8, 13, 14, 17, 19, 48). Two prospective feeding studies observed significant echocardiographic changes consistent with subclinical DCM after feeding a high pea diets (13, 14). In both studies peas were the primary protein source, and it is likely that the very high pea content (~60%) is behind the observed functional changes. In contrast, the current study only saw an increase in NT-proBNP after feeding our GF diet, which had pea content (30%) that more closely matched levels found in standard commercial diets (42). Also of note, Quilliam et al. (13) included a wrinkled pea variety in their diet that is known to have much higher amylose, but similar oligosaccharide content, whereas commercial pet food uses almost exclusively smooth-pea varieties.

The husbandry dental diet was selected to help manage periodontal disease that is common among Beagle dogs and deemed appropriate because it did not contain pulses or any other ingredients of concern (61, 62). The poor outcomes seen with the husbandry diet led us to reconsider the role of oligosaccharides in the pathogenesis of nutrition-related DCM and look more closely at other forms of fiber. Although we saw an increase in NT-proBNP after feeding the Oligo diet compared to after the GB diet, the impact of raffinose on cardiac health was minimal compared to the changes we observed after feeding the husbandry diet that was high in insoluble fiber. Since pulses are often higher in insoluble fiber compared to grain flours, our observations are still consistent with a high inclusion of pulses being behind the development of diet-related DCM (63). The mechanism underlying this possible link is still unclear, but the differences we saw in primary versus secondary bile acid excretion suggest the microbiome may be involved.

4.7. Limitations

We acknowledge that there are limitations in our study, particularly the small sample size of 8 Beagles and lack of washout period. To offset these factors, we conducted the study in a crossover design, which increased power and decreased variability. We also randomized treatment to counteract the latter limitation. The use of Beagles may also be seen as a limitation, given that they are not predisposed to developing DCM or taurine-deficiency, but several previous studies have now demonstrated diet-induced cardiac changes in this breed (13, 21). Supporting the utility of Beagles for nutritionally-mediated DCM studies, the current study also detected subclinical DCM changes, which was reversed with diet change.

A second limitation was the fact that the husbandry diet was not part of the randomized periods used for the other test diets. Moreover, proximate analyses performed after the pre-trial period where we fed the husbandry diet revealed that the energy density was much higher than the guaranteed analysis on the label for this commercial diet. This led to the dogs being fed significantly higher energy during the pre-trial period than intended. This husbandry diet also had higher sulfur amino acid levels, thus would have been predicted to protect cardiac health, but the opposite was observed.

Another limitation may have arisen from the inclusion of potato flour, which was originally listed as an ingredient of concern by the FDA (1). However a recent foodomic analysis concluded that potatoes were not significantly represented among diets associated with the initial nutrition-related DCM cases, and reaffirmed that peas, or possibly lentils, were the main ingredient(s) of concern (41). Potato flour was

chosen to balance the diet formulations due to its relatively low fiber and protein content. We recognize however, that potato flour is not devoid of protein or dietary fiber. Thus, the potato flour may have impacted amino acid balance, as well as modulated the secondary effects of extrusion and resulting kibble density or gelatinization on nutrient availability (64).

5. Conclusion

As seen previously in our lab, this study demonstrated cardiac outcomes consistent with early subclinical DCM in a Beagle model, which was reversed with dietary changes (13, 38). We observed that raffinose present in the diet, either added in purified form or due to inclusion of peas, is sufficient, but not necessary to increase NT-proBNP. However, no adverse cardiac function changes were coincident with this blood parameter change with either of the oligosaccharide-containing diets. This could be due to there being no link between raffinose and DCM in dogs, or alternatively, that higher pea inclusion or a longer feeding period is needed to see adverse cardiac changes from oligosaccharides. Most noteworthy from this study is that the poorer outcomes were observed after feeding the commercial dental diet, which contained no legumes or detectable oligosaccharides, but was instead high in IHMWDF from the addition of cellulose and beet pulp. We propose that the higher content of insoluble dietary fiber present in the dental diet may be linked to these poorer outcomes, but further research looking more directly at this relationship versus oligosaccharides is needed. An exploration of the role of the microbiome is also warranted considering the differences noted in primary and total bile acid excretion.

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 Research Ethics Board of the University of Saskatchewan.

Author contributions

EB and LW designed the study and drafted the article. EB formulated test diets, conducted the study, and performed the data analysis. All authors contributed to editing the article, as well as interpreting and verifying results.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2023.1183301/full#supplementary-material

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Dietary choline, but not L-carnitine, increases circulating lipid and lipoprotein concentrations, without affecting body composition, energy expenditure or respiratory quotient in lean and obese male cats during weight maintenance

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Introduction: Due to the involvement in one-carbon metabolism and lipid mobilization, choline and L-carnitine supplementation have been recommended to minimize hepatic lipid accumulation and support fat oxidation, respectively. This study investigated the lipotropic benefits of choline or L-carnitine supplementation in lean and obese cats maintaining body weight (BW).

Methods: Lean [$n = 9$; body condition score (BCS): 4–5/9] and obese ($n = 9$; BCS: 8–9/9) adult male neutered colony cats were used in a replicated 3 x 3 complete Latin square design. Treatments included choline (378 mg/kg $BW^{0.67}$), L-carnitine (200 mg/kg BW) and control (no supplement). Treatments were supplemented to the food for 6 weeks each, with a 2-week washout between treatments. Cats were fed once daily to maintenance energy requirements, and BW and BCS were assessed weekly. Fasted blood collection, indirect calorimetry, and dual-energy X-ray absorptiometry occurred at the end of each treatment period. Serum was analyzed for cholesterol (CHOL), high-density lipoprotein CHOL (HDL-C), triglycerides (TAG), non-esterified fatty acids (NEFA), glucose, creatinine (CREAT), urea, alkaline phosphatase (ALP) and alanine aminotransferase (ALT). Very low-density lipoprotein CHOL (VLDL) and low-density lipoprotein CHOL (LDL-C) were calculated. Data were analyzed using proc GLIMMIX, with group and period as random effects, and treatment, body condition, and their interaction as fixed effects, followed by a Tukey's *post-hoc* test when significance occurred.

Results: Cats supplemented choline had lower food intake ($P = 0.025$). Treatment did not change BW, BCS and body composition ($P > 0.05$). Obese cats had greater ALP, TAG, and VLDL, and lower HDL-C compared to lean cats ($P < 0.05$). Choline resulted in greater CHOL, HDL-C, LDL-C and ALT ($P < 0.05$). L-carnitine resulted in lower CREAT ($P = 0.010$). Following the *post-hoc* test, differences between treatment means were not present for ALP ($P = 0.042$). No differences were found for glucose, urea or NEFA ($P > 0.05$). Obese cats had a lower fed respiratory quotient (RQ), regardless of treatment ($P = 0.045$). Treatment did not affect fed or fasted RQ and energy expenditure ($P > 0.05$).

Discussion: Choline appeared to increase circulating lipid and lipoprotein concentrations regardless of body condition, likely through enhanced lipid mobilization and hepatic elimination. Neither dietary choline or L-carnitine altered body composition or energy metabolism in the lean or obese cats, as compared to control.

KEYWORDS

dual-energy x-ray absorptiometry, feline nutrition, indirect calorimetry, methionine, methyl donor, one carbon metabolism, pet obesity

1. Introduction

Feline hepatic lipidosis (FHL) is defined by the excess storage of hepatic lipids and is considered the most common liver disease affecting cats in North America (1). Although the exact pathophysiology of FHL has not been identified, there is evidence that the mobilization of free fatty acids from the adipose tissue leads to the accumulation of lipids within the livers of affected cats (2). Food deprivation, whether due to secondary anorexia or due to an imposed high degree of energy restriction, is considered a key factor in the development of FHL (1, 3–6). Left untreated, the prognosis for affected cats is considered poor and may result in liver failure and death (7). Obese cats are at an increased risk of developing FHL due to their increased levels of adipose tissue and subsequent stores of fatty acids available for mobilization (2, 8). Also, higher concentrations of hepatic triglycerides (TAG) and an increased risk of concurrent insulin resistance are believed to leave obese cats more susceptible to FHL (9, 10). Additionally, energy restriction is often recommended in obese cats in order to lose weight. However, a low degree of dietary energy restriction often does not result in weight loss in an obese cat, and therefore may result in a higher degree of energy restriction may be implemented (11, 12).

Under normal conditions, fatty acids within the hepatocytes can undergo β-oxidation within the mitochondria for the production of acetyl-CoA, or they can be re-esterified to TAG (13, 14). These TAG can be secreted into the circulation or they can continue to accumulate within the hepatocytes. It is unclear what leads to the accumulation of hepatic TAG during FHL. However, the metabolic idiosyncrasies that result from their obligate carnivorous nature put cats at risk for deficiencies in certain dietary essential nutrients. Insufficient intakes of dietary choline and carnitine during energy restriction have been proposed as potential risk factors in the pathogenesis of FHL (15).

Choline is a precursor for the biosynthesis of phosphatidylcholine (PC). Specifically, PC is required for packaging TAG and cholesterol into very low-density lipoproteins (VLDL) for export out of the liver (16). Betaine, a derivative of choline, also serves as a methyl group donor for one of the pathways through which homocysteine is remethylated to methionine. The production of methionine subsequently results in the methylation and synthesis of numerous metabolites, including carnitine (17). Previous research in mammals, including cats, found that choline deficiency leads to the development of fatty liver, which can be reversed and/or prevented when dietary choline is added back to the diet in sufficient quantities (18–20). In cats, increased dietary choline supplementation leads to increased concentrations of serum lipids and lipoproteins in both obese and overweight cats (21, 22), suggesting increased lipid hepatic mobilization through the synthesis and secretion of VLDL from the liver. Additionally, choline and its derivative betaine may reduce fat mass gain, as suggested in livestock species and growing kittens fed *ad libitum* post-gonadectomy (23–25).

In comparison, carnitine is required to facilitate the entry of fatty acids into the mitochondria for β-oxidation. This is considered the main pathway for the disposal of fatty acids under normal physiological conditions and is important for the production of ATP by the mitochondria (13). L-carnitine increases lipid oxidation in overweight cats, as suggested by increased palmitate oxidation (26), energy expenditure (EE) and lower post-prandial respiratory quotient (RQ) (27). Additionally, L-carnitine supplementation may decrease food intake and weight gain in *ad libitum* feeding scenarios (28). For these reasons, L-carnitine is commonly used by the pet food industry in the formulation of weight control and weight loss diets for cats. In human medicine, reduced β-oxidation has been suggested as a potential cause of lipid accumulation in the liver of patients with non-alcoholic fatty liver disease (NAFLD) (29). Thus, it has been proposed that an insufficient supply of carnitine may similarly be an important factor for the development of FHL, although the evidence to support this has been inconsistent (30–34).

Nutrients and nutraceuticals that can reduce the risk of hepatic lipid accumulation in obese cats and assist in mitigating weight gain would benefit the pet food industry and the veterinary community. Currently, there is evidence that dietary choline may provide such benefits to overweight and obese cats (21, 22). However, there have been no studies comparing the lipotropic benefits of dietary choline to L-carnitine, a dietary supplement commonly used by the pet food industry. Therefore, the objective of this research was to investigate the lipotropic effects of dietary choline compared to L-carnitine (a positive control) and no supplement (a negative control), on serum lipid and lipoprotein profiles, body composition, EE and RQ in obese and lean adult cats. We hypothesized that supplemental choline would result in greater serum lipid and lipoprotein concentrations, as compared to L-carnitine or control, whereas L-carnitine would increase EE and lower RQ only in the

obese cats, suggesting favored fatty acid oxidation. Additionally, we hypothesized that both choline and L-carnitine would improve body composition by decreasing fat mass and increasing lean body mass only in the obese cats, as compared to control.

2. Materials and methods

All procedures were approved by the University of Guelph Animal Care Committee (AUP#4496), in compliance with provincial and national guidelines regarding the care and use of animals in research.

2.1. Animals and housing

Eighteen domestic shorthair (DSH) male neutered cats (Marshall's Bio Resources, Waverly, NY, United States of America) were enrolled in this trial. The cats were between 1 to 2 years of age (mean ± SEM: 2.00 ± 0.11 years; range: 1.28–2.29 years) and were considered healthy prior to the start of the trial, based on physical examination, medical history, and the results of complete blood count (CBC) and serum biochemistry completed within 6 months of the trial. The cats in the present study were fed using a modified *ad libitum* protocol during growth (25). At the start of the trial, the 18 cats were classified into two groups, "obese" or "lean", based on assigned body condition score (BCS) (35). Nine of the 18 cats were classified as obese, with a BCS of ≥ 8/9 (36), and a mean body weight (BW) of 6.46 ± 0.15 kg. The remaining nine were classified as lean, with an assigned BCS of 4–5/9 and BW of 4.62 ± 0.15 kg.

All 18 cats were housed together in a free-living environment (23 ft x 19 ft) at the Animal Biosciences Cattery at the Ontario Agricultural College of the University of Guelph (Guelph, ON, Canada). The room had a controlled 12 h light 12 h dark cycle, with the lights turning on at 0700 h and off at 1,900 h. Humidity and temperature were maintained at 40% and 24°C respectively throughout the trial.

Water was available to the cats *ad libitum* in bowls and through an open tap. The room was cleaned daily and enrichment was provided through regular interaction with familiar people and within the environment, as previously described in Frayne et al. (37).

2.2. Diet

Four weeks leading up to the trial (adaptation period) and throughout the entire length of the trial, cats remained on a commercial extruded diet (Nutram Total Grain-Free® Chicken and Turkey Recipe, Elmira Pet Products, Elmira, ON, Canada) formulated for feline adult maintenance, per the Association of American Feed Control Officials (AAFCO) (35). To be fed individually, cats were separated and placed in individual cages for 1 h daily (08:00 h). Food was provided in quantities to maintain current BW based on historic dietary intake data. Individual orts were measured and recorded daily. Fasted BW and BCS were recorded weekly by the same assessor (A.R.).

As previously detailed in Rankovic et al. (22), nutrient analysis of the diet (Table 1) was performed using methods described by the Association of Official Analytical Chemists (AOAC) and the American Oil Chemist Society (AOCS) (Bureau Veritas, Mississauga, ON, Canada) (38, 39). This included the measurement of moisture (AOAC 935.29), crude protein (AOAC 990.03), crude fat (AOAC 920.39), crude fiber (AOCS Ba6a-05), ash (AOAC 942.05), total dietary fiber (AOAC 991.43, 985.29), pyridoxine [B6, AOAC 985.32 (modified)], folate (B9; AOAC 2004.5), cobalamin (B12; AOAC 986.23), choline (AOAC 999.14). Dietary carnitine concentrations were measured by liquid chromatography-mass spectrometry, according to AOAC 2012.17 (38). In addition, concentrations of dietary amino acids were measured using ultra-performance liquid chromatography (UPLC), as previously described by Cargo-Froom et al. (40). Nitrogen free extract (NFE) was calculated by difference (NFE % = 100-moisture-protein-fat-crude fiber-ash), and metabolizable energy (ME) was estimated using the Modified Atwater equation [ME = 10 x (3.5 x %crude protein) + (8.5 x %fat) + (3.5 x %NFE)] (41).

2.3. Study design and dietary choline and L-carnitine supplementation

All cats received each of the three treatments (choline, carnitine and control) in a 3 x 3 Latin square design. Prior to the trial, the 18 cats were split into three groups of six. Each group was balanced for BW and consisted of three obese and three lean cats. Each treatment was provided for a 6-week period, and cats were placed on a 2-week washout between periods where they received only the commercial cat food with no supplementation. The same food without supplementation continued to be provided in the same quantities to maintain BW during washout, as during the treatment periods.

As per previous research (22), choline (PuraChol, 70% aqueous choline chloride, 52.2% choline ion; Balchem Corporation, New Hampton, NY, United States of America) was supplemented to provide a daily choline intake of six times the recommended allowance (RA) for dietary choline published by the National Research Council (NRC) [6 x 63 mg/kg $BW^{0.67}$ (378 mg/kg $BW^{0.67}$)] (22, 41). The choline was measured daily, before feeding, with an adjustable volume pipette (Research plus™ Variable Adjustable Volume Pipette: 100–1,000 µL Single-Channel, Eppendorf Canada Ltd., Mississauga, ON, Canada). The estimated choline intake from the diet (4.3 mg choline/g diet as-fed) was accounted for when calculating the choline supplement dose for each individual cat. L-carnitine (48.5% L-carnitine tartrate, Bill Barr & Company, Overland Park, KS, United States of America) was supplemented at 200 mg/kg BW daily (26, 28). The L-carnitine was pre-measured daily before feeding and was mixed with a small amount of water. Each cat received their food once daily. Prior to feeding, each cat's daily food amount was split into two rations. The first ration provided ¼ of the daily food intake. The dietary treatments (choline or L-carnitine) were top-dressed onto this first ration and left to soak for 20 min prior to feeding. Once consumed, each cat was provided their second ration (3/4 daily food intake). Cats on the control treatment and during washout

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TABLE 1 The proximate analysis, energy content, fiber, choline, carnitine, selected B-vitamin and amino acid concentrations of a commercial extruded adult cat food fed at maintenance energy requirements to obese ($n = 9$) and lean adult cats ($n = 9$).

Moisture	% as-fed	4.3
Protein	% DM	41.7
Fat	% DM	17.5
Ash	% DM	8.8
Crude fiber	% DM	1.5
Total dietary fiber	% DM	4.5
NFE[b]	% DM	30.6
ME[a]	Kcal/kg	3,864.0
Choline	mg/100 g	428.4
Carnitine	mg/100 g	4.6
Cobalamin (B12)	mg/100 g	9.2
Pyridoxine (B6)	mg/100 g	1.4
Folate (B9)	mg/100 g	0.2
Alanine	% DM	2.4
Arginine	% DM	2.7
Aspartate	% DM	3.6
Cysteine	% DM	1.5
Glutamine	% DM	5.3
Glycine	% DM	3.4
Histidine	% DM	0.9
Isoleucine	% DM	1.6
Leucine	% DM	2.9
Lysine	% DM	2.5
Methionine	% DM	0.9
Phenylalanine	% DM	1.8
Proline	% DM	2.5
Serine	% DM	1.8
Taurine	% DM	0.2
Threonine	% DM	1.5
Tryptophan	% DM	0.2
Tyrosine	% DM	1.3
Valine	% DM	1.8

Reported on a dry matter basis (DMB) apart from moisture.

[a]Metabolizable Energy (ME) calculated using the Modified Atwater Equation: ME = 10 x [(3.5 x %crude protein) + (8.5 x %fat) + (3.5 x %NFE)] (41).

[b]Nitrogen Free Extract (NFE) % = 100–moisture–protein–fat-crude fiber-ash (41); Ingredients: Deboned Chicken, Deboned Turkey, Chicken Meal, Whole Eggs, Turkey Meal, Lentils, Peas, Chickpeas, Chicken Fat (preserved with Mixed Tocopherols), Split Peas, Flaxseed, Natural Chicken Flavor, Pumpkin, Broccoli, Quinoa Seed, Dried Cranberries, Choline Chloride, Pomegranate, Raspberries, Kale, Salt, Chicory Root Extract, Vitamins and Minerals (Vitamin E Supplement, Niacin (source of Vitamin B3), Vitamin A Supplement, Thiamine Mononitrate (source of Vitamin B1), d-Calcium Pantothenate (source of Vitamin B5), Pyridoxine Hydrochloride (source of Vitamin B6), Riboflavin (source of Vitamin B2), Beta-Carotene, Vitamin D3 Supplement, Folic Acid, Biotin, Vitamin B12 Supplement, Zinc Proteinate, Ferrous Sulfate, Zinc Oxide, Iron Proteinate, Copper Sulfate, Copper Proteinate, Manganese Proteinate, Manganous Oxide, Calcium Iodate, Sodium Selenite), DL-Methionine, Taurine, Yucca schidigera Extract, Spinach, Celery Seeds, Peppermint, Chamomile, Turmeric, Ginger, Rosemary.

similarly received two rations but did not have anything top-dressed onto their first ration.

2.4. Indirect calorimetry

Indirect calorimetry was performed on the last day of each 6-week period (day 42), to determine individual EE and RQ. Each 24-h session comprised of a gas equilibrium period of 30 min, pre-prandial (fasted) measurements (1.5 h), and fed and extended postprandial measurements (22 h). Cats were acclimated to the chambers, using the methods previously outlined by Gooding et al. (42). Calibration of gases and protocol followed the methods previously described (22, 25).

Briefly, an open circuit, ventilated system (Qubit C950 Multi Channel Gas Exchange, Qubit Systems Inc., Kingston, ON, Canada) was used, with plexiglass chambers measuring 53 x 53 x 79 cm (length x width x height). The volume of space within the chamber measured 221.91 L. To maintain levels of CO_2 within the chambers between 0.4% and 0.7%, room air was pulled through the chambers at a flow rate between 4.1–6.2 L/min.

The cats had access to a litter box, a water bowl and a hammock within the chamber. Water was filled before placing the cats in the calorimetry chambers and starting measurements. During each calorimetry session, cats continued to receive their food and treatment in the same 2-ration method as outlined previously.

Respiratory quotient and EE were calculated by the C950-Multi Channel Gas Exchange system (Qubit Systems Inc, Kingston, ON, Canada), using the following equations (43):

$$Respiratory\ Quotient\ (RQ) = CO2\ produced\ (L) / O2\ consumed\ (L)$$

$$Energy\ Expenditure\ (EE)\ (kcal) = 3.94 \times O2\ consumed\ (L) + 1.11 \times CO2\ produced\ (L)$$

2.5. Blood collection and laboratory analyses

Following the completion of each 24 h indirect calorimetry session, cats were anesthetized for blood and tissue sample collection. Tissue samples are subject to further analysis and are not part of this study. Cats were pre-medicated with hydromorphone (0.05 mg/kg BW IM) and acepromazine (0.04 mg/kg BW IM), and induced with alfaxalone (1–3 mg/kg BW IV, to effect) and midazolam (0.3 mg/kg BW IV) (44). Fasted blood samples were collected from the jugular vein (5 mL). Following collection, the whole blood was transferred to serum-separating tubes and stored at 5°C until centrifugation. Centrifugation of collected whole blood occurred within 10 h of collection. Samples were centrifuged at 2,500 g x 15 min at 4°C (LegendRT, Kendro Laboratory Products 2002, Germany). Serum was separated, aliquoted and submitted (0.5 mL) to the Animal Health Laboratory at the University of Guelph (Guelph, ON, Canada) for analysis of serum CHOL, TAG,

non-esterified fatty acids (NEFA), high-density lipoprotein CHOL (HDL-C), alkaline phosphatase (ALP), alanine aminotransferase (ALT), blood urea nitrogen (BUN), creatinine (CREAT) and glucose (GLUC), via photometry using a Roche Cobas 6000 c501 analyzer (Roche Diagnostics, Basel, Switzerland). The Friedewald equation was used to calculate very low-density lipoprotein CHOL (VLDL) and low-density lipoprotein CHOL (LDL-C) (45):

$$Very\ low-density\ lipoprotein\ CHOL\ (VLDL)\ (mmol/L) = TAG\ (mmol/L)\ /\ 2.2$$

$$Low-density\ lipoprotein\ CHOL\ (LDL-C)\ (mmol/L) = total\ CHOL\ (mmol/L) - HDL-C\ (mmol/L) - VLDL\ (mmol/L)$$

2.6. Dual energy x-ray absorptiometry

Following blood collection, on the same day, body composition was assessed by dual energy x-ray absorptiometry (DXA). If needed, cats were administered additional sedation using dexmedetomidine (0.3 mg/kg BW IM). Atipamezole (0.2 mg/kg BW IM) was used to reverse sedation following DXA scans (44). Scans were performed in duplicate for each individual cat using a fan-beam DXA device (Prodigy® Advance GE Healthcare, Madison, WI, United States of America) to estimate whole body fat % (BF %), total tissue mass (TTM), fat mass (FM), and lean soft tissue mass (LSTM) (enCORE Version 16; GE Healthcare, Madison, WI, United States of America). The Small Animal Mode with Thin Setting was used. Each cat was individually positioned onto the DXA scanner in dorsal recumbency with forelimbs extended cranially (46). The cats were repositioned as necessary between scans. Each scan lasted ~10 min.

2.7. Statistical analyses

Residuals of serum biochemistry and lipoprotein profile, body composition, indirect calorimetry, BW, BCS, and intake data were all assessed for normality with the Shapiro-Wilk test. Food intake, energy intake, BCS, fed EE, fed RQ, choline and carnitine intake (mg/day, mg/kg BW, and mg/kg $BW^{0.67}$) were not normally distributed and underwent a log transformation as a result. The data was back-transformed to obtain least square means (LSM) for each response variable.

The trapezoidal method was used to calculate area under the curve for RQ (AUC_{RQ}), both in the fasted (pre-prandial) and fed states (0–120 min, 120–480 min, 480–820 min, 820–1,300 min) (9.3.1, GraphPad Software, San Diego, CA, United States of America). Statistical analyses of serum ALP, ALT, BUN, CREAT, GLUC, lipid and lipoprotein concentrations (CHOL, NEFA, HDL-C, LDL-C, VLDL, and TAG), body composition data (BF %, TTM, FM, and LSTM), EE, RQ and AUC_{RQ}, were performed using SAS (SAS Studio 3.8, SAS Institute, Cary, NC, United States) as a generalized linear mixed model. The proc GLIMMIX procedure was used, with treatment, "body condition" (obese or lean), and treatment x body condition interaction set as the fixed effects, period and group as the random effects, and cat as the subject. Food intake and BW were included as covariates when assessing differences in RQ and EE. The covariance matrix resulting in the smallest Akaike information criterion value was used.

Differences in BW and BCS over time were similarly assessed through Proc GLIMMIX with treatment, body condition and their subsequent interaction as fixed effects, period and group as the random effects, week as the repeated term, and cat as the subject. Baseline BCS and BW were used as covariates when assessing change in BW and BCS.

Tukey's *post hoc* test was performed for all multiple comparisons where a significant effect of treatment, body condition, or treatment and body condition interaction was present. Results are expressed as LSM ± standard error of mean (SEM). A P-value < 0.05 was considered significant, and a P-value of < 0.10 was considered a trend.

3. Results

One obese cat was removed during period three due to a medical condition not related to the study. As a result, data from this cat were not included for period three (choline treatment). Supplements were accepted by the cats with no observed adverse health effects throughout the trial.

3.1. Food, energy, choline, and L-carnitine intake

Obese cats had greater food intake and subsequent energy intake compared to lean cats ($P_{Condition} < 0.001$; Table 2). Choline, but not L-carnitine, resulted in lower food and energy intake in both lean and obese cats ($P_{Treatment} = 0.025$), with no effect of treatment x body condition interaction ($P_{TreatmentxCondition} = 0.669$).

As expected, treatment affected choline and L-carnitine intake ($P_{Treatment} < 0.001$), where supplementation resulted in greater intake. Additionally, there was an effect of body condition when choline and L-carnitine intake were expressed on a mg/day and mg/kg BW basis (Mg/day: $P_{Condition} < 0.001$, and < 0.001, respectively; mg/kg BW: $P_{Condition} < 0.001$, and 0.003, respectively). When expressed as mg/day, choline and L-carnitine intake were greater in obese cats. However, obese cats had a lower intake of both choline and L-carnitine when expressed on a mg/kg BW basis. Treatment x body condition interaction also affected L-carnitine intake (mg/day: $P_{TreatmentxCondition} <0.001$; mg/kg BW: $P_{TreatmentxCondition} = 0.002$; mg/kg $BW^{0.67}$: $P_{TreatmentxCondition} = 0.001$), where obese cats receiving the L-carnitine treatment had the greatest intakes of L-carnitine. There was no effect of treatment x body condition interaction

TABLE 2 Food, energy, choline and L-carnitine intake of lean ($n = 9$) and obese ($n = 9$ for control and L-carnitine; $n = 8$ for choline) adult cats receiving supplemental dietary choline (378 mg/kg $BW^{0.67}$), supplemental L-carnitine (200 mg/kg BW), or control (no additional supplement) top-dressed onto a commercial extruded adult feline diet and fed to maintenance energy requirements in a 3 x 3 Latin square design for 6-week periods.

	Body condition (BC)	Treatment (T)			*P*-values		
		Control ($n = 18$)	Choline ($n = 17$)	L-carnitine ($n = 18$)	BC	T	BC X T
Food intake (G/Day)	Lean	53.84 ± 1.69	52.63 ± 1.65	52.21 ± 1.6	**<0.001**	**0.025**	0.669
	Obese	67.82 ± 2.13	65.27 ± 2.05	66.15 ± 2.08			
Energy intake (kcal/day)	Lean	207.03 ± 6.51	202.39 ± 6.36	200.76 ± 6.3	**<0.001**	**0.025**	0.669
	Obese	260.80 ± 8.20	250.97 ± 7.90	254.39 ± 8.00			
Choline intake (mg/day)	Lean	220.55 ± 5.46	1,049.38 ± 25.57	213.63 ± 5.2	**<0.001**	**<0.001**	0.696
	Obese	277.97 ± 6.89	1,300.90 ± 32.29	271.31± 6.73			
Choline intake (mg/kg BW)	Lean	48.18 ± 0.90	227.24 ± 4.20	46.76 ± 0.8	**<0.001**	**<0.001**	0.810
	Obese	43.61 ± 0.82	202.52 ± 3.80	42.44 ± 0.79			
Choline intake (mg/kg $BW^{0.67}$)	Lean	79.63 ± 1.41	376.57 ± 6.59	77.22 ± 1.3	0.770	**<0.001**	0.770
	Obese	80.36 ± 1.42	374.13 ± 6.62	78.28 ± 1.38			
Carnitine intake (mg/day)	Lean	2.48 ± 0.08^{Ad}	2.42 ± 0.07^{Ad}	917.46 ± 27.76^{A}	**<0.001**	**<0.001**	**<0.001**
	Obese	3.12 ± 0.10^{Bd}	3.00 ± 0.09^{Bd}	$1,282.28 \pm 39.31^{Bc}$			
Carnitine intake (mg/kg BW)	Lean	0.54 ± 0.01^{Ad}	0.52 ± 0.01^{Ad}	200.53 ± 4.41^{A}	**0.003**	**<0.001**	**0.002**
	Obese	0.49 ± 0.01^{Bd}	0.47 ± 0.01^{Bd}	200.58 ± 4.42^{Ac}			
Carnitine intake (mg/kg $BW^{0.67}$)	Lean	0.89 ± 0.02^{Ad}	0.87 ± 0.02^{Ad}	331.13 ± 7.47^{A}	0.127	**<0.001**	**0.001**
	Obese	0.90 ± 0.02^{Ad}	0.86 ± 0.02^{Ad}	369.95 ± 8.38^{Bc}			

Values expressed as LSM ± SEM. Down a column, different upper case letter superscripts (A,B), represent significant difference between body conditions within a treatment, where a *p*-value of less than 0.05 is considered significant (represented in bold); Across a row, different lower case letter superscripts (c,d), represent significant difference between treatments within a body condition, where a *p*-value of less than 0.05 is considered significant; Repeated measures ANOVA with Tukey *post-hoc* test. BW, body weight; T, effect of treatment; BC X T, effect of body condition by treatment interaction.

on choline intake (mg/day: $P_{TreatmentxCondition}$ 0.696; mg/kg BW: $P_{TreatmentxCondition} = 0.810$; mg/kg $BW^{0.67}$: $P_{TreatmentxCondition} = 0.770$).

3.2. Body weight, body condition score and body composition

Body weight, BCS and body composition data are presented in Table 3. Both BW and BCS were higher in the obese cats than in the lean cats ($P_{Condition} < 0.001$) and neither changed treatment or treatment x body condition interaction ($P_{Treatment} = 0.419$, and 0.667, respectively; $P_{TreatmentxCondition} = 0.885$, and 0.667 respectively). There were no changes in BW or BCS over time, in both lean and obese cats, or with any of the three treatments ($P > 0.05$; data not shown).

As expected, TTM, FM, and BF % were higher in the obese cats than in the lean cats ($P_{Condition} < 0.001$). However, treatment and treatment x body condition interaction did not change any of these parameters ($P_{Treatment} = 0.309$, 0.847, and 0.525, respectively; $P_{TreatmentxCondition} = 0.734$, 0.569, and 0.384, respectively). Obese cats had greater LSTM ($P_{Condition} = 0.001$). There was a trend for LSTM to change with treatment ($P_{Treatment} = 0.066$), but there was no effect of treatment x body condition interaction on LSTM ($P_{TreatmentxCondition} = 0.858$).

3.3. Serum biochemistry and lipoprotein profile

Mean serum biochemistry and lipoprotein values fell within reference intervals published by the Animal Health Laboratory (Guelph, ON, Canada), and are presented in Table 4. Serum TAG, HDL-C and VLDL concentrations differed between lean and obese cats ($P_{Condition} = 0.001$, 0.041, and 0.001, respectively). Concentrations of serum TAG and VLDL were greater in obese cats across all three treatments. Conversely, serum HDL-C was lower in obese cats, as compared to lean cats. Serum HDL-C concentrations were also affected by treatment ($P_{Treatment} = 0.005$); concentrations were higher with choline supplementation, as compared to control and L-carnitine. Serum CHOL and LDL-C concentrations were also greater with choline supplementation in all cats ($P_{Treatment} = 0.005$, and 0.042, respectively). However, neither CHOL or LDL-C were affected by body condition ($P_{Condition} = 0.104$, and 0.453, respectively). Concentrations of serum TAG and VLDL were not affected by treatment alone ($P_{Treatment} = 0.340$, and 0.340, respectively), but tended to increase with L-carnitine supplementation in obese cats ($P_{TreatmentxCondition} = 0.064$, and 0.064, respectively). There was also a trend for treatment to increase serum NEFA ($P_{Treatment} = 0.099$). However, neither body condition or treatment x body condition interaction affected NEFA ($P_{Condition} = 0.241$, and $P_{TreatmentxCondition} = 0.884$). No

TABLE 3 Body weight, BCS, and body composition of lean ($n = 9$) and obese ($n = 9$ for control and L-carnitine; $n = 8$ for choline) adult cats receiving supplemental dietary choline (378 mg/kg $BW^{0.67}$), supplemental L-carnitine (200 mg/kg BW), or control (no additional supplement top-dressed onto a commercial extruded adult feline diet) and fed to maintenance energy requirements in a 3 x 3 Latin square design for 6-week periods.

	Body condition (BC)	Treatment (T)			*P*-values		
		Control ($n = 18$)	Choline ($n = 17$)	L-carnitine ($n = 18$)	BC	T	BC X T
BW (kg)	Lean	4.59 ± 0.03	4.63 ± 0.03	4.58 ± 0.0	**<0.001**	0.419	0.885
	Obese	6.39 ± 0.03	6.44 ± 0.03	6.41 ± 0.03			
BCS	Lean	4.88 ± 0.12	4.88 ± 0.12	4.88 ± 0.1	**<0.001**	0.667	0.667
	Obese	7.88 ± 0.20	8.04 ± 0.20	7.93 ± 0.20			
Total Tissue mass (g)	Lean	4,360.89 ± 142.84	4,420.00 ± 142.84	4,353.72 ± 142.8	**<0.001**	0.309	0.734
	Obese	6,081.83 ± 142.84	6,128.50 ± 142.84	6,114.78 ± 142.84			
% Body fat	Lean	18.17 ± 1.25	18.28 ± 1.25	17.37 ± 1.2	**<0.001**	0.525	0.384
	Obese	32.30 ± 1.25	31.80 ± 1.25	32.12 ± 1.25			
Fat mass (G)	Lean	795.44 ± 84.88	807.33 ± 84.88	760.11 ± 84.8	**<0.001**	0.847	0.569
	Obese	1,965.72 ± 84.88	1,949.60 ± 84.88	1,968.61 ± 84.88			
Lean soft tissue mass (g)	Lean	3,565.50 ± 110.13	3,612.50 ± 110.13	3,593.61 ± 110.1	**0.001**	0.066	0.858
	Obese	4,115.61 ± 110.13	4,187.18 ± 110.13	4,146.06 ± 110.13			

Values expressed as LSM ± SEM; $P < 0.05$ (represented in bold), Repeated measures ANOVA with Tukey *post-hoc* test. BW, body weight; BCS, body condition score; BC, effect of body condition; T, effect of treatment; BC X T, effect of body condition by treatment interaction.

significant treatment x body condition interactions were noted for CHOL, HDL-C and LDL-C ($P_{TreatmentxCondition} = 0.619$, 0.242 and 0.586, respectively).

Treatment did affect serum ALP, ALT, and CREAT ($P_{Treatment} = 0.042$, 0.012, and 0.010, respectively). There were no differences between ALP means following a Tukey's *post-hoc*. Serum ALT concentrations were lower with control, as compared to the choline treatment. Conversely, L-carnitine supplementation reduced serum CREAT, as compared to both control and choline, in both lean and obese cats. Lean cats consuming choline had greater concentrations of serum ALT as compared to lean cats consuming control and L-carnitine, and obese cats consuming L-carnitine ($P_{TreatmentxCondition} = 0.002$). There was a trend for body condition to change serum GLUC ($P_{Condition} = 0.058$). However, GLUC did not change with treatment or treatment x body condition interaction ($P_{Treatment} = 0.476$, and $P_{TreatmentxCondition} = 0.827$). Serum BUN was not affected by treatment, body condition, or treatment x body condition interaction ($P_{Treatment} = 0.282$, $P_{Condition} = 0.213$, $P_{TreatmentxCondition} = 0.247$).

3.4. Indirect calorimetry

There was a trend for fasted EE to change with treatment ($P_{Treatment} = 0.087$; Table 5), but there was no change with body condition or treatment x body condition interaction ($P_{Condition} = 0.685$ and $P_{TreatmentxCondition} = 0.189$). These outcomes did not change when fasted EE was adjusted for BW ($P_{Treatment} = 0.088$, $P_{Condition} = 0.756$, and $P_{TreatmentxCondition} = 0.191$). Fed EE was not affected by treatment or treatment x body condition interaction ($P_{Treatment} = 0.915$, and $P_{TreatmentxCondition} = 0.874$), but tended to be lower in the obese cats ($P_{Condition} = 0.090$). Treatment, condition and treatment x body condition interaction did not change fed EE when adjusted for BW or food intake ($P_{Treatment} = 0.905$, and 0.761, respectively; $P_{Condition} = 0.264$, and 0.090, respectively; $P_{TreatmentxCondition} = 0.881$, and 0.767, respectively).

Fasted RQ did not change with treatment, condition or treatment x body condition interaction ($P_{Treatment} = 0.497$, $P_{Condition} = 0.905$, $P_{TreatmentxCondition} = 0.807$). When adjusted for BW, fasted RQ was lower in the obese cats ($P_{Condition} = 0.009$). The effects of treatment and treatment x body condition interaction remained insignificant ($P_{Treatment} = 0.587$, and $P_{TreatmentxCondition} = 0.712$). Fed RQ was similarly not affected by treatment or treatment x body condition interaction ($P_{Treatment} = 0.427$, and $P_{TreatmentxCondition} = 0.964$), but there was a trend for fed RQ to change with body condition ($P_{Condition} = 0.080$). These outcomes remained the same when fed RQ was adjusted for both BW ($P_{Treatment} = 0.354$, $P_{Condition} = 0.060$, and $P_{TreatmentxCondition} = 0.958$), and food intake ($P_{Treatment} = 0.729$, $P_{Condition} = 0.057$, and $P_{TreatmentxCondition} = 0.741$).

There was a tendency for AUC_{RQ} 0–120 min postprandial to change with treatment in both lean and obese cats ($P_{Treatment} = 0.055$). However, no effect of body condition or treatment x body condition interaction was observed ($P_{Condition} = 0.454$, and $P_{TreatmentxCondition} = 0.692$). Fasted AUC, and the remaining postprandial AUC_{RQ} intervals (120–480, 480–820, and 1,300 min) were not affected by body condition ($P_{Condition} = 0.894$, 0.914, 0.166, and 0.292, respectively), treatment ($P_{Treatment} = 0.538$, 0.676, 0.286, and 0.739, respectively), or treatment x body condition interaction ($P_{TreatmentxCondition} = 0.754$, 0.341, 0.940, and 0.694, respectively).

TABLE 4 Fasted serum biochemistry, lipid and lipoprotein profile values of lean ($n = 9$) and obese ($n = 9$ for control and L-carnitine; $n = 8$ for choline) adult cats receiving supplemental dietary choline (378 mg/kg $BW^{0.67}$), supplemental L-carnitine (200 mg/kg BW), or control (no additional supplement top-dressed onto a commercial extruded adult feline diet) and fed to maintenance energy requirements in a 3 x 3 Latin square design for 6-week periods.

Analyte	Units	Body condition (BC)	Treatment (T)			*P*-values		
			Control ($n = 18$)	Choline ($n = 17$)	L-carnitine ($n = 18$)	BC	T	BC X T
TAG	mmol/L	Lean	0.22 ± 0.45	0.27 ± 0.45	0.23 ± 0.4	**0.001**	0.340	0.064
		Obese	0.43 ± 0.45	0.41 ± 0.45	0.49 ± 0.45			
CHOL	mmol/L	Lean	5.91 ± 0.34	6.64 ± 0.34	6.01 ± 0.3	0.104	**0.005**	0.619
		Obese	5.45 ± 0.34	5.90 ± 0.34	5.16 ± 0.34			
HDL-C	mmol/L	Lean	4.80 ± 0.28	5.43 ± 0.28	5.03 ± 0.2	**0.041**	**0.005**	0.242
		Obese	4.38 ± 0.28	4.65 ± 0.28	4.37 ± 0.28			
LDL-C	mmol/L	Lean	1.01 ± 0.19	1.09 ± 0.19	0.87 ± 0.1	0.453	**0.042**	0.586
		Obese	0.87 ± 0.19	1.07 ± 0.19	0.57 ± 0.19			
VLDL	mmol/L	Lean	0.044 ± 0.01	0.053 ± 0.01	0.047 ± 0.0	**0.001**	0.340	0.064
		Obese	0.087 ± 0.01	0.081 ± 0.01	0.098 ± 0.01			
NEFA	mmol/L	Lean	0.31 ± 0.06	0.37 ± 0.06	0.44 ± 0.0	0.241	0.099	0.884
		Obese	0.40 ± 0.06	0.40 ± 0.06	0.49 ± 0.06			
ALP	U/L	Lean	14.56 ± 1.44	14.33 ± 1.44	14.67 ± 1.4	0.067	**0.042**	0.123
		Obese	19.00 ± 1.44	16.51 ± 1.44	18.78 ± 1.44			
ALT	U/L	Lean	40.44 ± 3.22^{Ad}	54.44 ± 3.22^{Ac}	43.33 ± 3.22^{A}	0.180	**0.012**	**0.002**
		Obese	39.44 ± 3.22^{Ac}	38.99 ± 3.22^{Bc}	43.11 ± 3.22^{Ac}			
CREAT	mmol/L	Lean	116.78 ± 6.83	115.78 ± 6.83	108.22 ± 6.8	0.701	**0.010**	0.103
		Obese	116.89 ± 6.83	118.90 ± 6.83	116.00 ± 6.83			
GLUC	mmol/L	Lean	5.56 ± 0.42	5.54 ± 0.42	5.70 ± 0.4	0.058	0.476	0.827
		Obese	6.48 ± 0.42	6.34 ± 0.42	6.83 ± 0.42			
BUN	mmol/L	Lean	7.58 ± 0.33	7.33 ± 0.33	7.19 ± 0.3	0.213	0.282	0.247
		Obese	7.97 ± 0.33	7.85 ± 0.33	8.00 ± 0.33			

Values expressed as LSM ± SEM. Down a column, different upper case letter superscripts (A, B), represent significant difference between body conditions within a treatment, where a *p*-value of less than 0.05 is considered significant (represented in bold); Across a row, different lower case letter superscripts (c, d), represent significant difference between treatments within a body condition, where a *p*-value of less than 0.05 is considered significant; Repeated measures ANOVA with Tukey *post-hoc* test. TAG, triglycerides; CHOL, cholesterol; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; VLDL, very low-density lipoprotein cholesterol; NEFA, non-esterified fatty acids; ALP, alkaline phosphatase; ALT, alanine aminotransferase; CREAT, creatinine; GLUC, glucose; BUN, blood urea nitrogen; BW, body weight; BC, effect of body condition; T, effect of treatment; BC X T, effect of body condition by treatment interaction.

4. Discussion

To the authors' knowledge, this is the first study to investigate the effects of dietary choline supplementation in comparison to L-carnitine supplementation in obese and lean cats fed to maintain BW. Specifically, we sought to investigate lipotropic changes, as determined by serum lipid and lipoprotein profile, EE, RQ, and body composition data, between the two supplements. Previously, we reported that dietary choline at 5 and 6 x NRC RA increased serum lipid and lipoprotein profiles in overweight and obese cats, likely representing increased mobilization of hepatic lipids into circulation (21, 22). However, to allow the pet food industry and veterinary clinicians to make informed decisions regarding dietary supplementation and feline weight loss, it is important to assess the lipotropic effects of choline at the previously determined dose of 6 x NRC RA against L-carnitine (22), a supplement that has been frequently researched and used by the pet food industry (26–28). The dose of L-carnitine supplemented to the cats in the present trial was similarly based on previous research in overweight and obese cats (26, 28, 47, 48).

As expected, obese cats had greater food intake, energy intake, BW, BCS, and body composition values (total tissue mass, BF %, fat mass and LSTM), when compared to lean cats. These obese cats were specifically enrolled in this study due to their increased adiposity and were fed at energy intakes to support their current BW and BCS. A limitation of the present study was the approach to defining adiposity at enrollment, as the cats were classified as obese based solely on BCS. Body composition was not assessed

TABLE 5 EE and RQ of lean ($n = 9$) and obese ($n = 9$ for control and L-carnitine; $n = 8$ for choline) adult cats receiving supplemental dietary choline (378 mg/kg $BW^{0.67}$), supplemental L-carnitine (200 mg/kg BW), or control (no additional supplement top-dressed onto a commercial extruded adult feline diet) and fed to maintenance energy requirements in a 3 x 3 Latin square design for 6-week periods.

	Body condition (BC)	Treatment (T)			*P*-values		
		Control ($n = 18$)	Choline ($n = 17$)	L-carnitine ($n = 18$)	BC	T	BC X T
EE fasted, kcal/kg BW/day	Lean	34.06 ± 2.91	32.96 ± 2.90	39.31 ± 2.9	0.685	0.088	0.189
	Obese	32.90 ± 2.86	34.63 ± 2.92	34.46 ± 2.86			
Adjusted for BW[$]	Lean	32.11 ± 4.57	31.02 ± 4.54	37.29 ± 4.6	0.756	0.088	0.191
	Obese	34.73 ± 4.37	36.62 ± 4.63	36.38 ± 4.49			
EE fed, kcal/kg BW/day	Lean	37.73 ± 2.90	35.53 ± 2.70	37.37 ± 2.8	0.090	0.915	0.874
	Obese	33.82 ± 2.42	33.76 ± 2.56	32.88 ± 2.35			
Adjusted for food intake[#]	Lean	35.49 ± 2.21	35.74 ± 2.31	38.24 ± 2.4	0.090	0.761	0.767
	Obese	33.19 ± 2.02	32.68 ± 2.10	33.01 ± 2.02			
Adjusted for BW[$]	Lean	39.07 ± 3.82	36.76 ± 3.69	38.77 ± 4.0	0.264	0.905	0.881
	Obese	32.92 ± 3.11	32.72 ± 3.40	31.96 ± 3.08			
RQ fasted	Lean	0.78 ± 0.01	0.79 ± 0.01	0.78 ± 0.0	0.905	0.497	0.807
	Obese	0.78 ± 0.01	0.78 ± 0.01	0.79 ± 0.01			
Adjusted for BW[$]	Lean	0.81 ± 0.01	0.82 ± 0.01	0.80 ± 0.0	**0.009**	0.587	0.712
	Obese	0.76 ± 0.01	0.75 ± 0.01	0.75 ± 0.01			
RQ fed	Lean	0.81 ± 0.01	0.80 ± 0.01	0.81 ± 0.0	0.080	0.427	0.964
	Obese	0.80 ± 0.01	0.79 ± 0.01	0.79 ± 0.01			
Adjusted for food intake[#]	Lean	0.81 ± 0.01	0.81 ± 0.01	0.81 ± 0.0	0.057	0.729	0.741
	Obese	0.80 ± 0.01	0.79 ± 0.01	0.79 ± 0.01			
Adjusted for BW[$]	Lean	0.83 ± 0.01	0.82 ± 0.01	0.82 ± 0.0	0.060	0.354	0.958
	Obese	0.79 ± 0.01	0.78 ± 0.01	0.78 ± 0.01			
AUC_{RQ} fasted, RQ*min	Lean	53.88 ± 3.51	56.24 ± 3.51	57.89 ± 3.6	0.894	0.538	0.754
	Obese	55.71 ± 3.51	54.33 ± 3.62	57.12 ± 3.51			
AUC_{RQ} 0–120, RQ*min	Lean	89.96 ± 1.21	92.18 ± 1.25	90.91 ± 1.2	0.454	0.055	0.692
	Obese	90.08 ± 1.21	91.79 ± 1.25	89.64 ± 1.21			
AUC_{RQ} 120–480, RQ*min	Lean	283.96 ± 5.91	289.05 ± 6.03	291.31 ± 6.0	0.914	0.676	0.341
	Obese	289.77 ± 5.91	287.41 ± 6.03	288.14 ± 5.91			
AUC_{RQ} 480–820, RQ*min	Lean	283.97 ± 4.77	278.88 ± 4.87	281.42 ± 4.8	0.166	0.286	0.940
	Obese	279.88 ± 4.77	276.28 ± 4.87	276.99 ± 4.77			
AUC_{RQ} 820–1,300, RQ*min	Lean	370.39 ± 14.61	376.65 ± 14.82	378.22 ± 14.8	0.292	0.739	0.694
	Obese	367.68 ± 14.61	371.79 ± 14.81	364.93 ± 14.61			

Values expressed as LSM ± SEM; $P < 0.05$ (represented in bold), Repeated measures ANOVA with Tukey *post-hoc* test. EE, energy expenditure; RQ, respiratory quotient; BW, body weight; BC, effect of body condition; T, effect of treatment; BC X T, effect of body condition by treatment interaction. [#]Individual food intake during calorimetry used as a covariate. [$]Individual BW at time of calorimetry used as a covariate.

by DXA at enrollment to evaluate BF % of the cats before group allocation and treatment. Although BF % differed between the lean and obese groups, the assigned mean BCS of the obese cats was higher than the respective mean BF % analyzed by DXA. The mean BF % of the obese cats was 32%, which corresponds with BCS 7/9 (36). Overall, all cats in the obese group could still be considered overweight or obese based on BF % (36), and BF % was still greater than in the lean cats. Because BCS is a subjective method of assessment, the BCS of the cats may have been overestimated due to localized fat deposits and/or abdominal fat pads which may have consisted of skin only. Similar limitations in investigator bias have previously been published in feline research

(49, 50). Furthermore, differences in DXA machine, protocol, and computer software used by researchers can lead to differences in body composition and fat mass measurements among feline obesity studies (51).

Obesity results in alterations in the secretion of adipokines, leading to the development of other secondary health concerns such as insulin resistance and dyslipidemia (52–54). The obese cats in the present study had differences in their serum lipid and lipoprotein profiles, independent of treatment, when compared to the lean cats. Specifically, obese cats had overall concentrations of TAG and VLDL that were 59% greater than the lean cats. Additionally, overall concentrations of serum HDL-C were 13% lower in the obese cats compared to lean cats. To the authors' knowledge, there are no published reference ranges in cats for serum TAG, VLDL or HDL-C. However, the concentrations of serum TAG, VLDL and HDL-C reported herein were similar to those previously reported in overweight and obese adult cats receiving supplemental choline (21, 22). Serum TAG concentrations were also similar to those previously reported in healthy adult cats (55, 56). The present results align with previous reports of similarly higher circulating TAG, VLDL and lower HDL-C in obese cats, as compared to a lean control group (57–60). However, said authors also reported greater circulating NEFA, CHOL, and LDL-C concentrations in obese cats, which was not observed in the present study. As the mean age of the cats in the present study was lower than the mean age reported in these previous studies (57, 60), it is unclear if age may have also had an impact. To our knowledge, the effect of age on serum lipid, biochemistry and/or metabolomic profiles in overweight or obese cats has not specifically been investigated. However, cats under the age of two were reported to have lower serum CHOL and LDL-C concentrations as compared to cats over the age of two (BCS unknown) (61). Additionally, as these cats gained weight during a 12-week modified *ad libitum* feeding protocol during growth (25), there is a question of whether acute vs. chronic obesity may also impact lipoprotein profile and circulating metabolites. It is also worth considering that the increase in serum LDL and CHOL with choline treatment in both the lean and obese cats in the present study may have masked an effect of body condition that may have otherwise been observed. The same is possible for serum NEFA, where L-carnitine had a tendency to increase concentrations across both lean and obese cats.

The choline treatment in the present study provided a daily dietary choline intake of 6 x NRC RA, whereas cats on the L-carnitine and control treatments consumed a daily average choline intake of 1.2 and 1.3 x NRC RA, respectively. Both lean and obese cats had greater concentrations of CHOL, HDL-C-and LDL-C with the choline treatment, as compared to L-carnitine treatment (11, 7 and 33 % greater, respectively) and control (9, 9 and 13% greater, respectively). Serum CHOL concentrations remained within reference range for all treatments (2–12 mmol/L), and serum HDL-C and LDL-C concentrations were similar to those previously reported in obese and overweight adult cats consuming supplemental choline (21, 22). The greater concentrations of CHOL, HDL-C and LDL-C with choline in the present study are not surprising as similar changes were previously observed in obese and overweight cats consuming choline at 5 and 6 x NRC RA daily, respectively (21, 22). Choline is a precursor for PC, which is considered essential in the formation of VLDL. The VLDL are responsible for packaging TAG and CHOL, and exporting them out of the liver and into circulation (16). Similarly, both HDL-C and LCL-C have important roles in CHOL transport and circulation throughout the body, and require PC for their assembly (62, 63). Nascent HDL-C also requires PC for its formation within the liver (64). Contrary to what was observed, it was expected that serum TAG and VLDL would increase with choline supplementation in the present study. The present findings are contrary to our previous findings in obese and overweight cats consuming choline at 5 and 6 x NRC RA, where both serum TAG and VLDL concentrations were greater as compared to control (21, 22). Instead, obese cats receiving the L-carnitine treatment tended to have the greatest concentrations of serum TAG and VLDL. These results contradict the current research that exists on L-carnitine supplementation in hyperlipidemic animals and obese human patients with non-alcoholic steatohepatitis, in which TAG concentrations were reduced or unchanged (65–69). Although Rahbar et al. (70) observed increases in fasting plasma TAG concentrations when L-carnitine was supplemented to human patients with type II diabetes mellitus, the number of cats enrolled in the current study and the differences in BF % between the lean and obese group may not have been sufficient to detect these differences.

Although fasted serum glucose concentrations remained within reference range (4.4–7.7 mmol/L) for all the cats, glucose tended to be 16% greater in the obese cats than in the lean cats in the present study. Impaired glucose tolerance and insulin resistance are common findings in obese cats (9, 71–73), and a 30% decrease in insulin sensitivity has been reported with each additional kg of BW in cats (73). Over time, the decrease in insulin sensitivity may result in the development of type II diabetes mellitus (74), which is considered four times more likely in obese cats, as compared to lean cats (75). It is unclear if the increased TAG concentrations in the obese cats receiving L-carnitine may have been reflective of decreased glucose tolerance. Similar results have not been published in cats, and the use of L-carnitine in the treatment of obesity and diabetes mellitus in cats requires further investigation.

Serum ALP concentrations in the present study were within reference range for all cats throughout all three treatment periods (12–60 U/L), but tended to be, on average, 22% higher in the obese cats when compared to the lean cats. To the author's knowledge, there have been no published reports of increased ALP concentrations in healthy obese cats. However, in humans, a significant positive linear relationship between ALP and body mass index has been reported (76); where obese subjects had ALP concentrations that were on average 15 and 16% greater when compared to the non-obese and lean subjects, respectively (76, 77). This is because adipose tissue can express and release ALP into circulation (77). In the present study, it is likely that the greater serum ALP concentrations in the obese cats were representative of their increased adiposity, instead of their liver health. None of the cats showed any clinical signs of hepatic malfunction at any point throughout the study. However, serum liver enzyme values alone cannot adequately assess feline hepatic health and/or function. In the future, ultrasonography and histopathology of liver

biopsies should be considered, along with pre- and postprandial bile acid profiles.

Conversely, lean cats consuming choline had the greatest concentrations of serum ALT. However, the concentrations similarly remained within reference range (31–105 U/L), suggesting that hepatobiliary diseases were absent in the present cats (78). Serum ALT concentrations were 26% greater in lean cats receiving the choline treatment as compared to control, and 20% greater as compared to the L-carnitine supplement. The present finding is unexpected, as ALT has been reported to increase in cases of non-alcoholic steatohepatitis resulting from choline deficiency in humans and in rodent models (79, 80). Additionally, these results contradict previous work by Verbrugghe et al. (81), where obese cats had higher ALT concentrations as compared to lean cats. Previously, choline supplementation did not affect serum ALT levels in overweight or obese cats (21, 22). The cats in the present study did not show signs of hepatic health concerns, regardless of treatment or body condition. However, as mentioned, hepatic health was not thoroughly investigated in the present study.

L-carnitine supplementation resulted in lower serum CREAT concentrations, as compared to the other two dietary treatments. This finding aligns with previous research in rodent models, where L-carnitine levels were inversely correlated with serum CREAT and renal function was improved with supplementation (82). The protective effects of L-carnitine on renal function are believed to be a result of its ability to reduce lipid peroxidation and free radical generation (83). Similarly, adult cats with chronic kidney disease (International Renal Interest Society Stages 1–2) consuming an average of 211 mg L-carnitine/kg BW from a test diet had serum CREAT levels that increased at a slower rate, as compared to the control group consuming an average of 2 mg L-carnitine/kg BW. However, the test diet and control diet differed in their overall nutrient profiles, and therefore the change in serum CREAT cannot be attributed to solely dietary L-carnitine intake (84). All cats in the present study were fed the same base diet, deemed in good health prior to enrollment with a complete blood count and serum biochemistry profile, and showed no signs of renal health concerns throughout the trial. Urinalysis and symmetric dimethyl arginine concentrations were not evaluated but should be considered for future L-carnitine studies.

Food and energy intakes were 0.3 % lower with choline treatment when compared to L-carnitine treatment, and 3% lower when compared to control. It is unclear whether the differences in food intake between treatments may have affected circulating metabolite concentrations, and to what effect. However, this reduction was not enough to influence BW, BCS, BF % and/or fat mass over the treatment periods. Similarly, it is also unlikely that this level of energy reduction was enough to put the cats in a negative energy balance and cause the observed lipid mobilization with choline treatment. It is unclear whether the change in food intake was due to palatability or due to a potential effect of satiety. A similar reduction in food intake was previously reported in growing kittens consuming choline at 3 x NRC RA. However, there were no changes in the analyzed fasted serum satiety hormone concentrations (ghrelin, leptin, glucagon-like peptide-1, peptide-YY or gastric inhibitory polypeptide) of said kittens with choline treatment (25). The mechanism through which choline may influence food intake in cats requires further elucidation.

Although there were no differences between dietary treatments on FM or BF %, there was a trend for greater LSTM in both obese and lean cats with treatment. On average, LSTM was 1.5% greater with choline treatment as compared to control, and 0.8% greater as compared to L-carnitine treatment. Supplementing betaine (a derivative of choline) in livestock species such as pigs has resulted in increased LSTM (23, 24). Although the exact mechanism has not been identified, it is presumably due to the role that betaine has as a possible methyl donor for the re-methylation of methionine; thus sparing methionine for protein synthesis and leading to increased s-adenosylmethionine production (85). There were no differences in LSTM with choline supplementation at 3 x NRC RA in growing kittens being fed *ad libitum* (25). However, supplemented kittens had lower food and subsequent protein intakes which may have impacted these results. In the present study, LSTM was 0.8% greater with L-carnitine compared to control. This observation aligns with previous research by Center et al. (26) in which overweight cats consumed a weight loss diet supplemented with 0 (control), 50, 100, or 150 mg carnitine/kg diet. Cats supplemented with carnitine achieved an increase in LSTM and a decrease in FM sooner (by day 42) compared to the cats receiving control (by day 84). These differences were attributed to lower RQ with treatment, indicating increased fat oxidation. Similarly, underweight and lean cats (BCS: 2.5–4/5) fed at 120% MER had lower fat deposition when supplemented 188 mg L-carnitine/kg diet compared to 121 mg L-carnitine/kg diet (28). However, the mechanism of action was unclear as there were no changes in EE, RQ or voluntary physical activity with dose throughout said 16-week trial.

Although EE is dependent on LSTM (86), the differences in LSTM between choline and the remaining treatments in the present study were minor and were likely not enough to elicit changes in EE. Instead, choline tended to increase RQ in the 120-min postprandial period, as compared to the other two treatments. The increase in RQ immediately post-feeding (0–120 min) may suggest a greater reliance on carbohydrate oxidation and less on gluconeogenesis with choline supplementation. This finding was not previously observed in overweight and obese cats or in guinea pigs supplemented with choline (21, 22, 87). It remains unclear why this may have occurred in the present study. There was a trend for greater fasted EE across all cats with the L-carnitine treatment. These findings align with previous research investigating L-carnitine supplementation in overweight cats. Overweight cats receiving 100 mg L-carnitine/kg BW similarly had greater fasted EE after 21 and 42 days of supplementation, as compared to overweight cats fed control (27). Although fed EE did not change with treatment in the present study, there was a trend for lower fed EE and fed RQ in the obese cats, as compared to the lean cats. Lower EE in overweight and obese cats as compared to lean cats is a common finding as an animal's surface area will influence EE (27, 86, 88). Perseghin et al. (89) similarly observed that overweight human participants had lower fed RQ when compared to lean participants, although not significant. The authors suggested that the increased leptin in the overweight participants may have played a role in promoting fatty acids toward oxidation instead of storage (90). However, said overweight study participants had

normal insulin and leptin sensitivity. Although there are reports of decreased insulin and leptin sensitivity with weight gain in cats (71, 91), neither leptin nor insulin were measured in the obese cats in the present study and we therefore cannot conclude if the same association was present.

In agreement with previous publications, the present study found clear differences in circulating lipid and lipoprotein concentrations of obese and lean cats. Specifically, obese cats had greater concentrations of circulating TAG and VLDL, and lower concentrations of circulating HDL-C. Despite the differences associated with obesity, choline supplementation at six times the published RA resulted in greater HDL-C, CHOL and LDL-C in all cats. These findings are in alignment with our previous research in overweight and obese cats, which similarly found greater circulating lipid and lipoprotein concentrations with choline supplementation. These results suggest that choline supplementation may be beneficial in mobilizing lipids out of the liver and into circulation. Based on the findings of the present research, choline shows more promise than L-carnitine as a supplement for combating the risk of FHL in cats. While choline decreased food intake and had a tendency to improve LSTM, it did not increase EE in the same manner that L-carnitine did. Thus, future research should focus on evaluating the synergistic benefits of supplementing dietary choline and L-carnitine together to obese cats on a weight loss program. Together, L-carnitine and choline may prove useful in promoting weight loss, while maintaining LSTM and minimizing hepatic lipid stores in these 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 University of Guelph Animal Care Committee (AUP#4496).

Author contributions

AV, MB, and GK secured funding for this research. AR, AS, MB, GK, and AV were responsible for designing this study. AR, SV-S, and AV were responsible for data collection. AR was responsible for statistical analysis and writing of the manuscript. All authors contributed to editing and reviewing this manuscript.

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Conflict of interest

AR declares that they have participated in paid engagements with and received scholarships from pet food companies within Canada. At the time of the study, SV-S held the position of Registered Veterinary Technician in Clinical Nutrition funded by Nestlé Purina Proplan Veterinary Diets, they were previously employed by Hill's Pet Nutrition, and received honoraria from pet food manufacturers. AS is the Champion Petfoods Chair in Canine and Feline Nutrition, Physiology and Metabolism, consults for Champion Petfood, was previously employed by P&G and Mars Pet Care, serves on the Scientific Advisory Board for Trouw Nutrition, and has received honoraria and research funding from various commodity groups, pet food manufacturers, and ingredient suppliers. AV is the Royal Canin Veterinary Diets Endowed Chair in Canine and Feline Clinical Nutrition, 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.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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Relative supersaturation values distinguish between feline urinary and non-urinary foods and align with expected urine analytes contributions to uroliths

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Introduction: Uroliths are concretions formed in the urinary tract. These can be problematic in humans and companion animals such as cats. Magnesium ammonium phosphate (struvite) and calcium oxalate (CaOx) are the most common forms of uroliths. The relative supersaturation (RSS) is a relative risk index of crystal formation. Here, an updated program for calculating RSS, EQUIL-HL21, was used to detect differences in RSS values when cats were fed foods formulated for urinary and non-urinary conditions. In addition, the contributions of urinary analytes to RSS values were examined via regression analyses.

Methods: Historical data from feeding trials including foods indicated for use in urinary or non-urinary conditions were analyzed for nutrient composition and urinary parameters. RSS was calculated by EQUIL-HL21. The relationship between RSS values calculated by EQUIL-HL21 and urinary analytes was examined by regression models, which were selected by R^2 and stepwise methods.

Results: Cats that consumed urinary foods had significantly greater levels of urinary sodium and chloride compared with those that consumed non-urinary foods, consistent with the greater amounts of sodium and chloride in the urinary foods. Those that consumed non-urinary foods had higher urine pH, ammonium, potassium, phosphorus, magnesium, oxalate, citrate, and sulfate. Struvite RSS value and number of urinary crystals were significantly lower in cats fed the urinary foods. Mean CaOx RSS values were similar in both foods, though the number of CaOx crystals were significantly higher in cats that consumed non-urinary foods. A model predicting the natural log of struvite RSS values indicated that these values would increase with increasing urine pH, ammonium, chloride, calcium, phosphorus, and magnesium, and would decrease with increasing urine citrate and sulfate. CaOx RSS was predicted to increase as urinary chloride, calcium, and oxalates increased, and would decrease as urine pH, sodium, phosphorus, citrate, and sulfate increased.

Discussion: These analyses demonstrate that the EQUIL-HL21 program can accurately detect expected differences between foods formulated for urinary and non-urinary indications. Regression models showed the eight urinary analytes that, respectively, contribute to the predicted RSS values for struvite and CaOx.

KEYWORDS

struvite, calcium oxalate, magnesium ammonium phosphate, feline, relative supersaturation, urolith

1. Introduction

Uroliths are concretions formed in the urinary tract under conditions that are influenced by pH conditions and concentrations of various types of inorganic and organic materials (1). Formation of uroliths is a common condition in cats, accounting for 13 to 28% of cases of feline lower urinary tract diseases (2). The most common uroliths reported in cats are composed of calcium oxalate (CaOx) or magnesium ammonium phosphate (struvite) (3–6). Age, sex, and breed are predisposing factors in the formation of certain types of uroliths (6, 7). Cats with kidney stones have a significantly shorter lifespan than those without (12.5 vs. 15.2 years) (8), and the prevalence of chronic kidney disease (CKD) in cats is significantly higher in cats with urolithiasis than in those without, though it is not known whether urolithiasis is a predictor or consequence of CKD (9).

The relative proportion of CaOx and struvite uroliths in affected cats has changed over time, with a decrease in the proportion of struvite uroliths and increase in CaOx uroliths (2). For example, an analysis of feline uroliths showed that the respective proportions of CaOx and struvite uroliths were 2 and 78% in 1981 and 41 and 49% in 2007 (4). Similar trends and proportions were found in different geographical locations, including Minnesota (4, 10), California (6, 10), Canada (5, 11), and the Benelux countries (12). Examination of age, sex, breed, and reproductive status of cats over this time period indicate that those factors are not responsible for the observed changes in the prevalences of struvite and CaOx uroliths (13).

In general, limiting the amounts of urolith precursors in food, controlling for urine pH, and diluting the urine can help to prevent urolith formation (1). Struvite uroliths can be prevented by feeding an acidifying maintenance food with low magnesium and phosphorus (14). From the early 1980s to the early 2000s, the pet food industry shifted to low magnesium, urine-acidifying foods, which may have caused the decrease in the proportion of struvite uroliths and increase in the proportion of CaOx uroliths. It is reported that feeding foods that acidify the urine results in increased urinary calcium excretion (15, 16), which raises the risk of CaOx uroliths (17). However, results from a more recent study indicated that acidification of the urine pH in ranges representative of those resulting from consumption of most commercial foods did not increase the risk of CaOx crystallization, although foods resulting in lower urine pH did reduce the risk of struvite crystallization (18).

Acidification of urine appears to reduce struvite formation but may increase the risk of CaOx urolith formation (2, 4). In fact, existing struvite uroliths can be dissolved in about 2–5 weeks by consumption of foods that are low in magnesium and acidify the urine (14, 19–21). Magnesium can form a complex with oxalate, which inhibits the association of oxalate with calcium and thus decreases CaOx urolith formation (22). In a large study evaluating food and urolith composition, an increased risk of struvite uroliths in cats was observed with foods containing high fiber, calcium, phosphorus, magnesium, sodium, potassium, or chloride and that were formulated to increase urine pH, whereas cats had a higher risk of CaOx uroliths when fed foods with significantly lower protein, sodium, potassium, or moisture and that were formulated to reduce urine pH (23). While CaOx uroliths cannot be dissolved using a food-based intervention (2), there is some evidence that CaOx uroliths can be prevented with specially formulated food (24) that also increases the concentration of glycosaminoglycans in urine, which inhibit the growth and aggregation of CaOx crystals (25).

Relative supersaturation (RSS) provides a risk index of crystallization via a computer algorithm into which the concentrations of several urinary solutes and pH are entered (1). Several different methods have been developed to calculate urine supersaturation (26–30). EQUIL is a program originally coded in FORTRAN (31) that has been considered the gold standard for RSS calculations in humans (32). EQUIL was updated to the BASIC coding language in 1985, yielding EQUIL2 (33). Another update, EQUIL93, added more ions to be evaluated (34), but those are beyond the number analyzed in standard practice, thus limiting its use in clinical settings. The EQUIL-HL21 program is a Windows-based update of EQUIL2 that can be used on modern computers, uses new coefficients for urinary solutes, has recently been shown to be similar with EQUIL2, and is available in the cited publication (35).

The present study tested the hypothesis that EQUIL-HL21 could show differences in CaOx and struvite RSS values for cat foods that are or are not indicated for urinary conditions. It was expected that consumption of urinary foods would result in RSS for struvite and CaOx at the lower end of the metastable range (1–2.5 for struvite and < 12 for CaOx uroliths in cats (36)) and that non-urinary foods would result in significantly higher struvite and CaOx RSS values. In addition, the relationship between urine analytes and the RSS values for CaOx and struvite was examined in order to evaluate the biological relevance and relationship between the urine analytes and their contributions to these RSS values in the context of animal-to-animal variation.

2. Materials and methods

2.1. Study design

A search of historical data from food trials conducted at Hill's Pet Nutrition, Inc. was completed to find those with available urinalysis data from July 30, 2010 to March 13, 2020. All trial protocols had been approved by the Hill's Institutional Animal Care and Use Committee and complied with Hill's Global Animal Welfare Policy and the United States National Research Council guidelines (37). Cats were housed individually at Hill's Pet Nutrition Center in condos that opened into a bigger room for unrestricted daily socialization on non-collection days. Food was provided once daily with access for approximately 22 h each day and water access was unlimited. Trials were selected based on foods from multiple brands that were either (1) indicated for use in cats

with urinary conditions, including those indicated to dissolve struvite stones ("urinary" foods), or (2) not indicated for use in cats with urinary conditions, including wellness foods from a diverse range of brands and therapeutic nutrition that was not specifically indicated for urinary conditions ("non-urinary" foods). Selected trials also included at least 10 cats fed over a period of at least 7 days (mean 20.7 days; range 7–28 days). Data for 130 observations from cats fed 10 urinary foods and 127 observations from cats fed 10 non-urinary foods were chosen for this analysis. One of the non-urinary foods was used in two trials. Some observations were from the same animals that were used in more than one trial, but because each trial or feeding period was independent of other trials, these data were included.

2.2. Sample collection and analysis

Samples of study foods were collected and analyzed prior to each trial. Nutrient compositions of the foods were determined by Eurofins (Lancaster, PA, United States) using standard AOAC methods (38).

On sample collection days, cats that were acclimated to urine collection and in their individual condos voluntarily voided urine into litter boxes with inert beads (Polypropylene Plastic Pellets Virgin PP only; SmartCat Box, Providence House Mfg. Inc., Lake Elsinore, CA, United States) over a 24-h period. [While the amount of collected urine was typically sufficient within 24 h, study protocols allowed the cats to remain in the study if they had not urinated within 24 h (up to 72 h). The sample was pulled when the collected urine volume was sufficient.] During collection periods, litter boxes were checked four times daily for feces, which was removed along with replacement of the beads to prevent contamination of urine. Litter boxes contained a drain hole and tubing to allow urine to directly collect in a thymol (Alfa Aesar, Thermo Fisher Scientific, Ward Hill, MA, United States)-treated container (to prevent bacterial growth) in a closed system (to prevent evaporation) that was housed within a 37°C water bath. Following the 24-h collection period, urine was moved to the laboratory and immediately analyzed for urine pH by potentiometric measurement using a pH meter (B30PCI, SympHony, VWR, Radnor, PA, United States) that was calibrated on each day of use. Urinalysis, including evaluation of urine sediment for crystals, was performed following pH analysis on the day of collection prior to freezing. Samples were then frozen at – 20° C until individual analyses were conducted using validated methods within 14 days of collection. Ammonium (39) and oxalate (40) were analyzed as previously described. Minerals (including calcium, magnesium, phosphorus, potassium, and sodium) were analyzed by inductively coupled plasma spectrometry (41), and sulfate and chloride were analyzed by ion chromatography (42), both adapted from AOAC methods. Citrate was determined via an enzymatic colorimetric assay (Boehringer Mannheim/R-Biopharm, Washington, MO, United States). These data were then used to calculate RSS via EQUIL-HL21 (Hill's Pet Nutrition, Inc., Topeka, KS) (35).

2.3. Statistical analysis

For analyses of urinary and non-urinary foods, summary statistics and linear model-based hypothesis tests of mean differences are presented. For the hypothesis test, two different linear models were used. If the variances were significantly different for the two categories of food, then a linear mixed model was used with each food category as a fixed effect and separate variances were estimated for each category. If the variances were similar for the two categories of food, then a linear mixed model was used with food category as a fixed-effect and animal and animal x food category as random effects. It was not possible to combine both models into one because the full model often did not converge. Effects were considered significant when $p \leq 0.05$.

To examine the relationship between CaOx or struvite RSS and urine analytes, multiple regression models were developed using PROC REG in SAS® version 9.4 (SAS Institute, Cary, NC, United States) that could predict RSS values for struvite and CaOx generated by EQUIL-HL21 software. An optimal model is one that, for a given number of predictor variables, produces the minimum error sum of squares, or, equivalently, the maximum R^2 statistic (43). To achieve this, two selection methods were used. One method was RSQUARE, which calculates the R^2 statistic of all one-variable models, then for all two-variable models, then three-variable models, etc. Because the R^2 statistic generally increases as more variables are added to a model regardless of their statistical significance, other criteria are needed to identify the optimal model. To achieve this goal, Mallows' Cp statistic was used. Mallows recommends the model where Cp first approaches p, which is the number of predictor variables plus the intercept. The parameter estimates are unbiased when the correct model is chosen, and this is reflected in Cp near p.

The other selection method employed to examine the relationship between RSS and urine analytes was a stepwise procedure in which variables are added one by one to the model. The F statistic for a variable to be added had to be significant at the SLENTRY = level. For this analysis, a variable had to be significant at the $\alpha = 0.10$ probability level to be included in the model. Variables were added to the model until no new variables meet the SLENTRY criteria. Variables were retained in the final model if the significance level to stay in the model was also < 0.10.

The inclusion of many predictor variables in a regression model can often result in multicollinearity, a high degree of correlation among two or more predictor variables (43). The existence of multicollinearity can inflate the variances of predicted values as well as the variances of the slope estimates and can result in estimates with unstable coefficients with incorrect signs or magnitudes. To check for multicollinearity in the models, the variance inflation factor (VIF) was used. The VIF is defined as $1/(1 - R^2i)$, where R^2i is the coefficient of determination for the regression of the ith independent variable on all other independent variables. The VIF statistic shows how multicollinearity has increased the instability of the coefficient estimates. Although there is no formal criteria for determining the magnitude of the VIF statistic that indicates poorly estimated coefficients, some suggest that values exceeding 10 may be cause for concern (43).

The procedure used to select the best model (PROC REG) uses least-squares regression, which does not allow for inclusion of random effects such as animal-to-animal variation. Therefore, to determine if the model could be improved, and whether slopes estimates were affected by accounting for animal-to-animal variation, the final model was reanalyzed using PROC GLIMMIX in SAS. The PROC GLIMMIX model included the final set of predictor variables as continuous covariates and animal as a random effect. Two criteria were used to assess the results: comparing the residual error variance from the model with and without a random animal term in the model, and the COVTEST option in PROC GLIMMIX, which produces a likelihood ratio test to determine whether the added variance component is significantly different from 0.

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TABLE 1 Characteristics of cats in the included studies.

Characteristic	Urinary foods ($n = 10$)	Non-urinary foods ($n = 10$)
Animals, *n*	43	35
Age, years	6.2 ± 2.4	6.0 ± 2.4
Sex, *n*		
NM	18	15
SF	25	20
Body weight, kg	4.9 ± 0.7	4.9 ± 0.8
Total observations, *n*	130	127
Feeding studies per animal, *n*	3.0 ± 1.9	3.6 ± 1.9
Feeding studies per animal, median (range)	1.9 (1–6)	1.9 (1–6)

Data are mean ± standard deviation unless otherwise indicated. One observation represents one collection from one particular cat.
NM, neutered male; SF, spayed female.

TABLE 2 Least-squares means ± standard errors for nutrient composition on a dry matter basis of feline foods that are or are not indicated for urinary conditions.

Nutrient composition	Urinary foods ($n = 10$)	Non-urinary foods ($n = 10$)	*p*-value
Energy, kcal/kg[1]	4,744 ± 56	4,333 ± 57	< 0.001
Crude protein	36.7 ± 0.59	38.1 ± 0.63	0.132
Crude fat	17.9 ± 0.27	20.5 ± 0.69	< 0.001
Carbohydrate (NFE)	36.3 ± 1.00	31.6 ± 1.06	0.002
Crude fiber	2.2 ± 0.17	2.5 ± 0.18	0.165
Ash	7.1 ± 0.18	7.3 ± 0.19	0.568
Calcium	0.85 ± 0.04	1.29 ± 0.04	< 0.001
Phosphorus	0.80 ± 0.01	1.07 ± 0.04	< 0.001
Potassium	0.93 ± 0.01	0.90 ± 0.01	0.001
Magnesium	0.07 ± 0.01	0.11 ± 0.002	< 0.001
Sodium	0.90 ± 0.04	0.45 ± 0.02	< 0.001
Chloride	1.62 ± 0.06	0.77 ± 0.03	< 0.001
Sulfate	0.96 ± 0.08	0.74 ± 0.12	0.122

Units are % unless otherwise indicated.
[1]In the event that energy content was not analyzed in a food, the published metabolizable energy value was used.NFE, nitrogen-free extract.

3. Results

3.1. Characteristics of cats used in the studies

A total of 78 cats, in whom 257 observations were recorded, were utilized in the studies chosen for this analysis (Table 1).

3.2. Comparisons of urinary and non-urinary foods

Comparison of the nutrient composition between urinary ($n = 10$ foods) and non-urinary ($n = 10$) foods showed that the urinary foods were significantly higher in energy, carbohydrate, potassium, sodium, and chloride, while non-urinary foods were higher in crude fat, calcium, phosphorus, and magnesium (Table 2). There was no significant difference between the two types of foods for crude protein, crude fiber, or sulfate. Nutrient compositions of the individual foods included in this analysis are in Supplementary Table 1.

TABLE 3 Least-squares means ± standard errors for urine parameters measured in cats consuming urinary or non-urinary products.

Urine parameters	Urinary food ($n = 130$)	Non-urinary food ($n = 127$)	*p*-value
Calcium oxalate RSS	3.33 ± 0.32	3.71 ± 0.37	0.071
Calcium oxalate crystals, count[1]	1	11	0.003
Struvite RSS	1.04 ± 0.17	4.48 ± 0.42	<0.001
Struvite crystals, count	8	47	<0.001
pH	6.15 ± 0.05	6.72 ± 0.05	<0.001
Ammonium, mM	190 ± 7.0	220 ± 7.0	0.002
Sodium, mM	220 ± 6.0	110 ± 6.0	<0.001
Potassium, mM	106 ± 5.0	200 ± 6.0	<0.001
Chloride, mM	250 ± 11.0	150 ± 6.0	<0.001
Calcium, mM	1.1 ± 0.1	1.2 ± 0.1	0.093
Phosphorus, mM	50.0 ± 2.0	80.0 ± 2.0	<0.001
Magnesium, mM	3.2 ± 0.2	4.5 ± 0.2	<0.001
Oxalate, mM	0.8 ± 0.04	1.2 ± 0.04	<0.001
Citrate, mM	1.2 ± 0.4	4.3 ± 0.4	<0.001
Sulfate, mM	80 ± 3.5	90 ± 0.4	0.016

[1]Actual count of the number of samples in which crystals were observed under microscopic observation.
RSS, relative supersaturation.

Cats that were fed urinary foods ($n = 130$ observations) had significantly higher sodium and chloride in urine compared with cats fed non-urinary foods ($n = 127$ observations; Table 3), which is consistent with the higher levels of sodium and chloride present in the urinary foods. Feeding non-urinary foods resulted in significantly higher urine pH, ammonium, potassium, phosphorus, magnesium, oxalate, citrate, and sulfate. There was no significant difference in urine calcium between the two groups.

The struvite RSS of urine from cats fed non-urinary foods was more than 4-fold greater compared with those fed the urinary foods ($p < 0.001$), and the number of struvite crystals present in urine were about 6-fold higher ($p < 0.001$; Table 3). Although the group means for CaOx RSS were not significantly different for the two food categories ($p = 0.071$), the CaOx crystal count was 11-fold higher ($p = 0.003$) in cats that had consumed non-urinary foods.

3.3. Predictive models for struvite RSS values

Both the RSQUARE and stepwise methods selected the same models for predicting struvite RSS values, with an R^2 of 0.58 (Table 4). The Cp statistic first decreased below the number of predictor variables with a five-predictor variable model. The slope estimates

TABLE 4 Urinary predictor variables selected and the associated R^2 and Mallows' Cp statistics for the RSQUARE selection method, and predictor urinary predictor variables selected by the stepwise selection method for predicting struvite relative supersaturation.

Number of variables	Selection method: RSQUARE	R^2	Mallows' Cp	Selection method: stepwise
1	pH	0.470	57.3	pH
2	pH, magnesium	0.520	30.6	pH, magnesium
3	pH, magnesium, citrate	0.558	12.0	pH, magnesium, citrate
4	pH, magnesium, oxalate, citrate	0.570	7.1	pH, magnesium, oxalate, citrate
5	pH, sodium, magnesium, oxalate, citrate	0.578	4.8	pH, sodium, magnesium, oxalate, citrate

TABLE 5 Slope estimates, standard errors (SE), *p*-values, and variance inflation factor (VIF) for a five-predictor variable model selected for predicting struvite relative supersaturation.

Variable	Slope estimates	Standard errors (SE)	*p*-value	Variance inflation factor (VIF)
Intercept	−32.26	2.26	<0.001	0
pH	5.16	0.35	<0.001	1.49
Sodium	−3.93	1.70	0.022	1.02
Magnesium	519	103.3	<0.001	1.91
Oxalate	1,278	518.3	0.014	1.92
Citrate	−286	51.3	<0.001	1.59

indicated that struvite RSS increased as urine pH, magnesium, and oxalates increased, and struvite RSS decreased as urine sodium and citrate increased (Table 5). All of the predictor variables in this model have a VIF value < 2, indicating that multicollinearity is not a problem with this model. There was no benefit in accounting for animal-to-animal variation with this model, as the animal variance component was not significantly different from 0 ($p = 0.325$), and the residual error variance remained largely unchanged with the inclusion of a random animal term.

For evaluating bias in the model, predicted versus observed struvite RSS values were plotted (Supplementary Figure 1). Struvite RSS values were relatively linear from 0 to 8, with the prediction equations underestimating the observed RSS beginning around 8. The curvature in a plot of the residuals versus observed values for struvite RSS indicate bias at both low and high values, with the model underestimating the predicted value at very low (< 0.5) RSS values and overestimating at very high (> 8) RSS values (Supplementary Figure 2).

In order to improve the predictive performance of the model, struvite RSS was natural log-transformed and the RSQUARE and stepwise selection methods were repeated. Both methods selected the same eight-predictor variable model with an R^2 of 0.94 (Table 6). The slope estimates indicated that predicted struvite RSS values increased with increasing urine pH, ammonium, chloride, calcium, phosphorus, and magnesium, and that struvite RSS decreased with increasing urine citrate and sulfate (Table 7). Two variables, urinary ammonium and urinary sulfate, had VIF values > 3, which are well below the threshold of 10. Although these two analytes were strongly correlated with each other ($r = 0.784$), the magnitude of this correlation was not strong enough to appreciably affect the coefficient estimates in the model, and so multicollinearity is not considered to be a serious problem with this model.

A plot of the natural log of predicted struvite RSS values and natural log of observed struvite RSS values showed good agreement between the two (Supplementary Figure 3). Since struvite RSS values on a non-logarithmic scale are of more interest to clinicians, the antilog values for predicted and observed struvite RSS were plotted (Supplementary Figure 4). These values generally followed the 1:1 line, particularly at lower values, indicating that there was an absence of bias in the model. The residuals plot shows that they were mainly randomly distributed around 0 (Supplementary Figure 5).

3.4. Predictive model for CaOx RSS values

The RSQUARE and stepwise methods were also used to select the best model for predicting CaOx RSS using urine analytes with an R^2 of 0.94 (Table 8). Mallows' Cp statistic first decreased below the number of predictor variables with an eight-variable model. Both methods selected the same models, with the exception of the seven-predictor variable model, for which the RSQUARE method chose urinary phosphorus but the stepwise method chose urinary potassium. The slope estimates indicate that CaOx RSS increased as urine chloride, calcium, and oxalates increased, and that CaOx RSS decreased as urine pH, sodium, phosphorus, citrate, and sulfate increased (Table 9). All of the predictor variables in this model had a VIF value < 10 (43), indicating that multicollinearity is not a problem with this model. There was no benefit from accounting for animal-to-animal variation with this model because the animal variance component was not significantly different from 0 ($p = 0.917$), and the residual error variance remained largely unchanged with the inclusion of a random animal term.

Bias in the eight-predictor variable model for predicting CaOx RSS was evaluated. The predicted and observed values mainly fell along a 1:1 diagonal line, with only a few predicted values below the line, indicating that the bias does not appear to be very strong (Supplementary Figure 6). Similarly, the residuals were generally randomly scattered along the x-axis, although above the value of 10 most of the residuals were positive, indicating that predicted values were slightly underestimated in this range (Supplementary Figure 7).

TABLE 6 Urinary predictor variables selected and the associated R^2 and Mallows' Cp statistics for the RSQUARE selection method, and urinary predictor variables selected by the stepwise selection method for predicting the natural log of struvite relative supersaturation.

Number of Variables	Selection method: RSQUARE	R^2	Mallows' Cp	Selection method: stepwise
1	pH	0.659	1,196	pH
2	pH, magnesium	0.845	414	pH, magnesium
3	pH, ammonium, magnesium	0.884	254	pH, ammonium, magnesium
4	pH, ammonium, magnesium, citrate	0.916	121	pH, ammonium, magnesium, citrate
5	pH, ammonium, phosphorus, magnesium, citrate	0.936	37	pH, ammonium, phosphorus, magnesium, citrate
6	pH, ammonium, chloride, phosphorus, magnesium, citrate	0.940	24	pH, ammonium, chloride, phosphorus, magnesium, citrate
7	pH, ammonium, chloride, phosphorus, magnesium, citrate, sulfate	0.943	12.2	pH, ammonium, chloride, phosphorus, magnesium, citrate, sulfate
8	pH, ammonium, chloride, calcium, phosphorus, magnesium, citrate, sulfate	0.944	8.6	pH, ammonium, chloride, calcium, phosphorus, magnesium, citrate, sulfate

TABLE 7 Slope estimates, standard error (SE), *p*-values, and variance inflation factor (VIF) for an eight-predictor variable model for predicting the natural log of struvite relative supersaturation.

Variable	Slope estimates	Standard errors (SE)	*p*-value	Variance inflation factor (VIF)
Intercept	−21.1	0.37	<0.001	0
pH	2.87	0.05	<0.001	1.35
Ammonium	6.26	0.61	<0.001	3.11
Chloride	0.97	0.27	<0.001	1.22
Calcium	69.1	29.1	0.018	1.20
Phosphorus	10.4	1.22	<0.001	1.29
Magnesium	301	15.2	<0.001	1.88
Citrate	−89.2	7.62	<0.001	1.54
Sulfate	−4.16	1.15	<0.001	3.04

4. Discussion

This study used the new EQUIL-HL21 program to compare CaOx and struvite RSS values and urine parameters in cats following the consumption of urinary or non-urinary foods. These data establish that EQUIL-HL21 can detect differences in urinary and non-urinary foods where expected. The ideal RSS is ≤1 for dissolution (undersaturation) of struvite uroliths in cats (36). For prevention, the metastable range has been reported as 1–2.5 for struvite (36), with a mean of ≤1.8 as the midpoint of the metastable saturation zone. The urinary foods tested here showed a struvite RSS of 1.04, while the non-urinary foods had a struvite RSS of 4.49. These results are consistent with the RSS values for dissolution and prevention as well as with the commercial claims that the urinary foods can dissolve struvite uroliths. For CaOx uroliths in cats, the ideal RSS for prevention is <12 (metastable) (36), with a mean of ≤6 as the midpoint of the metastable saturation zone. Both the urinary and non-urinary foods assessed in this study had CaOx RSS values <4 and so are in the metastable range. The CaOx RSS values for the urinary and non-urinary foods in this study did not significantly differ despite an 11-fold difference in CaOx crystal formation. A possible explanation for this is that foods designed to prevent CaOx uroliths may include inhibitors that are not captured in the RSS calculation. RSS includes a limited number of inputs, and thus does not capture all known inhibitors to crystallization often used in formulations. For example, some urine proteins, such as glycosaminoglycans, osteopontin, nephrocalcin, and prothrombin fragment-1, can inhibit CaOx urolith formation (44, 45). Future work should examine the contributions of these factors to RSS values.

Significantly higher levels of sodium and chloride in urine were observed when cats were fed urinary foods compared with non-urinary foods, consistent with the greater amounts of sodium and chloride in the urinary foods. In addition, significantly lower urine pH, ammonium, potassium, phosphorus, magnesium, oxalate, citrate, and sulfate were seen with consumption of urinary foods. Given the composition of struvite uroliths, it corresponds that consumption of foods formulated for urinary indications would lead to less magnesium, ammonium, and phosphorus in the urine. Further, these data are consistent with the prior observation that foods with higher levels of magnesium or phosphorus led to an increased risk of struvite urolith formation in cats (23).

No difference in urine calcium concentrations were observed in this study between the foods formulated for urinary and non-urinary indications. Prior observations established that higher dietary sodium results in increased urinary calcium excretion but a lower urinary calcium concentration due to urine dilution (46). This may explain the observation in the present study despite higher sodium in the urinary foods compared with the non-urinary foods and may be an area for future study. Urine dilution may also be contributing to the fewer observed crystals in the urine of cats fed the urinary foods. Since the RSS calculation utilizes fractional excretion of analytes rather than urine specific gravity, urine dilution may be a factor in reduced crystallization, but it is not captured in the RSS calculation.

The initial five-predictor variable model for examining the relationship between RSS and the urine analytes to predict struvite RSS was unsuccessful. However, an eight-predictor variable model selected for predicting the natural log of struvite RSS values was much more successful and indicated that predicted struvite RSS values would increase with increasing urine pH, ammonium, chloride, calcium, phosphorus, and magnesium, and that predicted struvite RSS values would decrease with increasing urine citrate and sulfate. For modeling of CaOx RSS, an

TABLE 8 Predictor variables selected and the associated R^2 and Mallows' Cp statistics for the RSQUARE selection method, and predictor variables selected by the stepwise selection method for predicting calcium oxalate relative supersaturation.

Number of Variables	Selection method: RSQUARE	R^2	Mallows' Cp	Selection method: stepwise
1	Calcium	0.762	642	Calcium
2	Calcium, citrate	0.799	511	Calcium, citrate
3	Calcium, oxalate, citrate	0.901	132	Calcium, oxalate, citrate
4	Potassium, calcium, oxalate, citrate	0.923	51.8	Potassium, calcium, oxalate, citrate
5	Sodium, potassium, calcium, oxalate, citrate	0.927	39.9	Sodium, potassium, calcium, oxalate, citrate
6	Sodium, potassium, chloride, calcium, oxalate, citrate	0.933	18.9	Sodium, potassium, chloride, calcium, oxalate, citrate
7	Sodium, chloride, calcium, potassium, oxalate, citrate, sulfate	0.936	11.7	Sodium, potassium, chloride, calcium, oxalate, citrate, sulfate
8	pH, sodium, chloride, calcium, potassium, oxalate, citrate, sulfate	0.937	7.9	pH, sodium, chloride, calcium, potassium, oxalate, citrate, sulfate

TABLE 9 Slope estimates, standard error (SE), *p*-values, and variance inflation factor (VIF) for an eight-predictor variable model selected for predicting calcium oxalate relative supersaturation.

Variable	Slope estimates	Standard errors (SE)	*p*-Value	Variance inflation factor (VIF)
Intercept	1.31	0.80	0.101	0
pH	−0.23	0.12	0.049	1.84
Sodium	−5.59	1.09	<0.001	4.59
Chloride	3.41	1.13	0.003	5.17
Calcium	2,970	59.2	<0.001	1.18
Phosphorus	−11.4	2.47	<0.001	1.27
Oxalate	2,820	178	<0.001	2.48
Citrate	−243	15.5	<0.001	1.57
Sulfate	−10.6	1.64	<0.001	1.45

eight-predictor variable model accurately predicted CaOx RSS, with predicted CaOx RSS values increasing as urine chloride, calcium, and oxalates increased, and decreasing as urine pH, sodium, phosphorus, citrate, and sulfate increased. Because existing CaOx uroliths cannot be dissolved by a food-based intervention, their prevention is key. Thus, the ability to predict CaOx RSS can help in the determination of which foods would result in less CaOx crystal formation and thus prevent CaOx uroliths. Of note, there is a lack of publications for comparison with this work, pointing to a potential need for further study.

One limitation of using RSS analysis is that there are parameters that are not considered in the RSS algorithm that can have an effect on urolith formation. Dietary protein levels also appear to influence crystallization, as feeding cats a high-protein food (55% crude protein) led to significantly lower urine pH and fewer struvite crystals compared with a lower-protein food (47). Since only one of the foods in the present analysis had a crude protein level > 55%, future work is needed to determine the contribution of high dietary protein to RSS values and crystallization. In addition, supplementation of cat food with long-chain polyunsaturated fatty acids led to a decreased RSS for struvite, fewer struvite crystals, and greater resistance to CaOx crystal formation compared with feeding a food without this supplementation (48). The optimal form of nutrients for urolith prevention may also not be clear, as $KHCO_3$ as a potassium source appeared to be more beneficial than KCl for the prevention of CaOx uroliths (49). As we could not determine the form of potassium used in the foods of the present analysis, we cannot evaluate whether this was a contributor to the observations of this study. This analysis was also limited due to its retrospective nature, so specific variables that may be of interest were not varied in the foods tested and so could not be examined as predictors of RSS. It should be noted that statistical significance (or lack of) does not indicate clinical significance, and the differences should be interpreted from a clinical perspective. Another limitation is that these feeding trials were performed on healthy cats, so the results may not be applicable to cats who form uroliths. Nonetheless, RSS as calculated by EQUIL-HL21 was able to show differences in struvite RSS values between consumption of urinary and non-urinary foods, which was supported by the nearly six-fold difference in the observed number of struvite crystals.

In summary, data from this analysis indicate that the RSS values determined via the EQUIL-HL21 program can discriminate between foods formulated for urinary and non-urinary indications. Regression modeling has indicated which urinary analytes contribute to the predicted RSS values for struvite and CaOx.

Data availability statement

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

Ethics statement

The animal studies were approved by Institutional Animal Care and Use Committee, Hill's Pet Nutrition, Topeka, KS,

United States. The studies were conducted in accordance with the local legislation and institutional requirements. Written informed consent was not obtained from the owners for the participation of their animals in this study because animals were owned by Hill's Pet Nutrition.

Author contributions

EM and DJ contributed to the conception and design of the study. EM and AM searched the historical trial data. JB performed the statistical analysis. All authors contributed to the development of the manuscript, including interpretation of data, and approved the submitted version.

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Conflict of interest

JB is an employee of Alpha Statistical Consulting, Inc., a subcontractor of Hill's Pet Nutrition, Inc., and DJ is a former employee of Hill's Pet Nutrition, Inc.

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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.2023.1167840/full#supplementary-material

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Modulation of digestibility of canine food using enzyme supplement: an *in vitro* simulated semi-dynamic digestion study

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Digestibility and nutrient availability are important parameters when estimating the nutritional quality of pet food. We have developed a simulated semi-dynamic *in vitro* canine digestion model to evaluate the digestibility of dry extruded canine food. Canine food was assessed for digestible energy, dry matter digestibility, protein digestibility, non-fibrous carbohydrate (NFC) digestibility, and total antioxidant capacity (TAC) in the absence and presence of an enzyme blend (DigeSEB Super Pet). Enzyme blend supplementation in canine food was found to increase the dry matter digestibility (18.7%, $p< 0.05$), digestible energy (18.1%, $p<0.05$), and protein digestibility (11%, $p< 0.1$) and reducing sugar release (106.3%, $p< 0.005$). The release of low molecular weight peptides (48.7%) and essential amino acids (15.6%) increased within 0.5h of gastrointestinal digestion due to enzyme blend supplementation. Furthermore, the TAC of the digesta was also increased (8.1%, $p<0.005$) in the canine food supplemented with enzyme blend. Overall, supplementation of enzyme blend in canine food is an effective strategy to enhance the food digestibility and nutrient availability for absorption.

KEYWORDS

canine food, enzyme blend, macronutrient digestion, semi dynamic *in vitro* digestion, antioxidant

1. Introduction

Companion animals positively affect the emotional and physical health of people with whom they are in contact. Anthropomorphism of canines makes owners more concerned about their pet's health and wellbeing (1). They are observant of their pets' diet in order to provide optimal nutrition and maintain their long-term health (2). Digestibility and nutrient availability are important parameters when estimating the nutritional quality of pet food (3). The diet composition, nutrient availability, and their interaction also regulates the cognition and behavior of canines (4, 5).

The pet food industry has introduced various commercial extruded kibble diets to the market (Pedigree adult chicken and vegetable, Ykibble oven baked premium canine food, IMS proactive health, etc.) with beneficial claims (improved skin health, strong bones and teeth, strong muscles, natural defense, optimum health, etc.). Health and nutrition are the foremost criteria of pet food selection; there are also quality, ingredients, freshness, taste, pet preference, and ease of preparation (6). A variety of plant-based (fruits, vegetables,

grains, legumes, nuts, and seeds) and animal-based (meat, eggs, dairy products, and organ meat) ingredients are added to the pet food (7) either individually or in combination to provide a 'complete and balanced' diet that meets the nutritional requirements. However, adequate digestion of the macromolecules (carbohydrate, protein, and fat) in the diet is imperative to disintegrate the food matrix and to release the required macronutrients, micronutrients, and minerals from the diet for absorption. While manufacturing commercial canine food products, processing methods positively or negatively affect the nutritional value (8). For instance, extrusion cooking positively influences palatability, digestibility, and destruction of undesirable factors but can also have a potentially negative impact on protein quality and vitamin availability (9). The inclusion of vegetable-based ingredients may add some anti-nutritional factors to the canine diet (10). Additionally, digestive health varies in each canine along with factors such as age and other illnesses. In this scenario, pet food supplemented with enzymes can enhance its digestibility and nutrient availability. A multitude of enzymes (β-mannanase, phytase, protease, xylanase, β-glucanase, cellulase, amylase, pectinase, lipase, and glucoamylase) have been studied to evaluate their effect on the digestibility of canine food (11–18). The inclusion of mannanase in the soybean meal increased protein and energy digestibility in dogs (11). The addition of proteases and lipases in feather meal showed enhanced digestible energy in dog trials (15). Diets supplemented with xylanase, β-glucanase, and amylase alleviated the anti-nutritive effect of non-starch polysaccharides (16). Further, multi-enzyme complexes are known to improve nutrient digestibility in pigs and poultry (19–23).

Here, we developed a simple and reproducible simulated semi-dynamic *in vitro* canine digestion model to study the canine food digestibility in presence of an enzyme blend (EB) supplement. We hypothesized that canine food supplemented with EB would enhance the digestibility and release of nutrients compared to its non-supplemented counterpart. DigeSEB Super Pet (a commercial enzyme blend) was used in this study as a model enzyme blend supplement.

2. Materials and methods

2.1. Materials

Extruded dry adult canine food [protein 21.5%, fat 7.4%, carbohydrate (nitrogen-free extract + crude fibers) 55.9%, moisture 8.9%, ash 6.3%, and gross energy 4.4 kcal/g; Ingredients: cereal and cereal by-product, chicken and chicken by-product, meat and meat by-product, soybean meal, di-calcium phosphate, soyabean oil, iodized salt, choline chloride, vitamins and minerals, antioxidant, carrot powder, pea powder, zinc sulfate monohydrate, preservative, and flavors] was purchased from the local market. Enzyme blend (DigeSEB Super Pet: acid proteases 10,500 HUT/g, alkaline proteases 1800 PC/g, amylase 2,135 SKB/g, and lipase 155 FIP/g) was a gift sample from Specialty Enzymes, United States. Pepsin (P6887) and pancreatin (P7545) were purchased from Sigma, India. Other chemicals used were of AR grade and purchased from Merck, India.

2.2. Simulated semi dynamic *in vitro* digestion model

A simulated semi dynamic canine *in vitro* digestion model was developed using the information obtained from the dynamic digestion model described by Smeets-Peeters et al. (3). The gastrointestinal digestion of the canine food was performed in a 2 L glass reactor with a temperature set to 39°C and a pH probe inserted in the reactor for pH monitoring. Briefly, food solution (150 mg of finely ground food powder/mL of distilled water, 300 mL) was mixed with simulated gastric fluid, pH 1.9 (10 mL; SGF-NaCl 3.5 g/L, KCl 1.3 g/L, $CaCl_2$ 0.2 g/L, $NaHCO_3$ 0.25 g/L, pepsin 75 mg/L, and lipase 90 mg/L), and EB (1% of the food). The control reaction was set up by replacing EB with an equal amount of distilled water. Gastric digestion was carried out at 39°C and 100 rpm for 3 h. Kinetic aspects of the dynamic model, such as gradual acidification and fluid and enzyme secretion, followed in the gastric phase of this model. After every 0.5 h of the gastric phase 15 mL of SGF pH 1.9 was added to the reaction mixture, and the pH of the system was adjusted using 1 N HCl. The pH was adjusted to 5.4, 5, 4.2, 3, 2.3, 2.1, and 1.9 at 0, 0.5, 1, 1.5, 2, 2.5, and 3, respectively (Supplementary Figure 1). Samples (20 mL) were removed at 0.5, 1, 2, and 3 h of the gastric phase, and the reaction mixture was replenished with the same amount of SGF. After 3 h of gastric digestion, the entire gastric digesta was shifted to another 2 L glass reactor (set at intestinal reaction conditions). Gastric digesta was mixed with the 417 mL of the simulated intestinal fluid (Supplementary Figure 1) (SIF-NaCl 7 g/L, KCl 0.5 g/L, and $MgCl_2.6H_2O$ 0.813 g/L), 135 mL of bile solution (60 g/L), and 67 mL of pancreatin (10 g/L). The pH of the reaction was adjusted to 6.5 using 1 N NaOH. The intestinal digestion was further carried out at 39°C and 100 rpm for 3 h. Samples (20 mL) were removed at 0.5, 1, 2, and 3 h of the intestinal phase (Gastrointestinal or GI phase), and the reaction mixture was replenished with the same amount of SIF. All the samples were centrifuged at 4°C and 3,000 rpm for 10 min. Pellet and supernatant were separated and used as undigested and digested fractions, respectively. Obtained undigested fractions were dried at 65°C until they reached a constant weight (24). All the digested fractions were stored at −20°C until required for analysis.

2.3. Analysis

2.3.1. Dry matter digestibility and energy digestibility

Moisture content of the undigested fraction was determined using IR balance, and dry matter digestibility was calculated using the following formula (24).

$$Dry\ matter\ digestibility\ (\%) = \frac{\text{Weight of raw sample} - \text{Weight of undigested fraction}}{Weight\ of\ raw\ sample} * 100$$

The energy content of the raw sample and undigested fraction was determined using an automatic Hamco 6E bomb calorimeter (25). Digestible energy was calculated using the following formula.

$$Digestible\ energy\ (kcal/kg) = Gross\ energy\ of\ raw\ sample - Gross\ energy\ of\ undigested\ fraction$$

2.3.2. Protein digestibility

EB blanks were run for each test but the protein was too low to contribute in any of the test results. The total protein present in the raw sample and undigested fraction was determined using the Kjeldhal method (6.25 conversion factor), and the protein digestibility was determined using the formula below (26, 27):

$$Protein\ digestibility\ (\%) = \frac{(Total\ protein\ in\ raw\ sample\ (g) - Total\ protein\ in\ undigested\ fraction\ (g))}{Total\ protein\ in\ raw\ sample\ (g)} * 100$$

The digested fraction (digesta) was analyzed for the degree of hydrolysis using an o-phthalaldehyde (OPA) assay. The sample (25 µL) was mixed with the OPA reagent (175 µL), and the reaction was incubated at room temperature (27 ± 2°C) for exactly 2 min. Absorbance was measured at 340 nm (28). Free amino groups were determined using slope of the standard curve (40–200 µg/mL of serine). The raw sample was hydrolyzed by acid and evaluated for total free amino groups by OPA assay. The degree of hydrolysis was determined as follows:

$$Degree\ of\ hydrolysis\ (\%) = \frac{Free\ amino\ groups\ in\ the\ digesta}{Free\ amino\ groups\ in\ acid\ hydrolysed\ raw\ sample} * 100$$

Amino acids released in the digesta were determined using HPLC with DAD detector. The column used was Agilent Zorbax Eclipse AAA at 40°C with a flow rate of 2 mL/min. The samples were derivatized using o-phthalaldehyde (OPA) and fluorenylmethyloxycarbonyl chloride (FMOC) as per the Agilent's instruction manual. The gradient system started from 98% of 40 mM phosphate buffer pH 7.8 and ended with 2% of an acetonitrile:methanol:water (45:45:10) mixture. Molecular weight distribution of the peptides in the digesta was determined using a SEC-HPLC system (28). Appropriately diluted samples were run on BioSep, 5 µm, SEC-s2000, 145 Å (Phenomenex Inc.) column at 25°C with a flow rate of 1 mL/min. The mobile phase used was a phosphate buffer (0.1 M, pH 6.8), and peptides were detected using DAD at 214 nm.

2.3.3. Non-fibrous carbohydrate digestibility

The total reducing sugars released in the digesta were quantified using 3,5-Dinitrosalicylic acid (DNSA) method (29) and dextrose (0.1–1 mg/mL) as a standard. Glucose released in the digested samples was detected using a GOD-POD kit (AUTOSPAN® liquid gold glucose kit).

2.3.4. Total antioxidant capacity

The total antioxidant capacity (TAC) of the digesta was determined using a phosphomolybdate assay and ascorbic acid (40–200 µg/mL) as a standard (30). Briefly, an appropriately diluted sample (0.1 mL) was mixed with the phosphomolybdate reagent (1 mL, 4 mM ammonium molybdate and 28 mM sodium dihydrogen phosphate in 0.6 M sulfuric acid). Reaction was incubated at 95 ± 2°C for 90 min, and absorbance was noted at 765 nm. TAC of the digesta was calculated as ascorbic acid equivalent in total digesta (mg) using following formula:

$$\text{Ascorbic acid equivalent in total digesta (mg)} = \left(\frac{(T - B) - constant}{Slope\ of\ standard\ curve} * D\right) * \frac{V}{1000}$$

where T and B-absorbance of test and reagent blank at 765 nm, D—dilution factor, and V—volume of the digested sample (mL).

2.3.5. Statistical analysis

All the experiments were performed in triplicates and represented as mean ± SD. Statistical analysis was performed on GraphPad Prism 9. Student's *t*-test and two-way ANOVA with Tukey's multiple-comparison test were used to analyze the data. $p \leq 0.05$ was considered as statistically significant.

3. Results and discussion

3.1. Simulated semi dynamic *in vitro* digestion model

In this study, a simulated semi-dynamic *in vitro* digestion model was used to evaluate the effect of EB (DigeSEB super pet) on canine food digestibility. The protein digestibility value obtained by our model was 55 ± 2.1% (Figure 1A), similar to the value obtained by Smeets-Peeters et al. (3) using a dynamic model (fast transit time), i.e., 62%. The variation in digestibility can be attributed to the difference in food composition as well as the nature of the model (semi-dynamic vs. dynamic). Digestion is a complex process that involves physicochemical, mechanical, and microbial parameters, which play a paramount role in canine health. *In vivo* canine food digestibility has been reported previously (31, 32) in literature. However, the restrictions imposed on the *in vivo* studies in canines are stringent due to ethical, regulatory, societal, and economical pressures. Alternatively, *in vitro* digestion models such as static and dynamic canine digestion model have also been used previously in various studies, including on protein digestibility, calcium availability (3), organic matter and energy digestibility (33), selenium accessibility (34), *in vitro* dissolution of formulation (35), the effect of supplementation of larvae meal in canine food on digestibility (36), and the effect of thermal processing on the digestibility of raw chicken meat (37). Although dynamic models closely mimic the complex nature of the digestive system, its laboratory practicality is constricted. On the other hand, the semi-dynamic model described in this study is simple, inexpensive, time saving, reproducible, and feasible in any lab. Although the results are not directly comparable to *in vivo* data in dogs, it can be used as a screening step/predictive system to optimize/compare the formulation/product before proceeding to the clinical study in dogs. The results of such *in vitro* studies can also envisage a prospective clinical study.

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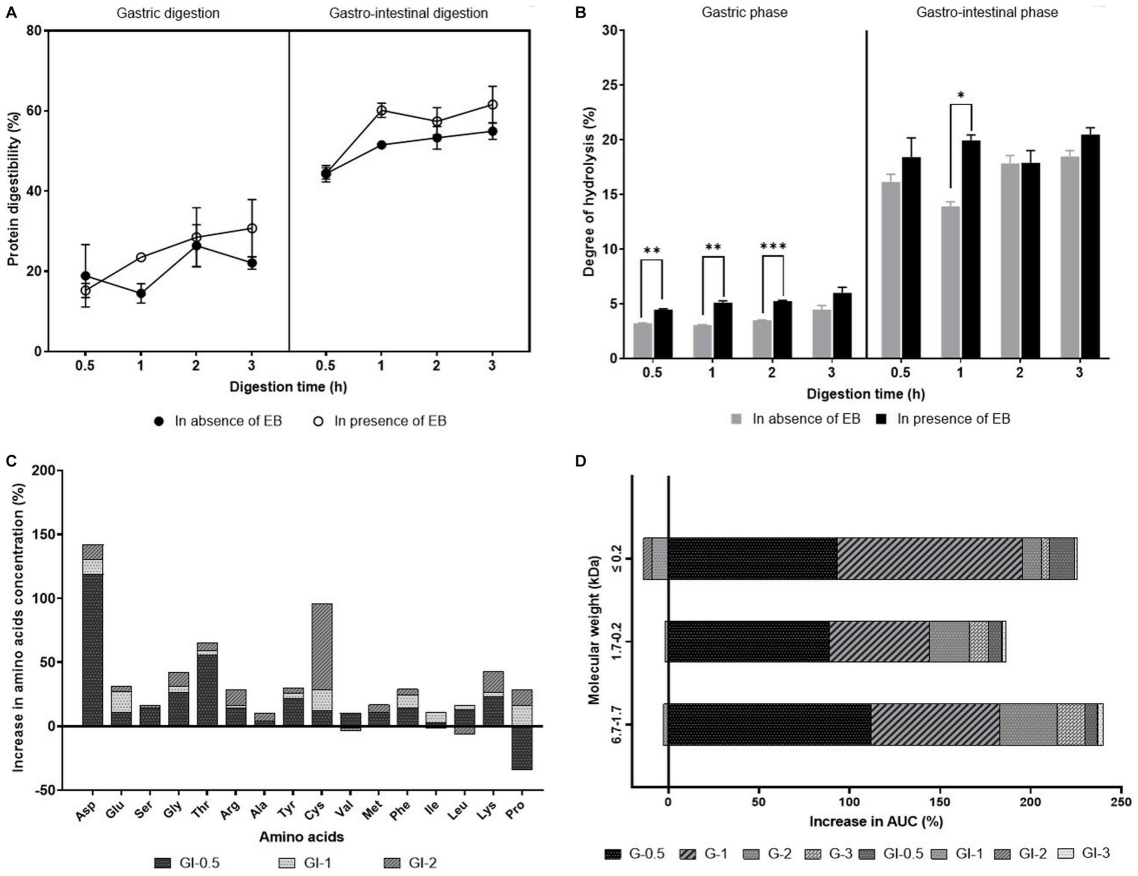

FIGURE 1
(A) Protein digestibility (%) and **(B)** Degree of hydrolysis (%) after gastric and gastro-intestinal digestion of the canine food in absence and presence of enzyme blend. **(C)** Increase in amino acid concentration (%) after gastro-intestinal (GI) digestion (digestion time—0.5, 1, and 2 h) of the canine food in presence of enzyme blend. **(D)** Increase in AUC (%) after gastric (G) and gastrointestinal (GI) digestion (digestion time—0.5, 1, 2, and 3 h) of the canine food in presence of enzyme blend. Values are represented as mean ± standard deviation. Two-way ANOVA with Tukey's multiple-comparison test was used to determine the *p* value. *, **, and *** represent significant difference at $p \leq 0.05$, $p \leq 0.005$, and $p \leq 0.001$, respectively.

A dynamic canine digestion model developed by Smeets-Peeters et al. (3) was computer controlled to simulate the pH, transit time, and secretion of digestive juices. While in this study, the semi-dynamic digestion model was manually altered for pH, addition of electrolytes, and enzymes based on the information (pH over the time, composition and concentration of electrolytes, and enzymes at any given time point) cumulated from the dynamic model. The pH was adjusted, and digestive juices (electrolytes and enzymes) were routinely added to the gastric phase post every 0.5 h. The intestinal phase, however, was static in nature—unlike the one used in the dynamic digestion model. While the dynamic digestion model spanned over 6 h consisting of various gastric and intestinal transit times, the semi-dynamic model differentiated into 3 h of gastric phase and 3 h of intestinal phase. Unlike dynamic digestion model, semi-dynamic digestion model does not include membrane absorption hence can not estimate/predict the bioavailability of the nutrients.

3.2. Effect of enzyme-blend supplementation in canine food on the dry matter and energy digestibility

Dry matter digestibility and the digestible energy of the dry extruded canine food were determined in the absence and presence of EB supplement (DigeSEB Super Pet). In the absence of EB, dry matter and energy digestibility increased slowly during gastric digestion but rapidly during the gastrointestinal digestion. Alternatively, the supplementation of EB contributed to the increased digestibility during the gastric digestion itself, indicating the improved digestion of food. The EB could enhance the dry matter digestibility from 48 to 58% ($p < 0.05$) and energy digestibility from 1,975 to 2,331 kcal/kg ($p < 0.05$) post complete gastrointestinal digestion of the canine food (Figure 2). Enzyme blend supplements containing amylase, protease, and lipase assist the endogenous digestive enzymes in the breakdown of macromolecules to release the nutrients from the food matrix, which in turn aids in increasing food digestibility and availability of nutrients for absorption. In prior *in vivo* studies, exogenous enzyme supplementation had not shown any effect on the canine food digestibility (18). The enzyme performance is dependent on its activity and specificity; hence, the careful selection of enzymes is necessary for its effect on digestibility. This also highlights the importance of an *in vitro* simulated digestion model in the optimization of formulation/product prior to designing an *in vivo* study.

Bourreau et al. (38) has previously explained that a high gastric emptying rate might overload the small intestine of smaller canines due to discharge of inadequately pre-digested food particles. These food particles are generally less susceptible to intestinal enzymatic

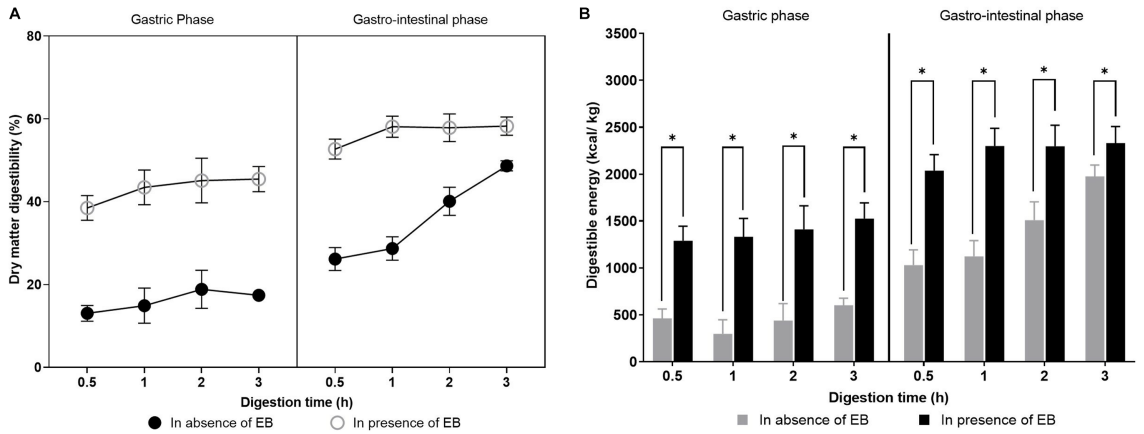

FIGURE 2
(A) Dry matter digestibility (%) and **(B)** Digestible energy (kcal/kg of sample) of the canine food in absence and presence of enzyme blend at gastric and gastro-intestinal phase. Values are represented as mean ± standard deviation. Student's *t-test* was used to determine *p* value. *represents a significant difference at $p \leq 0.05$.

hydrolysis and remain undigested. The undigested food then serves as a nutrient source for microbiomes through colonic fermentation (saccharolysis, proteolysis, and lipolysis). The metabolites produced during such colonic fermentation can have beneficial/detrimental effects on the host, depending on their nature. Proteolytic putrefaction produces proinflammatory uremic toxins, which have a negative impact on the host (39). Increment in dry matter digestibility of canine food observed after supplementation of EB can reduce the flow of undigested material to the colon and can further reduce the potential harmful impact on the host.

3.3. Effect of enzyme blend supplementation in canine food on the protein digestibility

Protein is an indispensable macronutrient required for optimum growth and maintenance. After digestion, smaller peptides and amino acids are released from proteins, which get absorbed in the intestine where they serve as an energy source and provide components necessary for metabolic functions. However, if they remain undigested, protein reaches the colon and negatively influences the canine intestinal ecosystem. It increases the ammonia levels, reduces the volatile fatty acids, lowers the lactobacilli and enterococci, and increases the *Clostridium perfringens* (40). In the absence of EB, protein digestibility was 22.1 ± 1.6% (gastric phase) and 54.9 ± 2.1% (gastrointestinal phase), which increased to 30.7 ± 7.1% (gastric phase, $p > 0.1$) and 61.6 ± 4.5% (gastrointestinal phase, $p > 0.1$) in the presence of EB (Figure 1A). The degree of hydrolysis (DH) is the measure of protein hydrolysis during digestion. Higher DH correlates with more solubility and higher availability of the protein for the absorption. The digesta obtained in the absence of EB showed a DH of 4.4 ± 0.4% and 18.4 ± 0.6% in the gastric and gastrointestinal digestion, respectively, whereas in the presence of EB, it increased to 5.9 ± 0.5% ($p < 0.1$) and 20.4 ± 0.6% ($p < 0.1$), respectively (Figure 1B). The DH data at 2 h gastro-intestinal digestion was not in line with the trend over the digestion time, which might be due to an error while sampling the reaction mixture. External enzyme supplementation of protease has shown increased apparent ileal crude protein and amino acid digestibility of over processed soybean meals in boilers (41). The improved (though not statistically significant) protein digestibility and hydrolysis observed in our study was owing to the presence of proteases in the supplements that work complementary to the endogenous proteases.

The nutritive value of the protein is dependent on the bioavailable peptides and amino acids. The amino acid profile of the digesta revealed that the EB supplement could increase the indispensable amino acid release by 15.62% within 0.5 h GI digestion (Figure 1C). Moreover, it also increased the release of phenylalanine, tyrosine, cysteine, and methionine content by 14.5, 21.71, 12.1, and 10.6%, respectively. The molecular weight distribution of the digesta demonstrated that the EB supplement increased the release of lower molecular weight peptides in the gastric stage itself, corroborating with the higher protein digestibility in the gastric phase (Figure 1D). The smaller molecular weight peptides are easily absorbed in the intestine (28). Overall, the EB supplement was found to increase the protein digestibility, release of lower molecular weight peptides, and free amino acids in the canine food. Previously amino acid supplementation had shown to reduce hair loss (42), induce intense and darker hair coat colors (43), and promotes normal cardiac function (44) in canines.

3.4. Effect of enzyme blend supplementation in canine food on non-fibrous carbohydrate digestibility

Carbohydrates are a major part of canine food that provide energy and fibers (45). The composition and structure of carbohydrates affects their digestibility. High oil maize, broken rice, sorghum, and millet showed better digestibility and greater metabolizable energy for canines than wheat bran, maize germ, and rice bran (46). In the current study, the effect of the EB supplementation on the NFC digestion was studied in terms of reducing sugars and glucose released in the digesta. In absence of EB, the total reducing sugar was 9.8 ± 0.1 mg/g at 0.5 h of gastric digestion and reached 101.8 ± 0.1 mg/g

TABLE 1 Total reducing sugar release (mg/g of sample) and glucose release (mg/g of sample) in absence and presence of enzyme blend (EB).

Reaction time (h)	Total reducing sugar release (mg/g of sample)						Glucose release (mg/g of sample)		
	Gastric phase			Gastro-intestinal phase			Gastro-intestinal phase		
	Absence of EB	Presence of EB	*p* value	Absence of EB	Presence of EB	*p* value	Absence of EB	Presence of EB	*p* value
0.5	9.8 ± 0.1	151.5 ± 5^{b}	0.0006	17.8 ± 1.1^{d}	186.0 ± 9.8^{b}	0.0017	4.8 ± 0.28	26.5 ± 1.5^{a,b}	0.0024
1	9.1 ± 0.5	174.4 ± 1.9^{a}	0.0001	38.2 ± 2.5^{c}	188.0 ± 12^{b}	0.0033	5.1 ± 0.22	23.9 ± 0.8^{b}	0.001
2	9.0 ± 0.4	163.5 ± 5.6^{a,b}	0.0006	72.3 ± 1.4^{b}	188.1 ± 0.9^{b}	0.0001	5.9 ± 0	27.3 ± 0.4^{a,b}	0.0001
3	13.1 ± 0.2	170.4 ± 4.7^{a}	0.0005	101.8 ± 0.1^{a}	210.1 ± 3.5^{a}	0.0005	6.3 ± 0.3	30.5 ± 0.1^{a}	<0.0001

Moisture was not subtracted while calculating nutrient release. Data represented as mean ± standard deviation. Two-way ANOVA with Tukey's multiple-comparison test was used to determine *p* value. Superscript lowercase letters present significant differences ($p \leq 0.05$) between the samples of the same column, whereas the value of *p* represents a statistical relation between the samples in the same row.

at end of the digestion. While that in presence of EB, it was 151.5 ± 5 mg/g at 0.5 h of digestion and reached 210.1 ± 3.5 mg/g at the end of the digestion. The increased reducing sugar concentration indicated improved NFC digestion ($p < 0.005$; Table 1). Furthermore, the glucose release was 6.3 ± 0.3 and 30.5 ± 0.1 mg/g in the absence and presence of EB, respectively, at the end of the digestion ($p < 0.0001$; Table 1).

Results demonstrated that EB supplementation promotes starch degradation as shown by the 2-fold increase in reducing sugar and 5-fold increase in glucose concentration. Amylase present in the EB supplement is hypothesized to work simultaneously or sequentially with pancreatic amylase in starch degradation to improve the digestion of NFC, which might have potential in management of the hypoglycemia in pets (47).

3.5. Effect of enzyme blend supplementation in canine food on the total antioxidant capacity

The total antioxidant capacity of the canine diet is useful to the regulation of the health status of the pet. The total antioxidant capacity of the digesta obtained in the absence and presences of EB was evaluated using a phosphomolybdate assay. The TAC of the digesta increased in the gastrointestinal phase compared to the gastric phase, which may be due to the larger degradation of the macromolecules in the gastrointestinal phase to release the antioxidants from the complex food matrix. The TAC of the digesta in the presence of EB was significantly higher than in the absence of EB at 0.5 h ($p < 0.05$) and 1 h ($p < 0.05$). Although not significantly different, at the end of the digestion (3 h), the TAC of the digesta in the presence of EB [1,306 ± 74 ascorbic acid equivalent (mg)] was higher than in the absence of the EB [1,208 ± 78 ascorbic acid equivalent (mg); Figure 3]. At the end of the digestion, EB supplement contributed to the increase in TAC by 8.1%. EB supplemented in the canine diet was found to release maximal antioxidant from the food matrix, potentially playing a vital role in the management of the oxidative status of the pet (48, 49). Antioxidant supplements in the diet of 62 Alaskan sled canines has previously shown resistance to exercise-induced oxidative damage (48). Antioxidant blends of vitamins, minerals, and carotenoids supplemented in the canine diet showed increased circulation of antioxidants and reduced DNA damage (49).

The positive impact of EB supplementation in the canine diet was illustrated in the simulated *in vitro* semi-dynamic digestion model with respect to dry matter and energy digestibility and release of macronutrients and antioxidants. Though *in vivo* studies in pets might shed more light on the importance of the enzyme supplements in the pet food, this model is beneficial as a predictive system in screening/optimizing formulations/products before proceeding for *in vivo* trials. However, this model has a few limitations, such as the results not being directly comparable with the *in vivo* results—it fails to incorporate the complexity of the dynamic nature of the digestive system, it cannot mimic the neuro-hormonal feedback mechanism, and it is unable to elucidate the bioavailability of the nutrients.

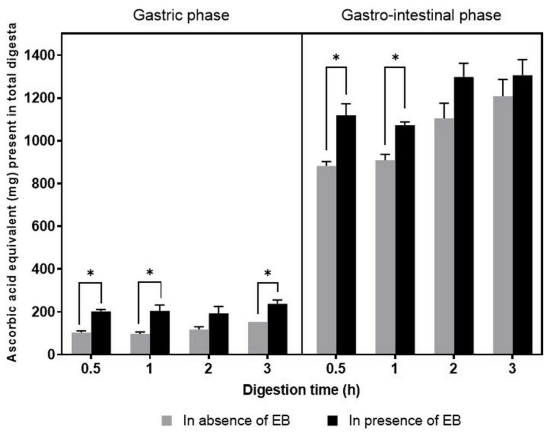

FIGURE 3
Total antioxidant capacity [ascorbic acid equivalent (mg) present in total digesta] after gastric (G) and gastro-intestinal (GI) digestion of the canine food in absence and presence of enzyme blend. Two-way ANOVA with Tukey's multiple-comparison test was used to determine *p* value. *represents a significant difference at $p \leq 0.05$.

4. Conclusion

A simulated semi-dynamic *in vitro* canine digestion model was used to evaluate the effect of external enzyme blend supplementation in canine food on the digestibility. DigeSEB Super Pet; an enzyme blend supplementation, not only increased the dry matter and energy digestibility but also improved the

protein and NFC digestion. Moreover, the total antioxidant capacity of the digested food was also found to be increased due to DigeSEB Super Pet. Overall, enzyme blend supplementation in the canine diet increased the food digestibility and the release of nutrients for absorption that would in turn ensure that the pet is adequately nourished.

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.

Author contributions

SJ and AR: conceptualization, methodology, validation, and writing—review and editing. MJ and TG: formal analysis and data curation. SJ and TG: writing—original draft preparation and visualization. SJ: supervision. All authors contributed to the article and approved the submitted version.

Conflict of interest

AR, SJ, MJ, and TG are paid employees of Advanced Enzyme Technologies, which has a corporate affiliation with Specialty Enzymes and Probiotics. Specialty Enzymes and Probiotics had no role in the study design and actual conduct of the study.

Publisher's note

Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2023.1220198/full#supplementary-material

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Feeding of fish oil and medium-chain triglycerides to canines impacts circulating structural and energetic lipids, endocannabinoids, and non-lipid metabolite profiles

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Introduction: The effect of medium-chain fatty acid-containing triglycerides (MCT), long-chain polyunsaturated fatty acid-containing triglycerides from fish oil (FO), and their combination (FO+MCT) on the serum metabolome of dogs (*Canis familiaris*) was evaluated.

Methods: Dogs ($N = 64$) were randomized to either a control food, one with 7% MCT, one with FO (0.18% eicosapentaenoate and 1.3% docosahexaenoate), or one with FO+MCT for 28 days following a 14-day washout period on the control food. Serum metabolites were analyzed via chromatography followed by mass spectrometry.

Results: Additive effects of serum metabolites were observed for a number of metabolite classes, including fatty acids, phospholipids, acylated amines including endocannabinoids, alpha-oxidized fatty acids, and methyl donors. Some effects of the addition of FO+MCT were different when the oils were combined compared with when each oil was fed separately, namely for acylcarnitines, omega-oxidized dicarboxylic acids, and amino acids. Several potentially beneficial effects on health were observed, including decreased circulating triglycerides and total cholesterol with the addition of FO (with or without MCT) and decreases in N-acyl taurines with the addition of MCT, FO, or FO+MCT.

Discussion: Overall, the results of this study provide a phenotypic characterization of the serum lipidomic response to dietary supplementation of long-chain n3-polyunsaturated and medium-chain saturated fats in canines.

KEYWORDS

canine, fish oil, medium-chain triglycerides, metabolome, lipidome, endocannabinoid

1. Introduction

The impact of the gut microbiome on host development, health, and metabolism has been well-studied in the last few decades, with dietary factors affecting the composition and function of the microbiome in both companion animals (including dogs) and humans (1–3). Several of these studies have linked the composition of the gut microbiome with circulating lipids in humans (3, 4). Medium-chain triglycerides (MCTs) and long-chain polyunsaturated triglycerides (LCPUTs) are dietary fatty acids with demonstrated positive health effects. While each can provide dietary energy, they can also affect physiology.

Prior study has shown that MCT can cause changes in the gut microbiome in a mouse model and in pigs (5, 6). In addition to the effects on the microbiome, MCT confers positive effects on the intestine. MCT supplementation appeared to protect rats from endotoxemia, preventing mortality, and injury to the gut and liver following lipopolysaccharide administration (7) and improving chemically induced colitis in rats (8). Supplementation with capric acid resulted in positive changes in the structure of the ileal mucosal epithelium (9) and protected against induced intestinal oxidative stress, inflammation, and barrier function, both studied in pigs (10). These effects may have implications on circulatory metabolites of microbial origin.

The n3 long-chain polyunsaturated fatty acids [LCPUFA(n3)] have frequently been tested in canine foods and have shown to be effective in changing many relevant biological outcomes. Both LCPUFA(n6) (11) and LCPUFA(n3) (12) are essential fatty acids in dogs. Fish oil (FO), which is high in LCPUT, can affect the composition of the gut microbiome as seen in studies on obesity (13, 14) and brain aging (15) in mice. Foods high in docosahexaenoic acid (DHA; C22:6n3) were shown to improve cognitive learning, immunological, and retinal function in puppies (16). FO with an eicosapentaenoate (EPA; C20:5n3)/DHA ratio of approximately 1.5 was effective at reducing urinary 11-dehydro thromboxane B_2 concentration in dogs, supporting its effectiveness in reducing inflammation (17). EPA and DHA have been shown to aid in the management of osteoarthritis (18–20), including reducing the needed medications in managing this disease (21). EPA and DHA also confer anti-inflammatory effects (22) and lead to modifications in the gut microbiome in both humans and animal models (13, 23). Consumption of FO by dogs enriches the composition of circulating complex lipids with DHA (24). As with MCT, LCPUFA(n3) appear to exert protective effects on gut epithelial barrier function in an *in vitro* model (25). The LCPUFA(n6) linoleate (18:2n6) is considered essential (26), and its levels decrease in the skin of dogs with ichthyosis (27). The ratio of n6/n3 LCPUFA may be a determinant of the degree to which these LCPUFAs are beneficial (28). In some contexts, LCPUFA(n6) may be detrimental to gut health, as shown through the modification of the gut microbiome in a mouse model (29). Whereas FO has been used therapeutically to manage canine disease related to immune status and inflammation, MCTs have been employed in companion animal dogs as a therapeutic intervention to aid in the management of seizures (30, 31) and cognitive decline (32). MCT combined with FO appears to decrease inflammation (33, 34) in mice, may modulate risk factors of cardiovascular disease (35) in a rat model, and may abate age-related changes in circulating concentrations of fatty acids and carnitine metabolites in dogs (36). The combination of MCT and FO has also been used to manage myxomatous mitral valve heart disease in dogs (37). However, in that study, FO was predominantly composed of EPA with relatively little DHA. A previous study in cats tested the effects of foods including MCT, FO, or both on the plasma metabolome and found a combined effect on several lipid classes, including those derived from gut microbial metabolism (38).

Canine physiological states share commonality with human counterparts; for example, there are parallels between the physiology of human and canine aging (39) and obesity (40, 41). As well, dogs are a model for human gestational diabetes (42), insulin-dependent diabetes mellitus (type 1) (43), and glucocorticoid therapy (44). Dogs are also prone to endocrine diseases that afflict humans, including non-insulin-dependent diabetes mellitus (type 2) (40), Cushing disease (45), and hypothyroidism (46). Dogs in these life stages, disease states, and conditions present with altered lipid profiles and dyslipidemia similar to humans. Aging and obesity elevate triglycerides in both humans (47, 48) and dogs (49, 50). Progression and outcome of human type 2 diabetes are influenced by diet and are associated with hypercholesterolemia (51); canine type 2 diabetes is also impacted by diet type (52), and experimental canine diabetes manifests hypercholesterolemia (43), although the organ-specific contributions to cholesterol accretion differ between species. Excess levels of glucocorticoids promote dyslipidemia (53) and changes in the circulating complex lipids (en toto, the "lipidome") that accompany acute and chronic glucocorticoid or adrenocorticotropin hormone provision have recently been described in dogs (54). Changes in the circulating canine lipidome accompanying Cushing disease and hypothyroidism have also recently been investigated in dogs (55). In contrast with humans, dogs are normally resistant to atherosclerosis even in the presence of obesity and dyslipidemia (56). Intriguingly, dogs with atherosclerosis were more likely to have concurrent diabetes or hypothyroidism (57), similar to observations for humans with hypothyroidism (58) and diabetes (59).

Representative lipidomes are similar between dogs and humans in ocular (60) and synovial (61) matrices. Despite these commonalities, variations in the circulating lipidome can differentiate breed types (62). Examinations of the lipidome have shown utility in studying genetic (63) and pharmacologic (54) canine models of disease. The canine lipidome has also been assessed in naturally occurring inflammatory diseases, including atopic dermatitis (64), chronic gastroenteritis (65), and the aforementioned endocrine diseases (55). The impact of dietary LCPUFA(n3), including DHA and EPA, on classes of metabolites within the canine lipidome has been reported (66), including a study that monitored the canine lipidome during a dietary feeding study with increased n6- and n3-PUFA in dogs with enteritis (67). In the present study, healthy dogs were chosen as test subjects as they have been previously assessed for response to intake of dietary lipids (36, 66, 68, 69). While these studies showed that food can modify some metabolomic parameters, the global lipidome response was not reported (36, 68, 69), control and test foods were not matched for ingredients and nutrition (66), and/or the effects of MCT with FO both alone and in combination were not compared (36, 66, 68, 69).

Here, dog food was supplemented with FO, MCT, or both as part of a complete maintenance food balanced for total dietary fat, protein, and carbohydrates in order to determine the impact of these dietary oils on the canine serum lipidome. This is the first study to report levels of several classes of lipid metabolites examined, including non-esterified fatty acids as well as their glycerides, acylcarnitines, amide endocannabinoids, and structural phospholipids. The fatty acid oxidation products canonically produced by the mitochondria (beta-hydroxy), endoplasmic reticulum (alpha-hydroxy), peroxisome (dioate), and membrane

(e.g., hydroxyeicosatetraenoate [HETE]) are also reported. Changes in circulating proxies of central metabolites including those of the tricarboxylic acid cycle (TCA) and amino acids were analyzed to gain insight into the degree to which the dietary fats were impacting associated energetic pathways. Circulating putrefactive postbiotics and their host-conjugated sulfates were also evaluated, particularly as our previous study showed that co-consumption of FO+MCT in domestic cats results in a decrease in circulating postbiotics of the indole and phenol classes (38).

2. Materials and methods

2.1. Ethics statement

The Institutional Animal Care and Use Committee, Hill's Pet Nutrition, Topeka, KS, USA (Protocol Number: FP578.1.2.0-A-C-D-ADH) reviewed and approved the study protocol. The study also complied with the National Institutes of Health guide for the care and use of laboratory animals and the guides from the US National Research Council and the US Public Health Service (70). Healthy dogs were included in the study, defined as those without chronic systemic disease based on physical examination, complete blood count, serum biochemical analyses, urinalysis, and fecal examination for parasites. No invasive procedures were used in this study.

2.2. Food formulation and production

The four dry extruded test foods used in the study were composed of the same base formula primarily of poultry byproduct meal, wet chicken meat, pork fat, barley, corn gluten meal, whole corn, wheat, and sorghum, as well as liver hydrolysate, fiber, vitamins, and minerals (Supplementary Table 1). MCT, FO, or both replaced pork fat levels in the test foods. As previously (38), CAPTEX-355 (ViaChem Inc, Plano, TX, USA) was the source of MCT, which is enriched for caprylate (C8:0) over caprate (C10:0), and caproate (C6:0) with negligible amounts of laurate (C12:0) and myristate (C14:0). Third-party testing (Eurofins Nutrition Analysis Center, Des Moines, IA, USA) showed the composition to be 51.4% caprylate (C8:0), 39.1% caprate (C10:0), <0.1% laurate (C12:0), and <0.01% each of all other fatty acids. Caproate (C6:0) was not reported but is projected to be approximately 8%. MEG-3™ 0355TG Oil (DSM Inc., Parsippany, NJ, USA) was used as the FO source of LCPUT(n3) as it is enriched for DHA (C22:6n3; 36.5%) over EPA (C20:5n3; 5%). All four foods used in this study met the canine maintenance nutrition requirements of the Association of American Feed Control Officials and National Research Council.

The test oils, MCT and FO, were added to the foods on a dry matter basis to be 7 and 2.85%, respectively. The MCT level was chosen to provide >20% of total dietary fat as MCT, similar to levels in prior publications that tested dietary MCT supplementation in dogs (71, 72). FO was fed at a dietary inclusion level previously found to be safe, as demonstrated in canine feeding trials that employed approximately 100 mg/kg body weight (73, 74). Based on certified analysis of ingredients and formulation levels, the MCT-containing foods had 3.7% caprylate (C8:0) and 4.3% caprate (C10:0), while the FO-containing foods had 0.18% EPA (C20:5n3) and 1.3% DHA (C22:6n3), all on a dry matter basis (Supplementary Table 2).

2.3. Study design and measurements

Animal care research technicians and sample analysts were blinded to the foods provided and to the group identity of dogs for purposes of sample collection and analysis. Dogs were beagles or mixed breeds (Supplementary Table 3), owned by the funders of this research, and acquired from on-site husbandry or licensed breeders. The sample size ($N = 64$) was based on effect sizes from a previous study (36). It was designed for 80% power to detect a 20% difference between groups for selected lipids while allowing for a potential dropout rate of 5% and a need for correction for multiple between-group testing. The study had a 2 × 2 factorial design. During the washout period, all dogs were fed the control (CON) food for 14 days. Dogs were then randomized into one of four foods ($n = 16$ each; CON, MCT, FO, FO+MCT) for 28 days by distributing the dogs into groups based on breed, sex, weight, and age; there were no significant differences in these parameters across groups ($P > 0.7$ for all). All pets had the opportunity for exercise and interaction together in large groups (~20 dogs) but were pair-housed for sleeping arrangements. Dogs remained in their preferred housing arrangement during the trial as previously determined by the colony veterinarian's assessment of temperament and social interactions. Dogs were fed daily at electronic feeders where each pet (through a radio frequency identification chip reader) was individually given access to food for 1 h of a controlled amount. These electronic feeders recorded food intake (g/day) for each dog. Dogs were fed to maintain body weights from the start of the study, which was a mean ± SD metabolizable energy of 1.69 ± 0.40 × 70 kcal × (kg body weight [BW])$^{0.75}$; water was available *ad libitum*.

Serum was collected prior to consumption of test foods at the end of the washout period to serve as a D0 baseline (D0) and again at day 28 (D28) at the end of the feeding period. Dogs were fasted for 23 h prior to serum collection, in which the total amount of blood drawn was 14 mL. Clinical blood chemistry was carried out on a COBAS c501 module (Roche Diagnostics Corporation, Indianapolis, IN, USA), and analysis of serum metabolomics was performed by Metabolon (Morrisville, NC, USA) as in previous studies (38, 75, 76).

2.4. Statistical analysis

As described previously (38), metabolite values were natural log (LN)-transformed, and LN(D0) baseline values were subtracted from LN(D28) end-of-feeding period values to create the difference of logs [LN(D28)–LN(D0)]. This difference of the log values is mathematically equivalent to, and is presented here, as the LN fold change [LN(D28)–LN(D0)] (77). This data normalization approach also conveniently results in positive values when the D28 value for a metabolite is greater than at the D0 baseline, while negative values on the y-axis indicate that a given metabolite has decreased from

the D0 baseline. As each dog had values for both D0 baseline and D28 end of feeding period, each dog served as its own control, which controlled for inter-animal variability and allowed for the reporting of the food effect on a given metabolite.

Changes from the D0 baseline across foods for the global serum metabolome were evaluated with the Metaboanalyst platform v4.0 (78). Sparse partial least squares analysis (SPLS) distinguished among test food groups (number of components = 2, validation method = 5-fold cross-validation, number of predictors = 20). Random Forest detected metabolite predictors of food group identity (number of trees = 2,000, number of predictors = 20, Randomness = On).

Multivariate analysis of variance (MANOVA) was used to assess the degree to which a discrete class of metabolites was altered by food type. Initially, an interaction term (FO × MCT) was included in a two-way ANOVA model, but the results indicated that none of the FO × MCT interactions reached significance when corrected for false discovery rate ($q > 0.1$). Thereafter, the interaction term was omitted and a one-way ANOVA was used to determine a univariate group effect. Dependent sample (paired) *t*-tests on the D0 baseline vs. D28 end-of-feeding natural log-transformed values were used to determine whether the change from the D0 baseline for a particular group was different than zero for a given metabolite. Tukey's *post hoc* test determined which changes from the D0 baseline were different among the groups. All of these analyses were carried out in JMP (Version 14.2-15.0. SAS Institute Inc., Cary, NC, 1989–2019). Whether the change from the D0 baseline of a class of metabolites differed across the test food groups was determined by MANOVA using the identity function, which individually fits a model for each metabolite and subsequently tests the models together. Supplementary Table 4 shows MANOVA *p*-values for Wilks' lambda, Pillai's trace, Hotelling-Lawley, and Roy's Max Root. The metabolite class was considered impacted by food type only when *p*-values for all of these metrics were <0.05. Two-way ANOVA in the Response Screening Platform with Cauchy robust fit in the JMP software package examined changes in individual metabolites within a class resulting from a dietary oil feeding study. In order to correct for multiple testing in the high-dimensional metabolomics data, ANOVA *p*-values were applied as an input, and q-values were generated for all metabolites (79) using the "qvalue" function in the R package qvalue v2.14.1 (80). Change in a metabolite was considered to be statistically significant when $p \leq 0.05$ and $q \leq 0.1$.

3. Results

3.1. Characteristics of dogs in the study

Sixty-four dogs (60 beagles and 4 mixed-breed) were randomized to one of four foods: CON, MCT, FO, or FO+MCT. The mean ± SE age was 5.9 ± 0.5 years, and the mean weight was 11.7 ± 0.4 kg; 41% were spayed females and 30% neutered males, while the rest were intact females (12%) and males (17%) (Supplementary Table 3). Following the study, all dogs were healthy and returned to the colony. There was no effect of food type on intake as quantified as kcal/kg $BW^{0.75}$ and analyzed by a mixed model with an animal as a random factor for repeated intake measurements ($p = 0.34$).

3.2. Impact of dietary oils on clinical blood chemistry

Clinical blood chemistry values remained in normal ranges for healthy canines (Supplementary Table 4). FO consumption resulted in decreased total triglycerides from the D0 baseline (−8.31 mg/dL; $p = 0.0453$). FO+MCT also decreased triglycerides (−13.44 mg/dL; $p < 0.0001$), while MCT alone had no effect. Circulating cholesterol decreased from the D0 baseline in the FO group (−33.06 mg/dL; $p = 0.0002$) but increased in the MCT group (20.13 mg/dL; $p = 0.0075$). There was an overall decrease in cholesterol with FO+MCT feeding (−20.13 mg/dL; $p = 0.0055$).

3.3. Impact of dietary oils on the global serum metabolome

3.3.1. Overview of metabolome

Metabolomic analysis performed on serum samples taken from dogs at the D0 baseline and week 4 identified 701 metabolites. Of these, one-way ANOVA showed that 354/701 metabolites (51%) were different across the four dietary groups (q-value FDR $p \leq 0.05$, $q \leq 0.1$). Individual dependent samples paired *t*-tests by food type to assess change relative to an individual's baseline indicated that 55/701 metabolites (8%) changed in the CON group from the D0 baseline values (31 increased, 24 decreased), 184/701 metabolites (26%) changed in the MCT group (59 increased, 125 decreased), 267/701 metabolites (38%) changed in the FO group (103 increased, 164 decreased), and 329/701 metabolites (47%) changed in the FO/MCT group (136 increased, 193 decreased) (Supplementary Table 4).

SPLS indicated differences among the groups' changes from the D0 baseline (Figure 1), with no overlap of the 95% confidence regions among the foods; however, little of the variation was explained by the first two components (component 1, 11.3%; component 2, 3.6%). SPLS loadings for components 1 and 2 are in Supplementary Table 4. Random Forest analysis (Supplementary Figure 1) provided discrimination between the dietary groups with an overall out-of-bounds class error of 4.7%, where 16/16 dogs were correctly assigned to the control group (class error 0%), 14/16 dogs were correctly assigned to the MCT group (class error 12.5%), 15/16 dogs were correctly assigned to the FO group (class error 6.3%), and 16/16 dogs were correctly assigned to the FO+MCT group (class error 0%). As expected, the top 20 ranked Random Forest predictors were lipid species. Among both SPLS and Random Forest, the lipid species fed in the foods were predominant predictors of a food group membership. The first component of the SPLS was composed of FO-derived DHA- and EPA-containing complex lipids along with arachidonoyl-containing lipids and was indicative of dogs that had been fed FO (or FO+MCT). Accordingly, higher values on the second component indicated increased MCT-derived caproate (C6:0) and

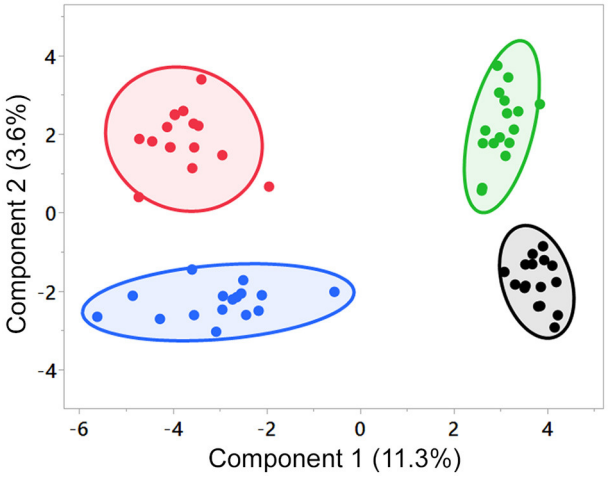


FIGURE 1
SPLS analysis of the test foods. Component 1 segregated groups by the presence of FO, and component 2 segregated groups by the presence of MCT in the food. Number of components = 2, validation method = 5-fold cross-validation, number of predictors = 20. Black, control group; green, MCT group; blue, FO group; red, FO+MCT group. Comp, component; FO, fish oil; MCTs, medium-chain fatty acid-containing triglycerides; SPLS, sparse partial least squares analysis.

caprate (C10:0) and were indicative of MCT (or FO+MCT)-fed dogs.

Major lipid classes were chosen for further analysis: catabolic-type lipids [non-esterified fatty acids (NEFAs), mono- and diglycerides (MDAGs), acylcarnitines, alpha-oxidized fatty acids, omega-oxidized fatty acids (dioates)], signaling-type N-acyl amino acids/neurotransmitters (NAAN), structural-type complex lipids [glycerophosphatidylcholines (GPCs), glycerophosphatidylethanolamines (GPEs), glycerophosphatidylinositols (GPIs), and sphingolipids/ceramides (SPHING)], metabolites involved in central energy metabolism (amino acids, TCA cycle, methylation), and gut microbial postbiotics (indoles, phenols).

3.3.2. Impact of MCT and FO on NEFAs

The metabolite class of NEFAs was different across groups in a multivariate manner (MANOVA $p < 0.001$; Supplementary Table 4), and 30/38 (79%) of the observed NEFA changes from the D0 baseline were different across foods by univariate ANOVA (median $p = 0.0035$). The CON group remained largely unchanged from the D0 baseline while the MCT group exhibited decreases in several NEFAs, and the FO group showed increases in LCPUFA(n3) including DHA (22:6n3) (Figure 2). Feeding of FO+MCT gave largely the same as seen with MCT alone. Together, the MCT and FO+MCT groups showed reduced levels of most NEFA of carbon chain length of 14 through 20.

The medium-chain fatty acid (MCFA) caprate (C10:0) was different across groups by ANOVA ($p \leq 0.0001$), and both the MCT and FO+MCT groups exhibited increased levels of caprate (C10:0) from the D0 baseline that were also different than changes seen in other groups. Another MCFA, caproate (C6:0), differed by food and increased from the D0 baseline in the MCT group, a change largely reproduced as a trend ($p = 0.1002$) in the combination of FO+MCT. The remaining MCFA, caprylate (C8:0), was different across the foods, driven largely by an increase solely in the FO+MCT group. The changes from the D0 baseline for the transition fat myristate (C14:0) were not different across the food groups. However, there was a decrease from the D0 baseline in this fatty acid in the MCT and FO+MCT groups.

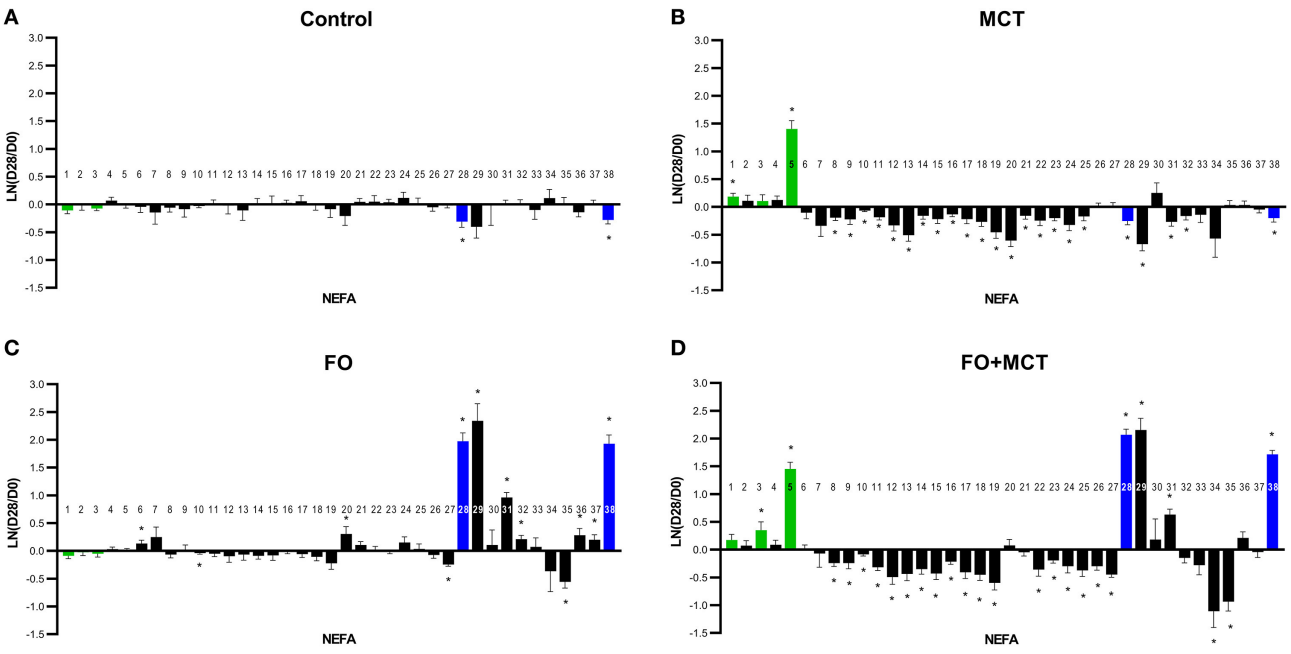
Food type strongly altered the following LCPUFA(n3): stearidonate (18:4n3), EPA (20:5n3), heneicosapentaenoate (21:5n3), docosapentaenoate (DPA, 22:5n3), and DHA (22:6n3). These increased from the D0 baseline in the FO group; EPA (20:5n3), heneicosapentaenoate (21:5n3), and DHA (22:6n3) also increased in the FO+MCT group.

ARA (C20:4n6), a precursor to lipid signaling mediators including prostaglandins, thromboxanes, and leukotrienes, was decreased by the LCPUFA(n3)-containing foods (FO, FO+MCT). Adrenate (22:4n6), the elongation product of ARA (C20:4n6), was also decreased in the FO and FO+MCT groups. In contrast, both docosadienoate (22:2n6) and docosapentaenoate (n6 DPA; 22:5n6) were increased in the FO group but not in the FO+MCT group. The MCT group showed decreased levels of the n6 NEFA, including hexadecadienoate (16:2n6), linoleate (18:2n6), dihomolinoleate (20:2n6), and docosadienoate (22:2n6).

3.3.3. Impact of MCT and FO on fatty acid glycerides, carnitines, endocannabinoid amides, and oxidation products

Multivariate analysis indicated that the MDAG class as a whole was changed by food type (MANOVA $p < 0.0001$; Supplementary Table 4). There were 21 metabolites detected in the data set, and of these, 16/21 (76%) were altered by food according to ANOVA (median $p = 0.0002$; Figure 3). The greatest effect was in the dogs consuming FO-containing foods; 19/21 (90%) of the MDAG were changed, and nearly all of these changes were decreases (1 up, 18 down). The FO+MCT group also had several changes in MDAG: 17/21 (81%) changed (1 up, 16 down). There was only 1/21 (5%) MDAG changed from the D0 baseline in the MCT group and 2/21 (10%) in the CON group.

Acylcarnitines, including carnitine and deoxycarnitine as a multivariate class, were different by food group (MANOVA $p < 0.0001$; Supplementary Table 4). ANOVA detected 20/30 (67%) individual carnitines (median $p = 0.0062$; Figure 4 MCT alone decreased several acylcarnitines (Figure 4), with 21/30 (70%) changed (20 acylcarnitines decreased and deoxycarnitine increased). FO had a more moderate effect on acylcarnitines, with a similar mixture of up (5) and down (4) shifts from the D0 baseline (9/30 changed; 30%). The FO+MCT group manifested 5 increased and 10 decreased (15/30 changed; 50%). Arachidonoylcarnitine was not affected by food, and neither FO nor FO+MCT showed changes from the D0 baseline in this ARA (C20:4n6) metabolite. Deoxycarnitine, precursor to carnitine and acylcarnitines, was increased in all three experimental groups: MCT, FO, and FO+MCT. Furthermore, carnitine itself was increased from the D0



1	caproate (6:0)	9	myristoleate (14:1n5)	17	oleate/vaccenate (18:1)	25	dihomolinoleate (20:2n6)	33	docosatrienoate (22:3n3)
2	heptanoate (7:0)	10	pentadecanoate (15:0)	18	linoleate (18:2n6)	26	dihomolinolenate (20:3n3 or 3n6)	34	docosatrienoate (22:3n6)
3	caprylate (8:0)	11	palmitate (16:0)	19	linolenate (18:3n3 or 3n6)	27	arachidonate (20:4n6)	35	adrenate (22:4n6)
4	pelargonate (9:0)	12	palmitoleate (16:1n7)	20	stearidonate (18:4n3)	28	eicosapentaenoate (EPA; 20:5n3)	36	docosapentaenoate (DPA; 22:5n3)
5	caprate (10:0)	13	hexadecadienoate (16:2n6)	21	nonadecanoate (19:0)	29	heneicosapentaenoate (21:5n3)	37	docosapentaenoate (n6 DPA; 22:5n6)
6	10-undecenoate (11:1n1)	14	margarate (17:0)	22	10-nonadecenoate (19:1n9)	30	behenate (22:0)	38	docosahexaenoate (DHA; 22:6n3)
7	5-dodecenoate (12:1n7)	15	10-heptadecanoate (17:1n7)	23	arachidate (20:0)	31	erucate (22:1n9)		
8	myristate (14:0)	16	stearate (18:0)	24	eicosenoate (20:1n9 or 1n11)	32	docosadienoate (22:2n6)		

FIGURE 2
Change from the D0 baseline to D28 end of feeding period (LN fold change) in serum levels of non-esterified fatty acids. Dogs were fed **(A)** control food, **(B)** food with MCT, **(C)** food with FO, or **(D)** food with FO+MCT. Lipid metabolites are presented in order of increasing chain length and, within a chain length, by increasing unsaturation. Green bars indicate fatty acids found in the MCT ingredient; blue bars indicate fatty acids in the FO ingredient. FO, fish oil; MCTs, medium-chain fatty acid-containing triglycerides. *$p < 0.05$ compared with the D0 baseline.

baseline in only the FO+MCT group, while it was unchanged in the MCT and FO groups.

The serum metabolomics dataset yielded acylated amides from ethanolamide ($n = 2$), taurine ($n = 3$), and choline ($n = 7$), together the NAAN class. As a class, NAAN was different by MANOVA ($p < 0.0001$) and 7/12 (58%) individual NAAN were different by ANOVA (median $p = 0.0129$; Supplementary Table 4). The most changes to members of the NAAN class were observed in the FO+MCT group (10/12, 83%) compared with MCT (5/12, 42%) or FO (4/12, 33%) alone (Figure 5). All changes to NAAN not containing either DHA (C22:6n3) or EPA (C20:5n3) were decreases. Only NAAN containing either DHA (C22:6n3) or EPA (C20:5n3) were increased with FO or MCT.

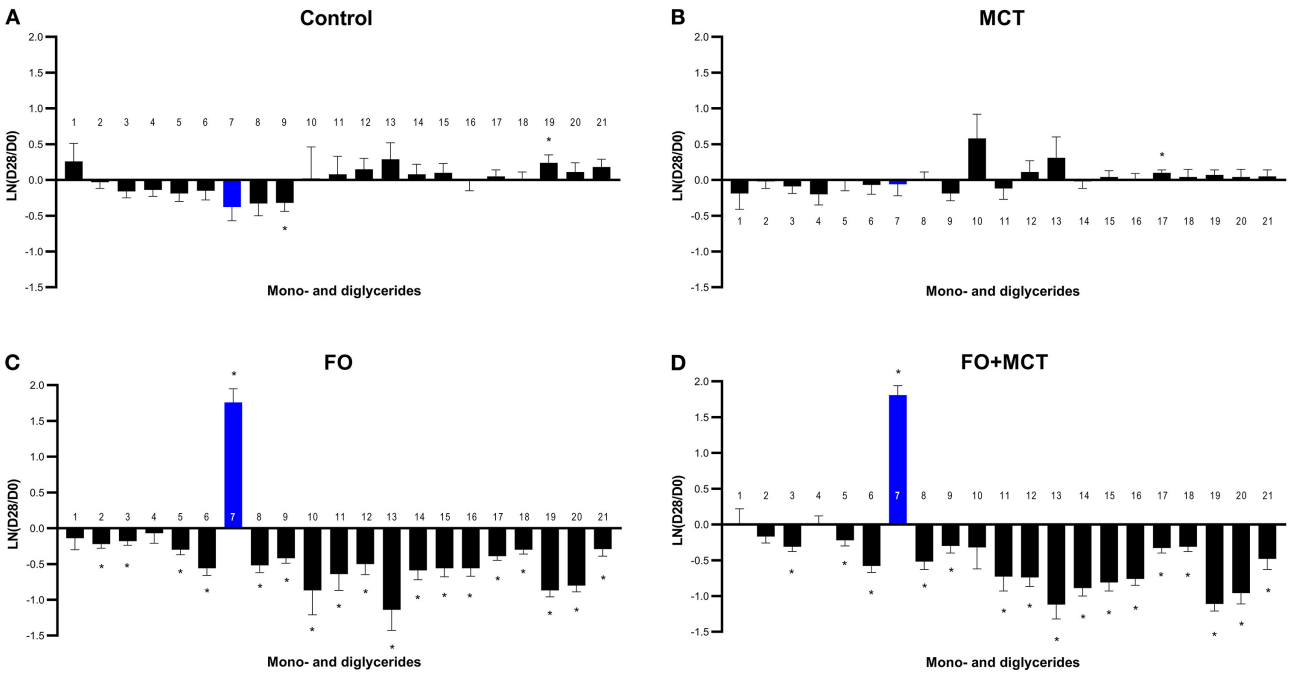
Alpha-oxidized products of monocarboxylic fatty acids (AHFA) are considered to be generated in the endoplasmic reticulum and are precursors of sphingolipids/ceramides. The class of AHFA was different by food type (MANOVA $p < 0.0004$), and 8/11 (73%) of the individual AHFA were different by ANOVA (median $p < 0.0001$; Supplementary Table 4). Both MCT and FO+MCT feeding led to a multifold increase in 2-hydroxylignocerate (C24:0) and a concurrent decline in its unsaturation product 2-hydroxynervonate (C24:1) as well as its chain-shortened congener 2-hydroxybehenate (C22:0).

Dicarboxylate fatty acids (dioates) are produced by omega oxidation of the terminal carbon of monocarboxylic fatty acids in peroxisomes; this metabolite class was different by food type (MANOVA $p < 0.0001$) with 9/15 (60%) of the individual dioates driving this effect by ANOVA (median $p = 0.0250$; Figure 5). When FO and MCT were fed together, there were changes to 11/15 (73%) of dioates, with several changes to dioates of 12 or fewer carbons. Feeding with MCT and FO individually each produced changes in 7/15 (47%) of dioates, with the effect less evident in the dioates of 12 or fewer carbons.

Alternate forms of oxidized fatty acids were assessed as well. The products of fatty acid beta oxidation (3-hydroxy fatty acids) formed in mitochondria and the products of membrane oxidation (9- and 13-hydroxyoctadecadienoate [9-HODE and 13-HODE], 9,10-dihydroxy-12Z-octadecenoate [9,10-DiHOME], 12,13-dihydroxy-9Z-octadecenoate [12,13-DiHOME], 12-hydroxyeicosatetraenoate [12-HETE], 12-hydroxyheptadecatrienoate [12-HHTrE]) were not different as classes by food type, and there were no significant between-group differences (Supplementary Table 4).

3.3.4. Impact of MCT and FO on phospholipids

The metabolite class of GPCs was different across food groups in a multivariate manner (MANOVA $p < 0.0001$; Supplementary Table 4). Generally, GPCs were broadly affected by FO and/or MCT in that 29/36 (81%) of observed GPC changes



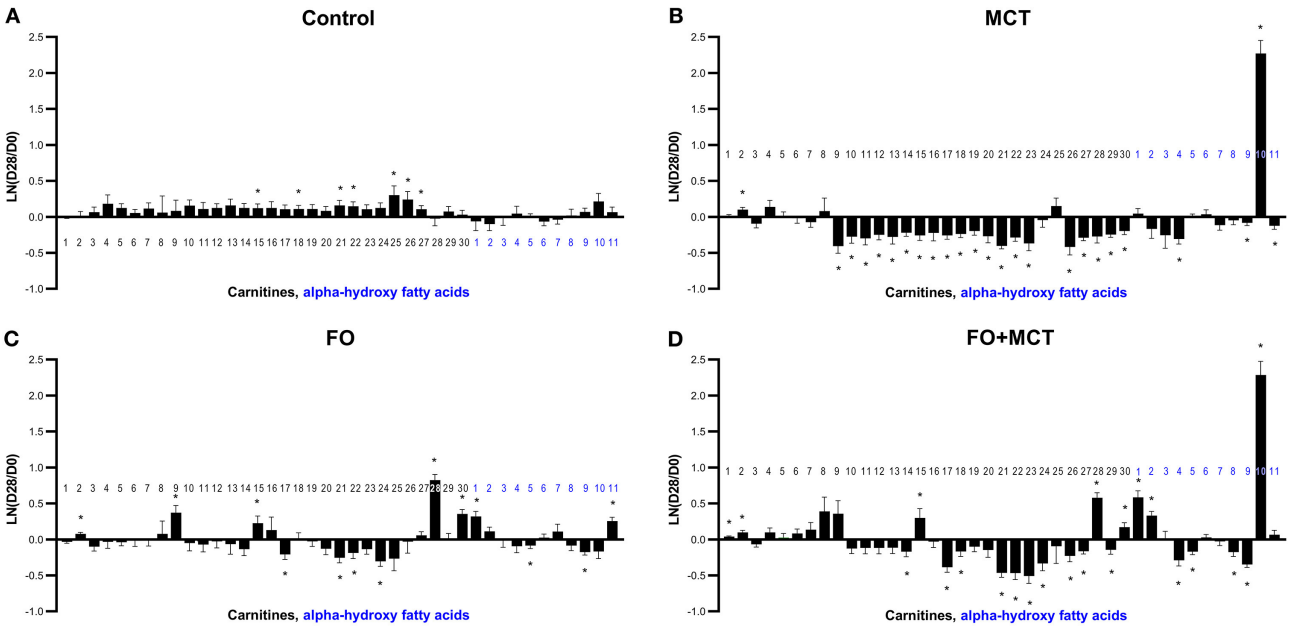
1	1-palmitoleoylglycerol (16:1)	6	1-arachidonylglycerol (20:4)	10	2-oleoylglycerol (18:1)	14	oleoyl-linoleoyl-glycerol (18:1/18:2) [1]	18	stearoyl-arachidonoyl-glycerol (18:0/20:4) [2]
2	1-oleoylglycerol (18:1)	7	1-docosahexaenoylglycerol (22:6)	11	palmitoleoyl-linoleoyl-glycerol (16:1/18:2) [1]	15	oleoyl-linoleoyl-glycerol (18:1/18:2) [2]	19	oleoyl-arachidonoyl-glycerol (18:1/20:4) [2]
3	1-linoleoylglycerol (18:2)	8	2-arachidonoylglycerol (20:4)	12	palmitoyl-arachidonoyl-glycerol (16:0/20:4) [1]	16	linoleoyl-linoleoyl-glycerol (18:2/18:2) [1]	20	linoleoyl-arachidonoyl-glycerol (18:2/20:4) [1]
4	1-linolenoylglycerol (18:3)	9	2-linoleoylglycerol (18:2)	13	palmitoyl-arachidonoyl-glycerol (16:0/20:4) [2]	17	stearoyl-arachidonoyl-glycerol (18:0/20:4) [1]	21	linoleoyl-arachidonoyl-glycerol (18:2/20:4) [2]
5	1-dihomo-linolenylglycerol (20:3)								

FIGURE 3
Change from the D0 baseline to D28 end of feeding period (LN fold change) in serum levels of mono- and diglycerides. Dogs were fed **(A)** control food, **(B)** food with MCT, **(C)** food with FO, or **(D)** food with FO+MCT. Blue bars indicate fatty acids in the FO ingredient. FO, fish oil; MCTs, medium-chain fatty acid-containing triglycerides. *$p < 0.05$ compared with the D0 baseline.

from the D0 baseline were different across the groups (ANOVA median $p < 0.0001$; Figure 6). Choline differed by food group, and the MCT and FO+MCT groups had increased choline levels relative to the D0 baseline. Trimethylglycine was increased from the D0 baseline in the MCT group and decreased in the FO group (Supplementary Table 4). There was a decrease from the D0 baseline of glycerophosphorylcholine in only the FO+MCT group. FO alone, and to a lesser extent MCT, decreased GPCs; with FO feeding, 27/36 (75%) GPC changed (21/36 [58%] decreased) while with MCT feeding, 20/36 (56%) GPC changed (15/36 [42%] decreased). With the FO+MCT group, 29/36 (81%) GPC changed, with 21/36 (58%) decreased in the dogs receiving the combined oils. In all instances, GPCs with ARA (C20:4n6) inclusion at the sn-2 position were decreased by FO feeding. Furthermore, all except 1-myristoyl-2-arachidonoyl-GPC (14:0/20:4) were decreased in the FO+MCT group as well, indicating that the effect of FO to decrease ARA-GPC was preserved in the presence of MCT. FO feeding also led to increased DHA (C22:6n3) at the sn-2 position.

The GPE phospholipids as a class were also impacted by food type (MANOVA $p < 0.0001$), and 18/23 univariate changes among these GPE appeared to be the drivers (ANOVA median $p < 0.0001$; Figure 7; Supplementary Table 4). As with GPC, the predominant effect was for both FO and MCT individually to decrease GPE with the effect of FO being greater (18/23 [78%] changed with FO; 12/23 [52%] changed with MCT). Regarding ARA (C20:4n6)-containing GPE, both FO and FO+MCT decreased all five of these species observed in the dataset. As well, FO and FO+MCT increased both observed DHA (C22:6n3)-containing GPE lipids.

The GPI class of phospholipids was also different according to food group (MANOVA $p < 0.0001$), with 9/11 (82%) of these changed according to individual ANOVA (median $p < 0.0001$; Figure 7; Supplementary Table 4). The results observed for GPI were largely similar to those observed for GPC and GPE. FO had a greater effect to decrease a number of GPI than did MCT (FO changed 7/11 [64%] while MCT changed 2/11 [18%]), while FO+MCT presented the same pattern of changes as FO (9/11 changed; 82%). The phospholipid 1-palmitoyl-GPI (16:0) was not changed from the D0 baseline in either the FO or MCT groups (MCT, $p = 0.0587$; FO, $p = 0.260$) but was decreased in the FO+MCT group ($p = 0.0096$). GPI substituted at the sn-2 position with ARA (C20:4n6) were decreased in the FO and FO+MCT foods. No DHA (C22:6n3)- or EPA (C20:5n3)-containing GPI were detected.



1	carnitine	6	hexanoylcarnitine (C6)	11	5-dodecenoylcarnitine (C12:1)	16	margaroylcarnitine (C17)	21	arachidoylcarnitine (C20)	26	behenoylcarnitine (C22)	1	2-hydroxyheptanoate	6	2-hydroxystearate	11	2-hydroxynervonate		
2	deoxycarnitine	7	octanoylcarnitine (C8)	12	myristoylcarnitine (C14)	17	stearoylcarnitine (C18)	22	eicosenoylcarnitine (C20:1)	27	lignoceroylcarnitine (C24)	2	2-hydroxyoctanoate	7	2-hydroxyoleate				
3	acetylcarnitine (C2)	8	decanoylcarnitine (C10)	13	myristoleoylcarnitine (C14:1)	18	oleoylcarnitine (C18:1)	23	dihomo-linoleoylcarnitine (C20:2)	28	nervonoylcarnitine (C24:1)	3	2-hydroxydecanoate	8	2-hydroxyarachidate				
4	propionylcarnitine (C3)	9	cis-4-decenoylcarnitine (C10:1)	14	palmitoylcarnitine (C16)	19	linoleoylcarnitine (C18:2)	24	dihomo-linolenoylcarnitine (20:3n3 or 6)	29	cerotoylcarnitine (C26)	4	2-hydroxylaurate	9	2-hydroxybehenate				
5	butyrylcarnitine (C4)	10	laurylcarnitine (C12)	15	palmitoleoylcarnitine (C16:1)	20	linolenoylcarnitine (C18:3)	25	arachidonoylcarnitine (C20:4)	30	ximenoylcarnitine (C26:1)	5	2-hydroxypalmitate	10	2-hydroxylignocerate				

FIGURE 4
Change from the D0 baseline to D28 end of feeding period (LN fold change) in serum levels of carnitines and alpha-hydroxy fatty acids. Dogs were fed **(A)** control food, **(B)** food with MCT, **(C)** food with FO, or **(D)** food with FO+MCT. FO, fish oil; MCTs, medium-chain fatty acid-containing triglycerides. *$p < 0.05$ compared with the D0 baseline.

As a class, SPHING was different by group (MANOVA $p = 0.0001$); 39/50 (78%) SPHING were individually different by ANOVA across the food types (median $p < 0.0001$). While FO produced changes in 37/50 (74%) SPHING, MCT only generated changes from the D0 baseline for 15/50 (30%) and FO+MCT feeding resulted in 41/50 (82%) changes (Figure 8; Supplementary Table 4). For the N-palmitoyl and N-stearoyl series, an increasing degree of unsaturation of the SPHING moiety (sphinganine → sphingosine → sphingadienine) resulted in more strongly reduced levels with FO and FO+MCT treatments. Thus, unsaturated N-palmitoyl and N-stearoyl sphingosine and sphingadienine were decreased by FO consumption, while saturated sphinganine was not. Sphingosine-1-phosphate was increased by FO and FO+MCT foods but not by MCT treatment.

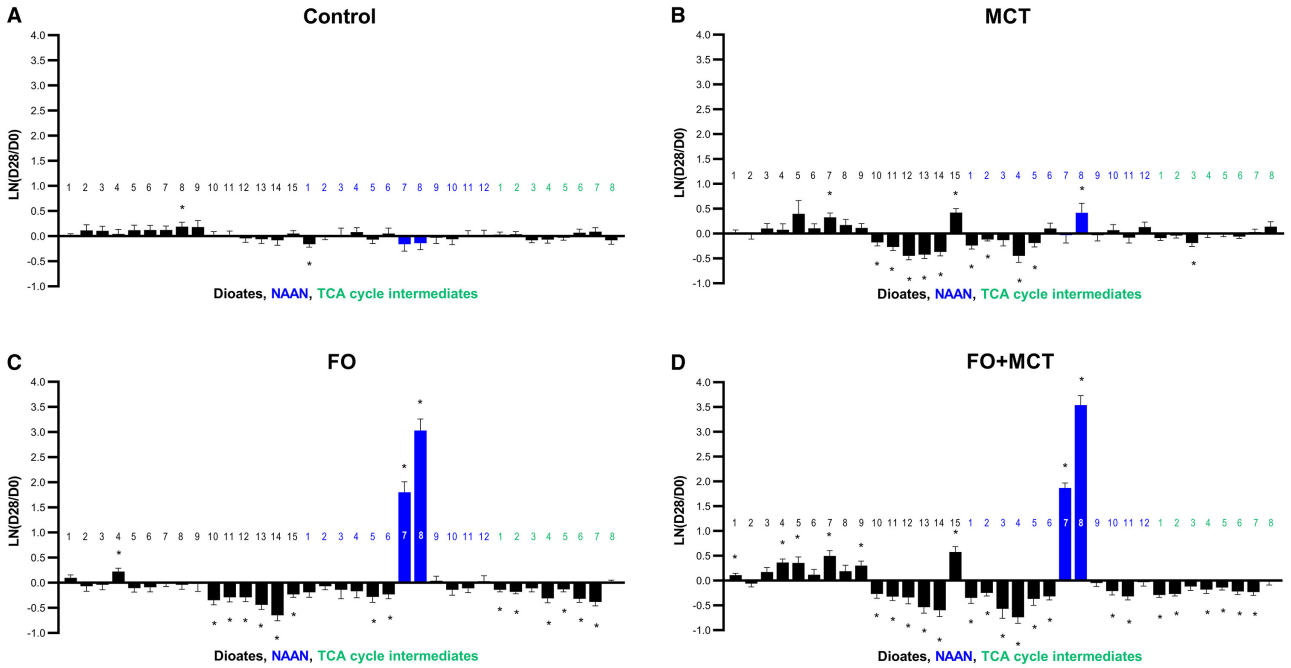
3.3.5. Impact of MCT and FO on products of central metabolism

TCA cycle intermediates were found to be different as a class of metabolites by group (MANOVA $p = 0.0003$), with 4/8 (50%) of the TCA metabolites individually different by food type (ANOVA median $p = 0.0533$; Figure 5). While there was only one change in a TCA metabolite in the MCT group (decreased isocitrate), 6/8 (75%) TCA metabolites were changed in both the FO and FO+MCT group; all were decreased.

Given that amino acids traffic nitrogen as well as carbon for energy, the levels of amino acids in response to feeding experimental foods were assessed. Proteogenic amino acids (plus taurine) as a class were different by group (MANOVA $p < 0.0001$), with 9/22 (41%) amino acids changed by food type according to ANOVA (median $p = 0.0890$; Figure 9; Supplementary Table 4). Most of the changes were present in the FO group (10/22; 45%), with fewer in the MCT group (6/22; 27%); these individual effects were compounded such that 14/22 (64%) amino acids were changed in the combination food FO+MCT. Most of these changes were increases, with only two amino acids decreased in each of the three experimental groups and the rest being increased circulating levels of amino acids.

3.3.6. Impact of MCT and FO on microbial postbiotics

The metabolite class of indoles and indolic sulfates was different across groups in a multivariate manner with 16 total metabolites detected (MANOVA $p < 0.0050$; Supplementary Table 4), 5/16 (31%) of which were altered by food type (ANOVA). The MCT group exhibited changes in 2 indoles, with 3-indoxyl sulfate decreased but 7-hydroxyindole sulfate increased. Similarly, the FO group decreased 5-hydroxyindole sulfate but increased indolepropionate. The FO+MCT group showed changes in two



1	oxalate (ethanedioate)	6	azelate (nonanedioate; C9)	11	hexadecenedioate (C16:1-DC)	1	linoleoyl ethanolamide	6	arachidonoylcholine	11	palmitoylcholine	4	alpha-ketoglutarate
2	glutarate (C5-DC)	7	sebacate (C10-DC)	12	octadecanedioate (C18)	2	oleoyl ethanolamide	7	docosahexaenoyl-choline	12	stearoylcholine	5	succinate
3	pimelate (C7-DC)	8	undecanedioate (C11-DC)	13	octadecenedioate (C18:1-DC)	3	N-oleoyltaurine	8	eicosapentaenoyl-choline	1	citrate	6	fumarate
4	heptenedioate (C7:1-DC)	9	dodecanedioate (C12)	14	octadecadienedioate (C18:2-DC)	4	N-palmitoyltaurine	9	linoleoylcholine	2	aconitate [cis or trans]	7	malate
5	suberate (C8-DC)	10	hexadecanedioate (C16)	15	eicosanodioate (C20-DC)	5	N-stearoyltaurine	10	oleoylcholine	3	isocitrate	8	2-methylcitrate/ homocitrate

FIGURE 5
Change from the D0 baseline to D28 end of feeding period (LN fold change) in serum levels of dioates, NAAN, and TCA cycle intermediates. Dogs were fed **(A)** control food, **(B)** food with MCT, **(C)** food with FO, or **(D)** food with FO+MCT. Blue bars indicate fatty acids derived from the FO ingredient. FO, fish oil; MCTs, medium-chain fatty acid-containing triglycerides. NAAN, N-acyl amino acids/neurotransmitters; TCA, tricarboxylic acid. *$p < 0.05$ compared with the D0 baseline.

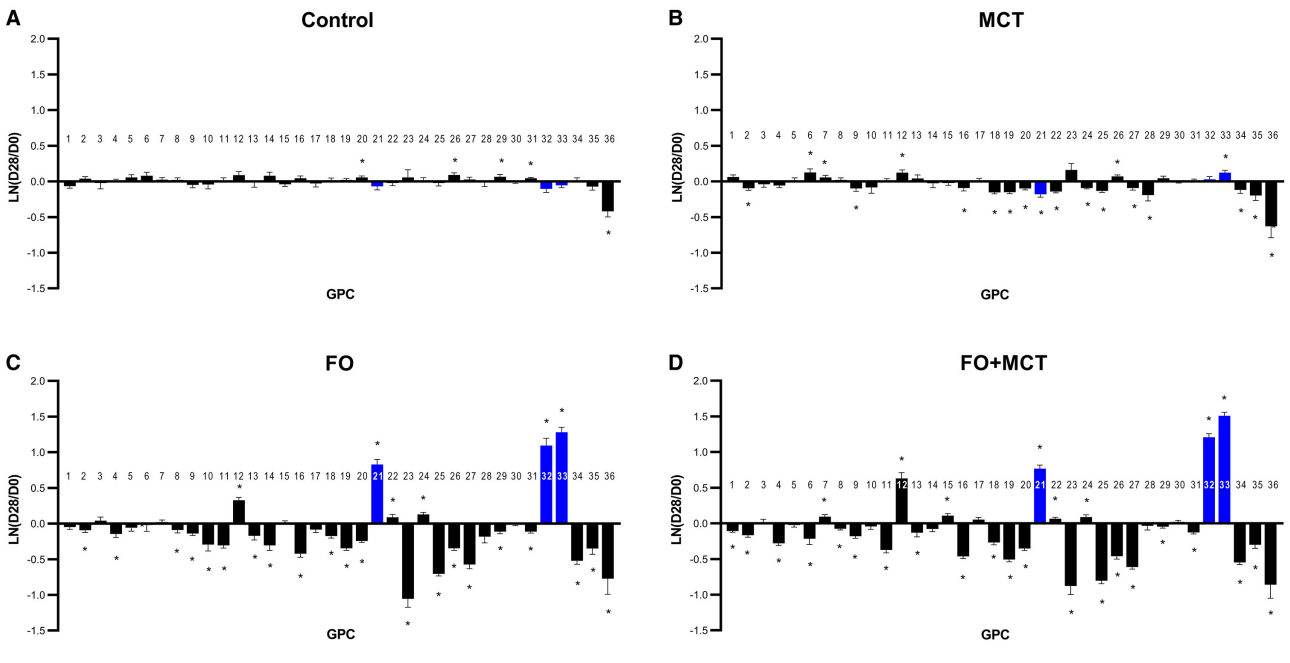
indoles as well, both of which were decreased (5-, and 6-hydroxyindole sulfates).

The class of phenols and phenolic sulfates was different across groups by multivariate analysis (MANOVA $p < 0.0005$; Supplementary Table 4), with 25 total metabolites detected and 8/25 (32%) altered by food (ANOVA). Two additional phenol postbiotic metabolites showed food effect p-values ≤ 0.0800 and q-values ≤ 0.100. The control group presented with two phenols decreased from the D0 baseline: 3-acetylphenol sulfate and 4-vinylphenol sulfate. The MCT group exhibited only a single changed phenol, with 2-aminophenol sulfate increased. The FO group manifested six decreased phenols, including 4-allylphenol sulfate, 4-aminophenol sulfate, 4-ethylphenyl sulfate, 4-hydroxyphenylpyruvate, 4-vinylphenol sulfate, and phenylacetylalanine. The FO+MCT group showed the same changes as the FO alone group, with the exception that neither 4-hydroxyphenylpyruvate nor phenylacetylalanine changed in the FO+MCT group.

4. Discussion

This trial evaluated the effects of the addition of MCT and FO to food via global metabolomics to characterize the serum levels of several classes of energetic, structural, and signaling lipid metabolites. Changes in central metabolites, including those from the TCA cycle and amino acids, were analyzed to gain insight into the degree to which the dietary fats impact associated energetic pathways. Serum samples were drawn from 23-h fasted dogs. With regard to the metabolism of dietary fat, protein, and starch/sugar, available reports document that 23 h is sufficient time to become post-absorptive for dogs. The half-life of circulating triglycerides in dogs is 22 min, so dietary fat-derived fatty acids from a meal would no longer be circulating 23 h later but would instead be mobilized and trafficked from adipose stores or generated by hepatic *de novo* lipogenesis (81). In addition, blood urea nitrogen derived from postprandial amino acid catabolism returns to baseline between 16 and 24 h after a meal in dogs (82). For dietary carbohydrates, consumption of glucose leads to a return to baseline blood glucose levels approximately 90 min in dogs, while consumption of various starches leads to blood glucose returning to baseline by 3 h after a meal (83). Taken together, there is evidence that 23 h post-feeding can be considered a post-absorptive state in the dog.

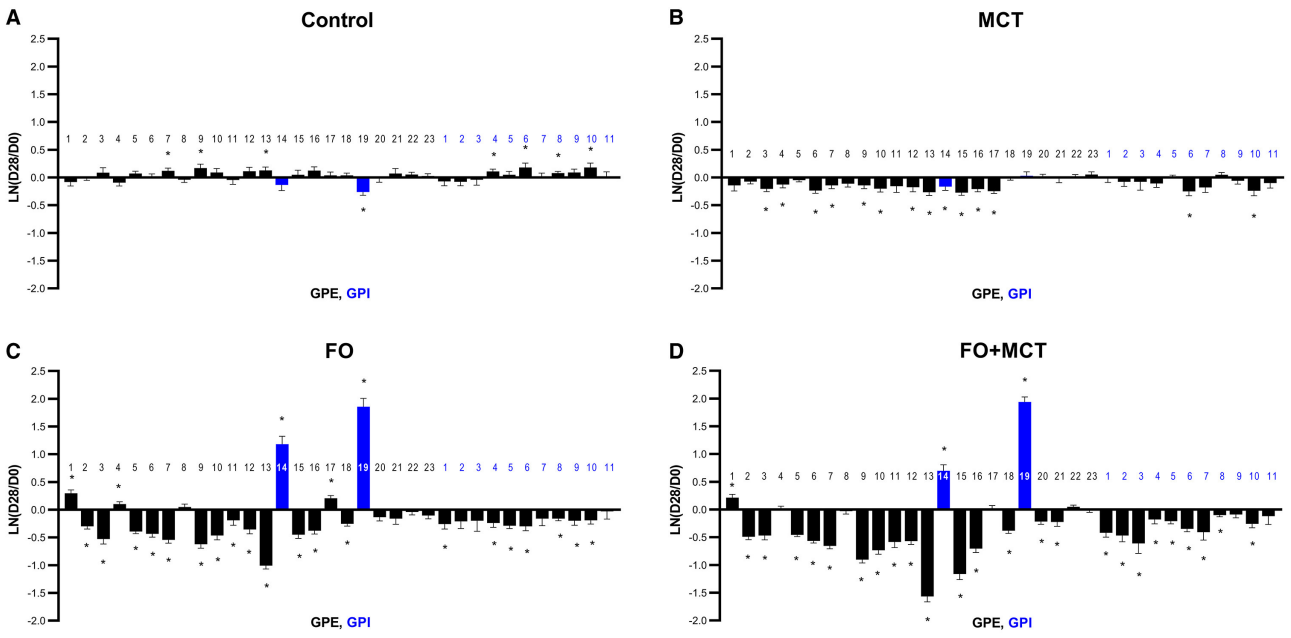
In the current trial, dietary oil feeding in dogs led to reduced triglycerides. Consistent with trials in both dogs (66, 84) and humans (85), there was a decrease in circulating triglycerides with FO feeding. This decrease was also apparent in the FO+MCT



1	glycerophosphor-ylcholine (GPC)	5	1-palmitoleoyl-GPC (16:1)	9	1-linoleoyl-GPC (18:2)	13	1-myristoyl-2-palmitoyl-GPC (14:0/16:0)	17	1-palmitoyl-2-stearoyl-GPC (16:0/18:0)	21	1-palmitoyl-2-docosahexaenoyl-GPC (16:0/22:6)	25	1-(1-enyl-palmitoyl)-2-linoleoyl-GPC (P-16:0/18:2)	29	1-stearoyl-2-oleoyl-GPC (18:0/18:1)	33	1-oleoyl-2-docosahexaenoyl-GPC (18:1/22:6)
2	1-palmitoyl-GPC (16:0)	6	2-palmitoleoyl-GPC (16:1)	10	1-linolenoyl-GPC (18:3)	14	1-myristoyl-2-arachidonoyl-GPC (14:0/20:4)	18	1-palmitoyl-2-oleoyl-GPC (16:0/18:1)	22	1-(1-enyl-palmitoyl)-2-palmitoyl-GPC (P-16:0/16:0)	26	1-(1-enyl-palmitoyl)-2-arachidonoyl-GPC (P-16:0/20:4)	30	1-stearoyl-2-linoleoyl-GPC (18:0/18:2)	34	1,2-dilinoleoyl-GPC (18:2/18:2)
3	1-(1-enyl-palmitoyl)-GPC (P-16:0)	7	1-stearoyl-GPC (18:0)	11	1-arachidonoyl-GPC (20:4)	15	1,2-dipalmitoyl-GPC (16:0/16:0)	19	1-palmitoyl-2-linoleoyl-GPC (16:0/18:2)	23	1-(1-enyl-palmitoyl)-2-palmitoleoyl-GPC (P-16:0/16:1)	27	1-palmitoleoyl-2-linoleoyl-GPC (16:1/18:2)	31	1-stearoyl-2-arachidonoyl-GPC (18:0/20:4)	35	1-linoleoyl-2-linolenoyl-GPC (18:2/18:3)
4	2-palmitoyl-GPC (16:0)	8	1-oleoyl-GPC (18:1)	12	1-lignoceroyl-GPC (24:0)	16	1-palmitoyl-2-palmitoleoyl-GPC (16:0/16:1)	20	1-palmitoyl-2-arachidonoyl-GPC (16:0/20:4n6)	24	1-(1-enyl-palmitoyl)-2-oleoyl-GPC (P-16:0/18:1)	28	1-palmitoleoyl-2-linolenoyl-GPC (16:1/18:3)	32	1-stearoyl-2-docosahexaenoyl-GPC (18:0/22:6)	36	1,2-dilinolenoyl-GPC (18:3/18:3)

FIGURE 6
Change from the D0 baseline to D28 end of feeding period (LN fold change) in serum levels of GPCs. Dogs were fed **(A)** control food, **(B)** food with MCT, **(C)** food with FO, or **(D)** food with FO+MCT. Blue bars indicate fatty acids derived from the FO ingredient. FO, fish oil; GPC, glycerophosphatidylcholine; MCTs, medium-chain fatty acid-containing triglycerides. *$p < 0.05$ compared with the D0 baseline.

group but not in the MCT group. The level of FO included was nearly 3% of the food dry matter in the FO and FO+MCT groups and replaced pork fat that was composed of ~35% saturated fat. Thus, the inclusion of FO led to an approximately 1% decrease in saturated fat in the FO and FO+MCT foods. Although decreasing dietary saturated fat is known to reduce circulating triglycerides in other species (86), it has been proposed that n3 fatty acid inclusion in the food can independently decrease triglycerides as well (86, 87). However, in contrast to the reports that indicate that FO can reduce triglycerides in dogs (66, 84), three FO feeding trials in dogs showed no effect of FO on triglycerides (36, 88, 89). The levels of total FO (or EPA + DHA) offered to dogs as well as the DHA-to-EPA ratio were lower in all of those trials than in the current trial. In the three trials that showed no effect of FO on triglycerides, one tested FO as a supplement in client-owned, mixed-breed dogs with an intake of 0.03–0.04 g FO/kg BW (88), while the other two studies used FO in food form at 0.11 g EPA+DHA/kg BW in Belgian Shepherd working dogs (89) and 0.06 g FO/kg BW in beagles (36). In the two trials that showed a reduction in triglycerides with FO, one tested FO as a supplement in miniature Schnauzers with primary hyperlipidemia (intake of 0.10 g EPA+DHA/kg BW, though it is unclear how this amount was present in one FO capsule) (84) and the other tested a food form of FO (0.10 g EPA+DHA/kg BW) in beagles (66). In the current study, the offering of FO to dogs was much higher (0.45 g FO/kg BW), and 0.19 g of EPA+DHA/kg BW was fed. As well, the ratio of DHA to EPA in the studies was 0.59 (88), 1.2 (89), 0.77 (84), 1.42 (66), 0.7 (36), and 7.3 in the currently reported trial. There is some evidence in humans that DHA more potently reduces triglycerides compared with EPA (90), although data on the effects of EPA vs. DHA on triglycerides in dogs are lacking. It may be that both the increased level of FO fatty acids and the increased ratio of DHA to EPA in the current report led to the observations that FO decreases triglycerides. The reason for the discordancy of results among previous reports, however, is not clear. It may be that breed or activity level was a determinant in those studies, although estimations of caloric intake and weight maintenance from these trials (where possible) indicate an activity factor of ~1.4–1.6 for all dogs. Another factor may be that when controlling for total fat level in the food (as was done in the current study), the inclusion of n3-rich FO will necessarily change fatty acid composition (68), a variable that cannot be deconvoluted in our study design. Thus, the ratio of FO-derived EPA and/or DHA to total fatty acids may be a determinant of the degree to which FO reduces triglycerides in dogs as well. In our previously published study in cats (38), the FO group also showed decreased triglyceride levels, although in that study the combination of FO+MCT did not decrease triglycerides, whereas it did in the present canine study. In the current study, consumption of MCT alone increased total



1	1-(1-enyl-oleoyl)-GPE (P-18:1)	6	1-(1-enyl-stearoyl)-2-linoleoyl-GPE (P-18:0/18:2)	11	1-oleoyl-2-linoleoyl-GPE (18:1/18:2)	16	1-palmitoyl-2-oleoyl-GPE (16:0/18:1)	21	1-stearoyl-2-oleoyl-GPE (18:0/18:1)	3	1-oleoyl-GPI (18:1)	8	1-stearoyl-2-arachidonoyl-GPI (18:0/20:4)
2	1-(1-enyl-palmitoyl)-2-arachidonoyl-GPE (P-16:0/20:4)	7	1-(1-enyl-stearoyl)-2-oleoyl-GPE (P-18:0/18:1)	12	1-oleoyl-GPE (18:1)	17	1-palmitoyl-GPE (16:0)	22	1-stearoyl-GPE (18:0)	4	1-palmitoyl-2-arachidonoyl-GPI (16:0/20:4)	9	1-stearoyl-2-linoleoyl-GPI (18:0/18:2)
3	1-(1-enyl-palmitoyl)-2-oleoyl-GPE (P-16:0/18:1)	8	1-(1-enyl-stearoyl)-GPE (P-18:0)	13	1-palmitoyl-2-arachidonoyl-GPE (16:0/20:4)	18	1-stearoyl-2-arachidonoyl-GPE (18:0/20:4)	23	2-stearoyl-GPE (18:0)	5	1-palmitoyl-2-linoleoyl-GPI (16:0/18:2)	10	1-stearoyl-2-oleoyl-GPI (18:0/18:1)
4	1-(1-enyl-palmitoyl)-GPE (P-16:0)	9	1-arachidonoyl-GPE (20:4)	14	1-palmitoyl-2-docosahexaenoyl-GPE (16:0/22:6)	19	1-stearoyl-2-docosahexaenoyl-GPE (18:0/22:6)	1	1-arachidonoyl-GPI (20:4)	6	1-palmitoyl-2-oleoyl-GPI (16:0/18:1)	11	1-stearoyl-GPI (18:0)
5	1-(1-enyl-stearoyl)-2-arachidonoyl-GPE (P-18:0/20:4)	10	1-linoleoyl-GPE (18:2)	15	1-palmitoyl-2-linoleoyl-GPE (16:0/18:2)	20	1-stearoyl-2-linoleoyl-GPE (18:0/18:2)	2	1-linoleoyl-GPI (18:2)	7	1-palmitoyl-GPI (16:0)		

FIGURE 7
Change from the D0 baseline to D28 end of feeding period (LN fold change) in serum levels of GPEs and GPIs. Dogs were fed **(A)** control food, **(B)** food with MCT, **(C)** food with FO, or **(D)** food with FO+MCT. Blue bars indicate fatty acids derived from the FO ingredient. FO, fish oil; GPE, glycerophosphatidylethanolamine; GPI, glycerophosphatidylinositols; MCTs, medium-chain fatty acid-containing triglycerides. *$p < 0.05$ compared with the D0 baseline.

cholesterol; in contrast, this lipid was decreased by FO feeding alone and FO+MCT. Indicative of the interaction of FO with MCT, the FO+MCT feeding was less potent in decreasing cholesterol than was FO alone, perhaps offset by the inclusion of MCT. Given that elevated triglycerides are associated with aging in dogs (49, 91), the current data add context to considerations that diets of older dogs are supplemented with sources of DHA and EPA such as FO.

There are few comparisons available for the canine circulating lipidome. Some publications examine non-serum matrices such as ocular fluids (60) or red blood cell membrane (65) and thus may not reflect circulating levels or include both structural and energetic lipids. Other publications have examined the impact of dietary oils on the circulating lipidome in dogs with inflammatory dermatological (64) or gastrointestinal (67) disease, and these disease states may perturb lipid metabolism relative to healthy canines of the sort enrolled in the current study. Some prior reports in this area of investigation have examined the changes in circulating lipid metabolites with LCPUFA and MCT feeding, but some of these studies did not include control foods (lacking both types of fats) or lacked a food that combined both fats (92–94). Previous publications have documented that feeding LCPUFA(n3) and MCFA-containing foods can increase the fatty acid, carnitine, and phospholipid fractions with these fats (17, 36, 38, 69). In the current trial, it was observed that consumption of LCPUFA(n3) and MCFA also enriched levels of these fats in the broader circulating lipidome. These phenomena included catabolic intermediates of MCT-derived fatty acids (e.g., the dioate sebacate [C10:0, MCT]) and signaling-type lipids derived from FO LCPUFA(n3) (e.g., docosahexenoylcholine [C22:6n3, FO]). This current report is novel in the factorial design of the oil feedings (alone or in combination) and the extensive reporting of the canine lipidome including structural, energetic, and signaling lipids.

When dietary levels of LCPUFA(n3) are increased, these fats increase at the sn-2 position of structural-type lipid classes including phospholipids, while LCPUFA(n6) fatty acids decrease at this position (24, 95). That phenomenon was particularly evident here with FO, which decreased phospholipids with an ARA (C20:4n6) at the sn-2 position. FO also decreased ARA (C20:4n6) as a free fatty acid (NEFA), ARA-containing MDAG, and NAAN arachidonoylcholine. Concurrently, FO also increased levels of DHA in phospholipids at the sn-2 position. A recent publication documented the impact of FO on circulating lipids in the plasma and erythrocytes from active dogs and noted that provision of the LCPUFA(n3) increased the incorporation of these lipids into phospholipids from both matrices at the expense of

LCPUFA(n6) incorporation (89). When the total fatty acid makeup of serum was examined in healthy dogs after consumption of diets high in C18:2n6 or C18:1n9, the fatty acid contingent of the circulating lipidome was enriched in these respective fats (96). In a separate study, dogs consuming sources of two types of n3 fatty acids, linoleate (C18:3n3) from flaxseed oil, and EPA + DHA (C20:5n3 + C22:6n3) from FO, had increased n3 fatty acid content in the phospholipid fraction of the circulating lipidome (66). In the current study, the acylcarnitine derivative of ARA was not changed by FO or FO+MCT, indicating that increased beta oxidation of ARA to energy did not lead to the observed decreases in ARA and ARA-containing lipids. The levels of dietary ARA were not meaningfully different between the FO and CON groups, so differences did not arise from ARA intake variations. It appears then that the current study is consistent with a model of competitive interaction between n3-type and n6-type LCPUFA for incorporation into complex lipids. The fate of the decreased ARA is still lacking clarity; although circulating complex structural-type lipids manifested a decrease in ARA content, the energetic-type catabolic intermediate ARA-carnitine was not changed in compensation. ARA may be sequestered into a lipid fraction that is not in equilibrium with circulating lipids.

Considering energetic-type lipids in the post-absorptive canine subjects at the time of collection, the NEFA observed here were likely derived from lipolysis in adipose stores rather than from remnant circulating dietary fat. Although the dogs were increasingly reliant on fat for metabolism as glycogen stores are reduced over time without food, it is clear that they were not yet in ketosis. Circulating NEFA can be fated for energetic catabolism (direct beta oxidation in peripheral tissues or ketogenesis in the liver) or anabolic esterification processes that lead to phospholipids and triglycerides. Increased levels of NEFA (97) or acylcarnitines (98) are indicative of reduced capacity for central lipid metabolism. In the current study, MCT feeding produced greater decreases than did FO on energetic-type fatty acid metabolites. MCT led to changes in more end-stage catabolic products (e.g., NEFA and acylcarnitines) while FO impacted upstream intermediates of triglyceride catabolism (MDAG). It is unlikely that carnitine availability limited acylcarnitine levels in the MCT-fed dogs as carnitine was not changed by this oil. Interestingly, beta-oxidized fatty acids were not different as a class and there were few differences by diet. It may be that there was a decrease in NEFA and acylcarnitines without a concurrent increase in beta oxidation or that there was increased

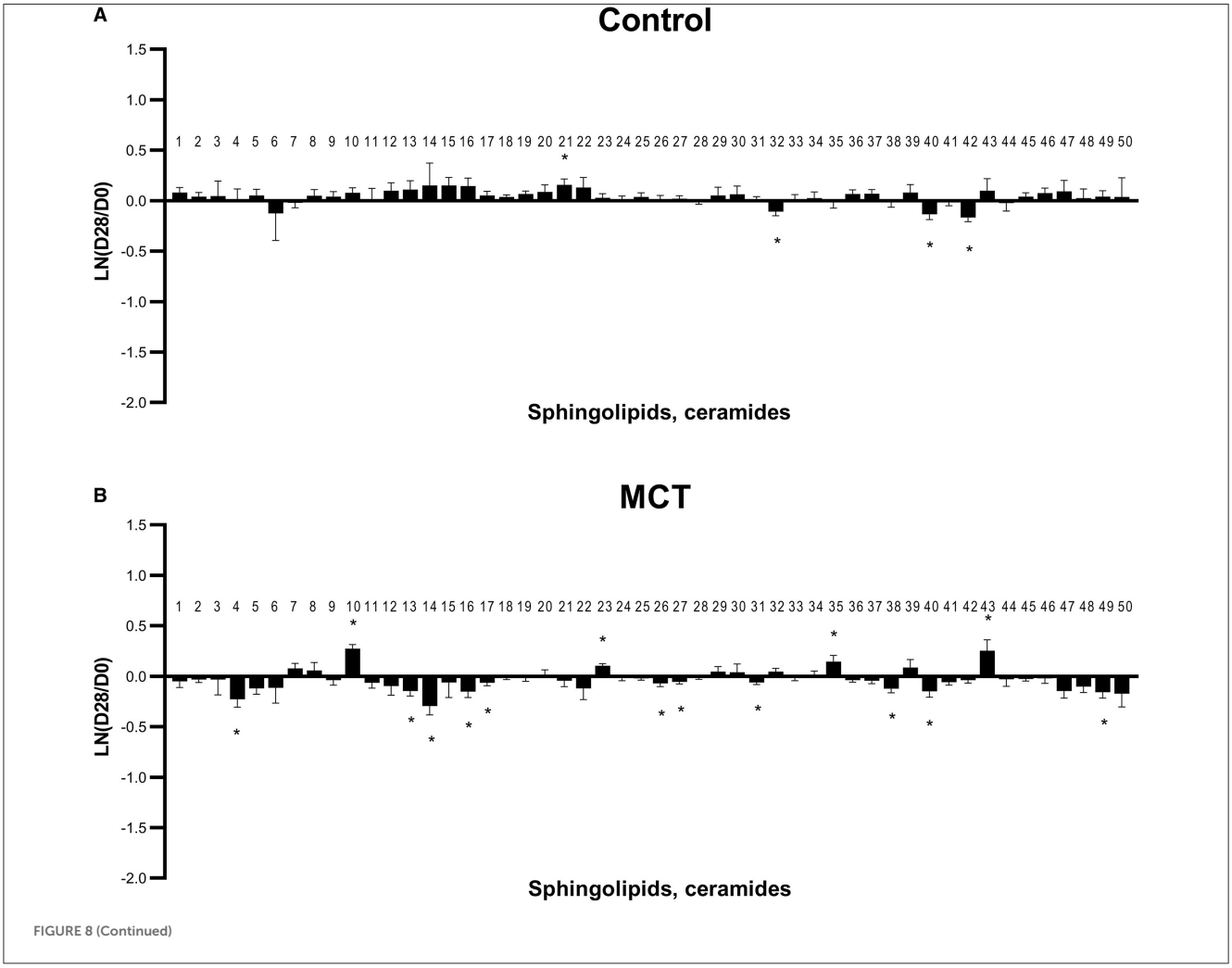
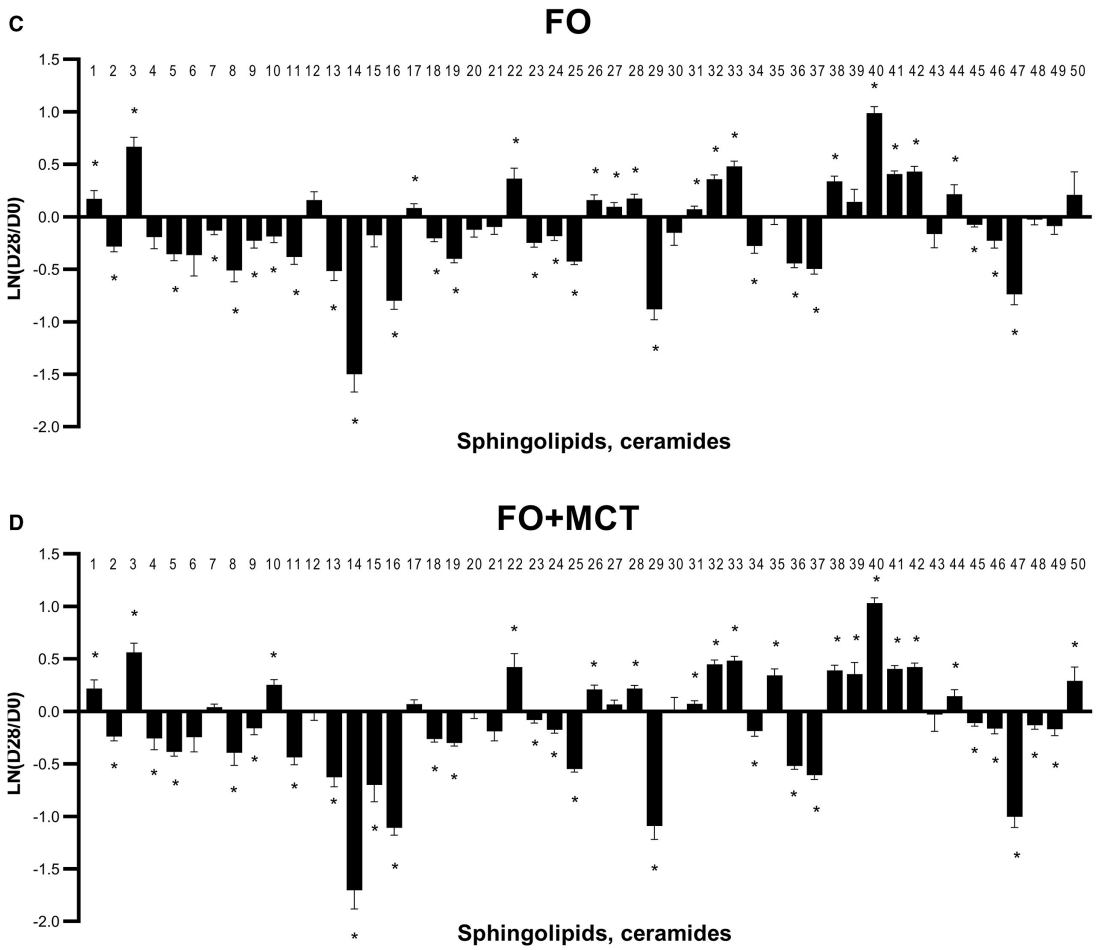
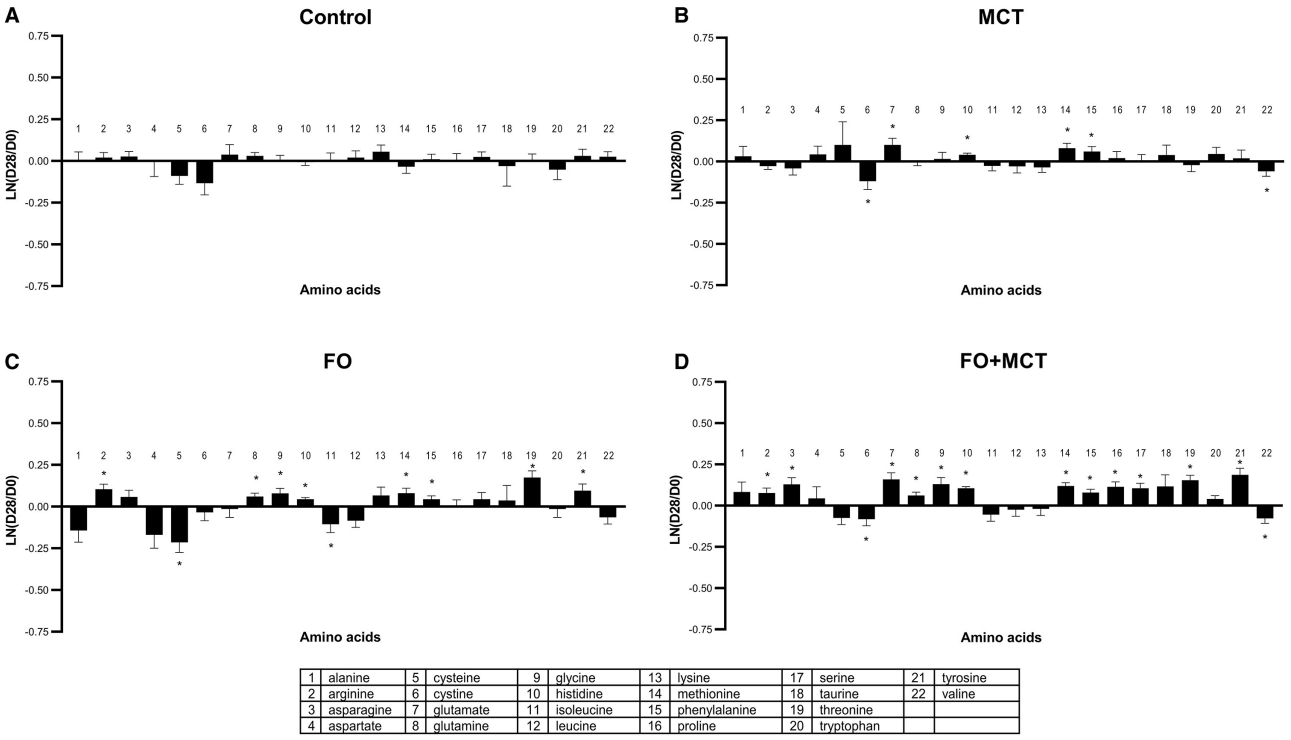


FIGURE 8 (Continued)



1	behenoyl dihydrosphingomyelin (d18:0/22:0)	14	N-stearoyl-sphingadienine (d18:2/18:0)	27	sphingomyelin (d18:1/20:0, d16:1/22:0)	39	sphingomyelin (d18:2/23:0, d18:1/23:1, d17:1/24:1)
2	behenoyl sphingomyelin (d18:1/22:0)	15	N-stearoyl-sphinganine (d18:0/18:0)	28	sphingomyelin (d18:1/20:1, d18:2/20:0)	40	sphingomyelin (d18:2/23:1)
3	glycosyl-N-(2-hydroxynervonoyl)-sphingosine (d18:1/24:1(2OH))	16	N-stearoyl-sphingosine (d18:1/18:0)	29	sphingomyelin (d18:1/20:2, d18:2/20:1, d16:1/22:2)	41	sphingomyelin (d18:2/24:1, d18:1/24:2)
4	glycosyl-N-palmitoyl-sphingosine (d18:1/16:0)	17	palmitoyl dihydrosphingomyelin (d18:0/16:0)	30	sphingomyelin (d18:1/21:0, d17:1/22:0, d16:1/23:0)	42	sphingomyelin (d18:2/24:2)
5	glycosyl-N-stearoyl-sphingosine (d18:1/18:0)	18	palmitoyl sphingomyelin (d18:1/16:0)	31	sphingomyelin (d18:1/22:1, d18:2/22:0, d16:1/24:1)	43	sphingosine
6	lactosyl-N-behenoyl-sphingosine (d18:1/22:0)	19	sphingomyelin (d17:1/16:0, d18:1/15:0, d16:1/17:0)	32	sphingomyelin (d18:1/22:2, d18:2/22:1, d16:1/24:2)	44	sphingosine 1-phosphate
7	lactosyl-N-palmitoyl-sphingosine (d18:1/16:0)	20	sphingomyelin (d17:2/16:0, d18:2/15:0)	33	sphingomyelin (d18:1/24:1, d18:2/24:0)	45	stearoyl sphingomyelin (d18:1/18:0)
8	lactosyl-N-stearoyl-sphingosine (d18:1/18:0)	21	sphingomyelin (d18:0/18:0, d19:0/17:0)	34	sphingomyelin (d18:1/25:0, d19:0/24:1, d20:1/23:0, d19:1/24:0)	46	tricosanoyl sphingomyelin (d18:1/23:0)
9	lignoceroyl sphingomyelin (d18:1/24:0)	22	sphingomyelin (d18:0/20:0, d16:0/22:0)	35	sphingomyelin (d18:2/14:0, d18:1/14:1)	47	ceramide (d18:1/17:0, d17:1/18:0)
10	myristoyl dihydrosphingomyelin (d18:0/14:0)	23	sphingomyelin (d18:1/14:0, d16:1/16:0)	36	sphingomyelin (d18:2/16:0, d18:1/16:1)	48	ceramide (d18:2/24:1, d18:1/24:2)
11	N-palmitoyl-sphingadienine (d18:2/16:0)	24	sphingomyelin (d18:1/17:0, d17:1/18:0, d19:1/16:0)	37	sphingomyelin (d18:2/18:1)	49	glycosyl ceramide (d18:1/20:0, d16:1/22:0)
12	N-palmitoyl-sphinganine (d18:0/16:0)	25	sphingomyelin (d18:1/18:1, d18:2/18:0)	38	sphingomyelin (d18:2/21:0, d16:2/23:0)	50	glycosyl ceramide (d18:2/24:1, d18:1/24:2)
13	N-palmitoyl-sphingosine (d18:1/16:0)	26	sphingomyelin (d18:1/19:0, d19:1/18:0)				

FIGURE 8 (Continued)
Change from the D0 baseline to D28 end of feeding period (LN fold change) in serum levels of sphingolipids and ceramides. Dogs were fed **(A)** control food, **(B)** food with MCT, **(C)** food with FO, or **(D)** food with FO+MCT. FO, fish oil; MCTs, medium-chain fatty acid-containing triglycerides. *$p < 0.05$ compared with the D0 baseline.



1	alanine	5	cysteine	9	glycine	13	lysine	17	serine	21	tyrosine
2	arginine	6	cystine	10	histidine	14	methionine	18	taurine	22	valine
3	asparagine	7	glutamate	11	isoleucine	15	phenylalanine	19	threonine		
4	aspartate	8	glutamine	12	leucine	16	proline	20	tryptophan		

FIGURE 9
Change from the D0 baseline to D28 end of feeding period (LN fold change) in serum levels of amino acids. Dogs were fed **(A)** control food, **(B)** food with MCT, **(C)** food with FO, or **(D)** food with FO+MCT. FO, fish oil; MCTs, medium-chain fatty acid-containing triglycerides. *$p < 0.05$ compared with the D0 baseline.

flux through beta oxidation without changes in levels of the members of this metabolite class. For non-lipid energetic and central metabolites, FO (with or without added MCT) showed broad changes to members of the TCA cycle class. In contrast, both FO and MCT individually and in combination impacted circulating amino acids. Omega oxidation is mostly carried out in the endoplasmic reticulum and produces dicarboxylic acids (dioates). It can occur when fatty acid beta oxidation is unable to keep pace with fatty acid flux (99). In the current trial, both FO and MCT feeding decreased all omega-oxidized C16 and C18 dioates. The MCFA (C6:0, C8:0, C10:0) are metabolized by microsomal cytochrome P45 enzymes to medium-chain dioates of the same chain length (100) and are reported to increase with MCT feeding in a manner distinct from that observed with fasting or abnormal fatty acid oxidation (101). The current results show that with MCT feeding sebacate dioate (C10:0) increased while suberate (C8:0) did not. Alpha-oxidized fatty acids can arise from peroxisomal oxidation of lipids involved in sphingolipid synthesis. In the current study, MCT feeding led to large increases in a single alpha-hydroxy fatty acid (2-hydroxylignocerate), which is incorporated into sphingomyelin that accumulates in the liver (102). The impact of FO and MCT feeding appeared to be minimal on HETE, HODE, HOME, and HHTrE-type lipid oxidation products, indicating some specificity of the actions of FO and MCT to impact structural, energetic, and some classes of signaling lipids. Taken together, it would appear that combined FO+MCT oil feeding may benefit metabolic status as indicated by improved lipid levels of NEFA, acylcarnitines, MDAG, omega and alpha-oxidized fatty acids, and non-lipid energetic intermediates.

Postbiotics are metabolites generated by gut microbial catabolism of food that bypassed small intestinal absorption. These metabolites can be absorbed by the host and appear in circulation to impact host physiology (103). Microbial putrefaction of tyrosine or tryptophan and phenylalanine produces the postbiotic phenols and indoles, respectively (104), and these can have negative effects on host health, including on renal function and inflammation (105). In the current study, no endpoints were measured from feces, and thus, the origin of the molecules observed in the circulating metabolomics dataset that are canonically considered postbiotics cannot unambiguously be known. However, some of the putative postbiotic molecules have been observed to change in blood in previous studies and assessment of their response to FO and/or MCT in dogs helps to inform design and criteria for future studies. It was recently published that food can decrease levels of potentially detrimental postbiotics in dogs (106), and the current findings indicate that the spectrum of foods that might decrease these postbiotics is broader than previously understood. In the previously published feline study (38), microbial putrefaction products of aromatic amino acids were decreased with combined FO+MCT feeding, although this was not evident to the same degree in the current study with canine subjects. In the current study, the foods produced changes in 32% of observed microbiome putrefactive postbiotics of both the indole and phenol classes. This is a less pervasive effect for canines than that observed in the feeding of these oils to felines, where 73% of observed

indoles and 83% of observed phenols were altered by food type (38). Furthermore, whereas the FO+MCT condition provided a starkly evident effect for the FO+MCT combination to decrease postbiotics in cats, in the current study with dogs no such effect was apparent.

In most instances, the qualitative effects of FO or MCT alone appeared to be additive such that lipids appearing upon consumption of one oil would still be present at similar levels when the oils were fed together. For example, the signaling-type lipid sphingosine-1-phosphate (107) was increased by the FO-containing foods with no impact by MCT. Similarly, MCT appeared to not impact the effect of FO on TCA metabolites as both FO and FO+MCT groups responded similarly. This additive nature of the effects of FO and MCT was evident for some phospholipids. The lysophospholipid 1-(1-enyl-palmitoyl)-GPE (P-16:0) was decreased by MCT but increased by FO with the overall effect in the FO+MCT group being no change. The same was true for 1-palmitoyl-GPE (16:0); MCT decreased while FO increased levels and FO+MCT showed no change. In some cases, the combination of FO+MCT feeding produced more marked changes for energetic and signaling lipids than when either oil was fed alone, including alpha- and omega-oxidized fatty acids (AHFA and dioates) and the endocannabinoid NAAN class. On balance, though, there was little statistical interaction apparent for lipid metabolites.

We previously published the results of a similar feeding trial in cats noting the effects of FO and/or MCT on the same classes of lipids (38). This provides an opportunity to assess species differences in canine and feline responses to the same dietary levels of the same bioactive fats. As examples of species differences in responses, in dogs MCT had a broad effect to decrease several NEFA even when FO was fed at the same time (FO+MCT). In cats, however, while MCT feeding also broadly decreased NEFA, the combination of FO+MCT increased NEFA (38). Another dissimilarity in response was that in dogs MCT decreased acylcarnitines regardless of the presence of FO, while in cats this lipid class was largely not impacted by MCT feeding and was increased by FO+MCT (38). There was also some concordance in response of some lipid types in cats and dogs. Both the cat and dog studies showed that FO+MCT led to decreases in N-acyl taurines, the accumulation of which (along with acylcarnitines) appears to lead to beta-cell dysfunction and type 2 diabetes (108). There was extensive agreement in cat and dog responses for phospholipids (GPC, GPE) and sphingolipids; in both species, the patterns observed for these lipid classes with FO+MCT were remarkably similar to the pattern with FO alone. Also seemingly concordant between the cat and dog findings was the decrease in several members of the NAAN class of endocannabinoid signaling lipids with combination FO+MCT oil feeding that was less responsive to either individual oil alone.

A strength of this study was the longitudinal design, which allowed a comparison of the changes induced by oil feeding rather than only providing a cross-sectional post-feeding assessment. Furthermore, the 2 × 2 study design allowed for the assessment of the interaction between the feeding of MCT and FO. A limitation of this design, however, was that the study was not performed in a crossover or Latin square design due to the constraints perceived around the long-term carryover effects of oil feeding. Thus, as each dog only consumed one of the oil formulations throughout the study, a comparison of different oil formulations within each subject was not possible. A further limitation of this study was that feces were not collected, and thus, analysis of the source of putative circulating postbiotics is not possible; the current data are thus only minimally useful in drawing firm conclusions on the source of putative postbiotics and their response to the dietary oil feeding.

In summary, feeding dogs MCT, FO, or FO+MCT demonstrated responsiveness of several simple and complex lipid classes and characterized the patterns of metabolites that drove these class-wise changes. The current study provides valuable insights into canine physiology in response to feeding dietary oils that can be employed therapeutically (FO for mobility and MCT for seizures). In this trial, dogs responded to FO consumption with a reduction in triglycerides. Consumption of MCT largely led to changes in lipids associated with energy metabolism, while FO consumption produced changes dominated by structural-type lipids. Both of these observations are consistent with the known biology of these lipids, where MCT are employed for metabolic disease and FO can be employed to alter membrane fluidity and triglycerides. These data confirm previous reports that consumption of LCPUFA(n3) decreases the incorporation of LCPUFA(n6) into circulating phospholipid fractions. Taken together, it can be concluded that lipidomic signatures relevant to the clinical efficacy of FO or MCT are maintained when these oils are fed in combination, and this report provides insights into which classes of lipids are most responsive to either dietary oil.

Data availability statement

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

Ethics statement

The animal studies were approved by Institutional Animal Care and Use Committee, Hill's Pet Nutrition, Topeka, KS, USA. The studies were conducted in accordance with the local legislation and institutional requirements. Written informed consent was not obtained from the owners for the participation of their animals in this study because animals were owned by Hill's Pet Nutrition.

Author contributions

MJ and DJ designed the project and methodology, acquired the funding and resources, curated the data, and performed the formal analysis. MJ wrote the first draft of the manuscript. MJ and DJ reviewed and edited the manuscript. All authors have read and agreed to the published version of the manuscript.

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Conflict of interest

The research was performed at the Pet Nutrition Center, Topeka, KS, where the authors currently work (MJ) or formerly worked (DJ).

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2023.1168703/full#supplementary-material

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Early life programming by diet can play a role in risk reduction of otitis in dogs

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Introduction: Otitis in dogs is often chronic while local treatment primarily consists of flushing, antibiotics, and/or antifungals. We were interested in finding early life variables that associate with otitis later in life, preferably some that could be modified.

Methods: A cross-sectional hypothesis-driven study with longitudinal data was performed to search for associations between pre- and postnatal exposures, and the incidence of owner-reported otitis in dogs at over 1 year of age. The multivariate logistic regression analysis study included data from 3,064 dogs and explored 26 different early life variables at four early life stages: prenatal, neonatal, postnatal, and puppyhood. We compared two feeding patterns, a non-processed meat-based diet (NPMD, raw) and an ultra-processed carbohydrate-based diet (UPCD, dry).

Results: We report that eating a NPMD diet significantly decreased the risk of otitis later in life, while eating a UPCD diet significantly increased the risk. This was seen in different life stages of mother or puppy: The maternal diet during pregnancy (p=0.011) and the puppies' diet from 2 to 6months of age (p=0.019) were both significantly associated with otitis incidence later in life, whereas the puppies' first solid diet, was associated in the same way, but did not reach significance (p=0.072). Also, analyzing food ratios showed that when puppies were consuming >25% of their food as NPMD it significantly decreased their incidence of otitis later in life, while a ratio of >75% UPCD in their diet significantly increased their risk of otitis. Also, if the dog was born in the current family, was exposed to sunlight for more than 1hour daily, and was raised on a dirt floor during puppyhood, there was a lower risk of otitis development later in life.

Discussion: The findings only suggest causality, and further studies are required. However, we propose that veterinarians, breeders, and owners can impact otitis risk by modifying factors such as diet and environment.

KEYWORDS

DogRisk, nutrition, ear, inflammation, canine, early, programming, DOHaD

Introduction

Otitis in dogs is a frequent inflammatory disease, seldom diagnosed as a primary disease, and it may affect any of the ear parts, either external ear canal (otitis externa) (1), middle ear (otitis media), and/or the inner ear (otitis interna) (2). Otitis in dogs is associated with alterations in the ear microbiota (3). The prevalence of the disease has been estimated in Europe to range from 8.7 to 20% (4–6). The etiology of otitis in dogs is multifactorial where various factors have been contributed to its pathogenesis (7–9). Recently its etiology has been classified into Primary, Secondary, Predisposing, and Perpetuating causative factors (2) which are abbreviated into the PSPP system (10). The main reasons for otitis development in dogs are allergies, atopic dermatitis (AD), food hypersensitivity, allergen contact, otoacariasis, and autoimmune and endocrine diseases (2, 9, 11–13), which are perpetuated by secondary yeast and/or bacterial infections (8). The age of onset of otitis in dogs is highly variable, since it differs based on the underlying primary cause (11).

The genetic nature and breed predisposition to develop otitis have been reported in specific dog breeds [(14); Supplementary Table S1]. A higher incidence of otitis is often seen within breeds with pendulous/long ears (7), although the reduced incidence of otitis within some breeds with pendulous ears indicates that congenital factors alone do not determine disease incidence. The Developmental Origin of Health and Disease (DOHaD) hypothesis suggests that early life exposures, especially early diet, during the critical developmental periods; pre- and postnatal, modulate developmental programming via epigenetics and the establishment of the early microbiome, thereby stimulating the immune system and determining the dog's susceptibility to diseases later in life, including allergies and autoimmune diseases (15, 16).

There are numerous potential mechanisms involved in early life programming by the diet, among them genetics and epigenetics, microbiome establishment, and fetal organogenesis (15–18). Previously we investigated the importance of early life nutritional and environmental exposures in several studies. We have looked at time periods starting from prenatal life till one and a half years old and analyzed the risk of canine atopic dermatitis and inflammatory bowel disease/canine chronic enteropathy in dogs (19–22). Thus, identification and elimination of the most primary causes of otitis such as allergies, canine atopic dermatitis, and other primary etiologies early in life, through early life programming of the individual's immune system, are possible and highly practical preventive approaches for reducing the risk of otitis incidence in dogs. The current study aimed to investigate the role of the early life modifiable and non-modifiable exposures on later onset of otitis in dogs.

Materials and methods

Data source and outcome measure

We scrutinized data from the validated (23) owner-reported DogRisk food frequency questionnaire (FFQ) with a cross-sectional and longitudinal design (available in Finnish at: http://bit.ly/427aGBa). The FFQ was developed at the University of Helsinki, Finland and was available for dog owners online from 2009 to 2021. The questionnaire was widely disseminated to Finnish dog owners via several professional, public, and social platforms as described in a previously published study (24). As an epidemiological tool of preventive medicine, the FFQ was screening potential causes of non-communicable diseases in Finnish dogs. The FFQ included different categories of questions regarding the dogs' disease diagnoses and life-long exposures, including nutrition of the dog and its dam, environmental indoor and outdoor exposures, history of maternal diseases, breed, sex, age, coat color, etc. More information on the FFQ has been presented in prior research (19–22, 24). The FFQ was approved by the ethical board of Viikki campus, University of Helsinki (29.4.2016).

The dichotomized (yes/no) outcome question to the owner was: Has your dog suffered from otitis/inflammation of the ear? From a total of 10,4601 participants that answered the FFQ between 2009 and 2018, a study sample of 3,064 dogs (1,237 cases and 1,827 controls) was analyzed. To avoid reverse causality all cases under 1 year of age and all controls under 3 years of age were excluded (shown in the study flowchart, Figure 1).

Study setting, design, and early-life tested variables

A cross-sectional epidemiological study with longitudinal data was performed to look for the associations between early life exposures and the development of otitis in dogs at over 1 year of age. The study included one dichotomous dependent variable and 26 dichotomous and categorical independent covariates (Supplementary Table S2). Modifiable and non-modifiable exposures from the four early periods of the dog's life were selected based on previous research and classified into five groups of analysis according to their time of exposure: group I included the prenatal non-modifiable exposures, group II included the prenatal modifiable exposures, group III included the neonatal modifiable exposures, group IV included the early postnatal modifiable exposures, and group V included the late postnatal modifiable exposures (Table 1, Figure 2). The early life variables included in the analysis were heterogeneous, obtained from different categories of exposure such as genetic, hereditary, demographic, dietary, environmental, domestic, and immune-related (Supplementary Table S2). Two breed-related variables were created based on the breed information available at the Federation Cynologique Internationale (FCI)[1]: (i) the dog's ear shape (erect, semi-erect, or dropped ears) and (ii) the presence of hair in the ears ("hairy/pilose ears" or "non-hairy or pilose ears"). Based on that and breed disposition literature mentioned in Supplementary Table S1, the DogRisk FFQ breeds have been classified into two groups: otitis prone and otitis non-prone breeds. The methods used for data preparation have been described previously (19). Three open answers questions were

Abbreviations: FFQ, food frequency questionnaire; AD, atopic dermatitis; CAD, canine atopic dermatitis; AASS, atopy/allergy skin symptoms; NPMD, non-processed meat based diet; UPCD, ultra-processed carbohydrate based diet.

1 https://www.fci.be/

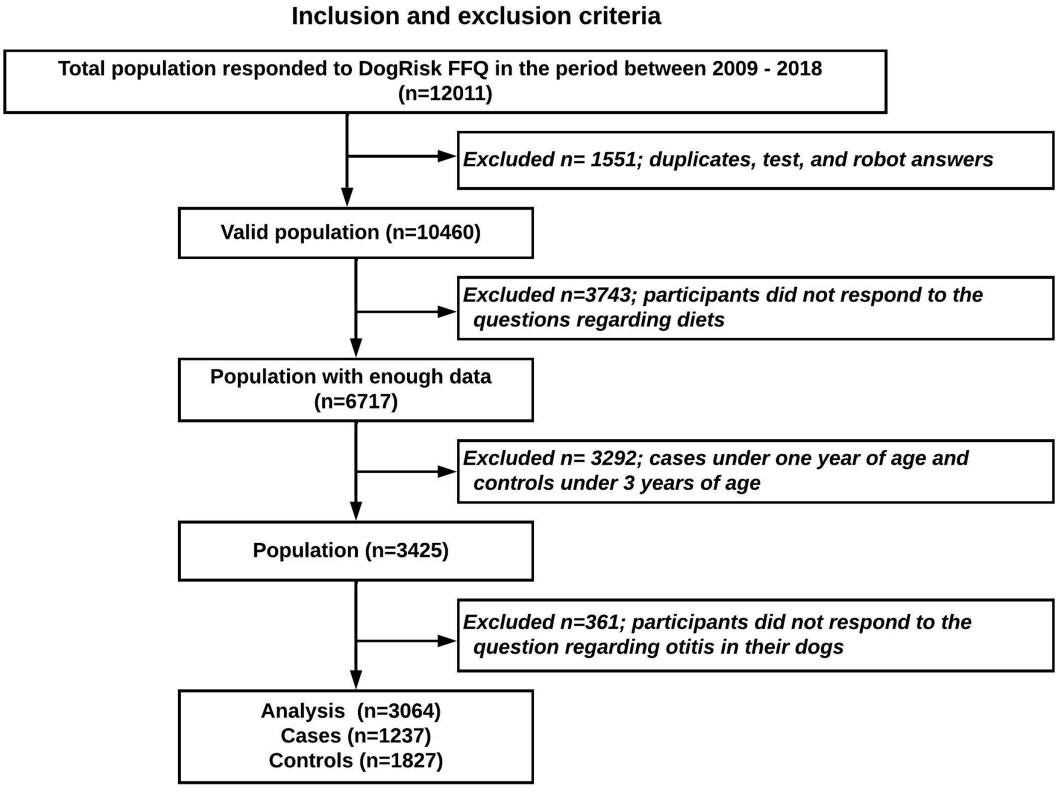

FIGURE 1
Study population flowchart.

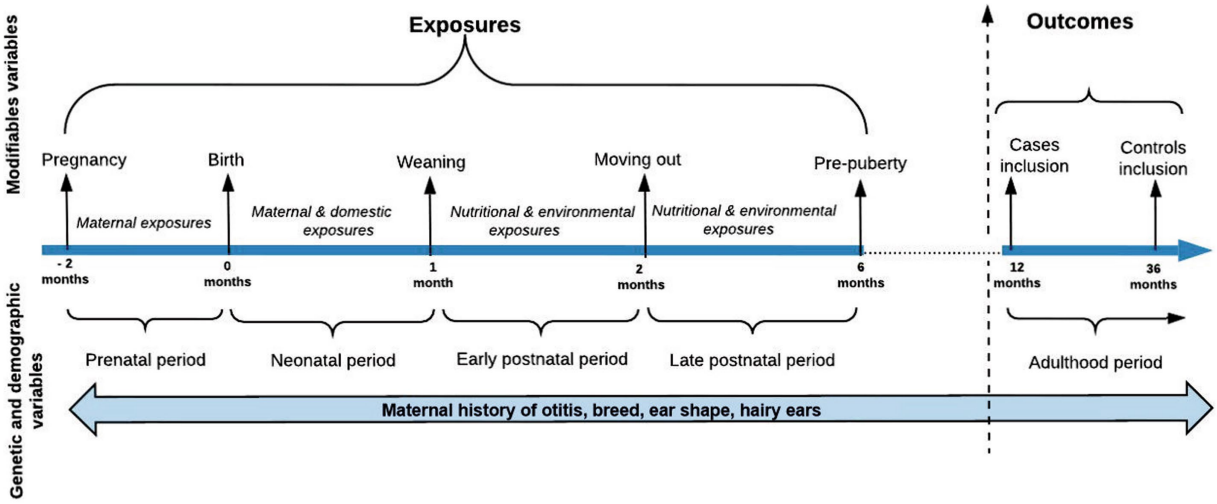

FIGURE 2
Study timeline. The diagram modified from Hemida et al. (19).

asking about the maternal diets during pregnancy and lactation, and the puppy's first solid diet. Based on the answers, the dams and puppies were divided into either ultra-processed carbohydrate-based diet (UPCD) or non-processed meat-based diet (NPMD) eating dogs. Dogs that were on other diets were not included in this study. The puppies' diets from 2 to 6 months of age were extracted from a diet ratio question where the owner could choose the consumed % from four kinds of diets; UPCD, NPMD, processed wet, and home-cooked diets. From the latter we only analyzed two extreme groups; the dogs that had either been eating over 80% of UPCD or over 20% of NPMD in their diets. These percentages were chosen based on previous research (21). Additionally, we examined two ratio scales to evaluate the prevalence of otitis among the study sample when consuming different ratios (0%, 1–25%, 26–50%, 51–75%, 76–100%) of the two, NPMD and UPCD, feeding patterns.

TABLE 1 Associations between pre-, neo-, early post-, and late postnatal period variables and otitis disease based on univariate logistic regression analyses.

Covariates	Total population for analysis (n = 3,064)		Crude effect estimates	
	Included dogs (n)	Missing dogs (n)	cOR (95% CI)	p-value
I. Prenatal period				
Genetic and demographic factors; non-modifiable				
1. Maternal history of otitis	1,295	1,769		
Mothers without otitis vs. mothers with otitis			0.114 (0.082–0.158)	**<0.001***
Mothers with otitis vs. mothers without otitis			8.760 (6.312–12.157)	
2. Dog's AASS	2,936	128		
Dogs without AASS vs. dogs with AASS			0.264 (0.219–0.317)	**<0.001***
Dogs with AASS vs. dogs without AASS			3.795 (3.156–4.564)	
3. Dog's ear shape (Ref. Erect ears)	2,394	670		
Semi-erect vs. erect ears			1.343 (1.039–1.736)	**0.024***
Dropped vs. erect ears			2.668 (2.208–3.225)	**<0.001***
4. Hairy/pilose ears	2,394	670		
Hairy/pilose vs. non-hairy/pilose ears			1.301 (1.043–1.622)	**0.020***
Non-hairy/pilose vs. hairy/pilose ears			0.769 (0.616–0.959)	
5. Dog breed	2,340	724		
Otitis non-prone vs. prone breed			0.400 (0.337–0.474)	**<0.001***
Otitis prone vs. non-prone breed			2.503 (2.112–2.967)	
6. Dog color	2,875	189		
<50% white colored coat vs. >50%			0.927 (0.770–1.116)	0.425
>50% white colored coat vs. <50%			1.078 (0.896–1.298)	
7. Dog sex	2,980	84		
Female vs. male			0.817 (0.705–0.946)	**0.007***
Male vs. female			1.224 (1.057–1.418)	
Maternal factors; modifiable				
8. Mother's diet during pregnancy	1,896	1,168		
NPMD vs. UPCD			0.607 (0.417–0.882)	**0.009***
UPCD vs. NPMD			1.648 (1.133–2.396)	
9. Was the mother dewormed during pregnancy?	1,878	1,186		
Yes vs. no			1.128 (0.630–2.019)	0.685
No vs. yes			0.886 (0.495–1.586)	
10. Was mother vaccinated during pregnancy?	1,082	1,982		
Yes vs. no			1.143 (0.893–1.463)	0.289
No vs. yes			0.875 (0.683–1.120)	
II. Neonatal period (0–1month); modifiable factors				
11. Mother's diet during lactation	1,798	1,266		
NPMD vs. UPCD			0.760 (0.525–1.099)	**0.144**
UPCD vs. NPMD			1.317 (0.910–1.905)	
12. Was the dog born in the same family?	3,064	0		
Yes vs. no			0.431 (0.315–0.590)	**<0.001***
No vs. yes			2.321 (1.695–3.177)	
13. Season of birth (Ref. Autumn)	3,016	48		

(Continued)

TABLE 1 (Continued)

Covariates	Total population for analysis (n = 3,064)		Crude effect estimates	
	Included dogs (n)	Missing dogs (n)	cOR (95% CI)	p-value
Winter vs. autumn			1.175 (0.945–1.461)	**0.147**
Spring vs. autumn			0.998 (0.810–1.231)	0.988
Summer vs. autumn			0.929 (0.741–1.164)	0.520
III. Early postnatal period (1–2months); modifiable factors				
14. Puppy's first solid diet	1,853	1,211		
NPMD vs. UPCD			0.714 (0.496–1.028)	**0.070**
UPCD vs. NPMD			1.400 (0.972–2.016)	
15. Frequency of outdoor activity (Ref. Not at all)	2,318	746		
Many times / day vs. not at all			0.733 (0.542–0.991)	**0.043***
Once/day vs. not at all			0.913 (0.645–1.292)	0.607
A few times / week vs. not at all			1.058 (0.735–1.523)	0.763
A few times / month vs. not at all			1.334 (0.839–2.123)	0.224
16. Sunlight exposure, hours / day	1,672	1,392		
≥ 1 vs. not at all			0.697 (0.572–0.849)	**0.001***
Not at all vs. ≥ 1			1.435 (1.178–1.749)	
17. Type of flooring	2,420	644		
Dirt / lawn vs. non-dirt / lawn floor			0.735 (0.526–1.026)	**0.07**
Non-dirt / lawn vs. dirt / lawn floor			1–361 (0.975–1.901)	
18. Body condition Score	2,570	494		
Normal weight vs. underweight			0.819 (0.625–1.072)	**0.145**
Underweight vs. normal weight			1.222 (0.933–1.600)	
Normal weight vs. overweight			1.045 (0.833–1.311)	0.703
Overweight vs. normal weight			0.957 (0.762–1.201)	
Overweight vs. underweight			0.783 (0.564–1.088)	**0.145**
Underweight vs. over weight			1.277 (0.919–1.773)	
IV. Late postnatal period (2–6months); modifiable factors				
19. Puppy diet	1,471	1,593		
NPMD vs. UPCD			0.627 (0.431–0.911)	**0.014***
UPCD vs. NPMD			1.595 (1.098–2.318)	
20. Outdoor activity, hours / day (Ref. < 0.5)	2,560	504		
0.5–1 vs. < 0.5			1.144 (0.664–1.971)	0.629
1–2 vs. < 0.5			1.134 (0.664–1.934)	0.646
> 2 vs. < 0.5			0.866 (0.496–1.510)	0.611
21. Sunlight exposure, hours / day	2,342	722		
≤ 1 vs. > 1			1.121 (0.930–1.350)	0.230
> 1 vs. ≤ 1			0.892 (0.741–1.075)	
22. Type of flooring	2,927	137		
Dirt / lawn vs. non-dirt / lawn floor			0.703 (0.548–0.904)	**0.006***
Non-dirt/ lawn vs. dirt /lawn floor			1.421 (1.107–1.826)	
23. Body condition score	2,671	393		
Normal weight vs. underweight			0.864 (0.723–1.033)	**0.108**

(Continued)

TABLE 1 (Continued)

Covariates	Total population for analysis (n = 3,064)		Crude effect estimates	
	Included dogs (n)	Missing dogs (n)	cOR (95% CI)	p-value
Underweight vs. normal weight			1.157 (0.968–1.383)	
Normal weight vs. overweight			0.791 (0.578–1.083)	**0.144**
Overweight vs. normal weight			1.264 (0.923–1.730)	
Underweight vs. over weight			0.916 (0.655–1.281)	0.607
Overweight vs. underweight			1.092 (0.781–1.527)	
24. Was the puppy vaccinated 2–4 times under 1 year of age?	3,014	50		
Yes vs. no			1.226 (0.564–2.666)	0.607
No vs. yes			0.815 (0.375–1.773)	
25. Was the puppy dewormed 2–10 times under 1 year of age?	2,966	98		
Yes vs. no			2.339 (0.861–6.358)	**0.096**
No vs. yes			0.427 (0.157–1.162)	

(n), number of dogs; included dogs, the number of valid answers for the corresponding question; crude effect estimates, one covariate in the model each time; cOR, crude odds ratio; CI, confidence interval; AASS, atopy/allergy skin symptoms; bolded, $p < 0.2$; *, $p < 0.05$; NPMD, non-processed meat based diet; UPCD, ultra-processed carbohydrate based diet; vs., versus; Ref., reference.

Data analysis

Data analyses were conducted using IBM SPSS Statistics 28 for Windows. Cross-tabulation was used to describe the study population and covariates' characteristics. The prevalence of otitis within the study sample when consuming different ratios of NPMD or UPCD was also calculated using crosstabulation. Univariate logistic regression analysis was performed individually for the quantitative estimation of the association between the different covariates and the outcome by entering one independent variable at a time. The variables which had a liberal association ($p < 0.2$) with the dependent variable were selected and used for further modeling. The multivariate logistic regression analysis was done using a multi-model design to get adjusted odds ratios with 95% CI of the predictors using the backward stepwise regression method with entry and removal probability (0.1) and (0.4), respectively. Five models stretched over the four time periods (Figure 2) were analyzed, the first period included one model of non-modifiable and one of modifiable variables. Models were adjusted for age and sex at all four time points. The missing values were not imputed but handled by listwise deletion. The fitness of the models was tested and established by the Omnibus test (p-value < 0.05), Hosmer and Lemeshow test (p-value > 0.05), and Nagelkerke's R for the largest value.

Results

The prevalence of owner-reported otitis within the DogRisk FFQ total population after excluding the duplicates, test, and robot answers (n = 10,460) was 27.74%. The characteristics of the study population and the tested variables are presented in Supplementary Table S2. The study sample's average age ± SD was 5.44 ± 2.8, in cases 5.25 ± 2.94, and in controls 5.56 ± 2.7 years old.

Breeds' predisposition for otitis in Finland

The otitis breed predisposition was tested among the DogRisk FFQ population's breeds. From a total of 205 breeds (including mixed breeds), we found that 53 breeds, with a total number of 5,297 dogs, showed a significant difference between dogs with otitis and dogs without otitis in Finland. The breeds are presented in a descending order starting from breeds with the highest ratio of dogs with otitis in Table 2.

Logistic regression analysis

Twenty variables from a total of 26 variables were found to be associated with owner-reported otitis incidence in dogs later in life with a $p < 0.2$ using the univariate logistic regression analysis, from which 12 variables were significant with $p < 0.05$ (Table 1). All twenty were forwarded for the final modeling using the multivariate analysis. From the five final models of the multivariate logistic regression, we found that seven variables were significantly associated with owner-reported otitis incidence in dogs later in life and two variables showed a tendency towards the association ($p < 0.10$ but did not reach significance at $p < 0.05$) (Figure 3).

The effect of consuming different food ratios on the prevalence of owner-reported otitis within the DogRisk study population

From the crosstabulation analysis for determining the prevalence of owner-reported otitis in dogs when consuming different ratios from both tested feeding patterns in the current study; NPMD and UPCD, we found that the prevalence of otitis was significantly decreased when consuming >25% of their diets as NPMD. On the other hand, the

TABLE 2 Percentages of dogs with otitis within breeds showing significant difference between study cases and controls ($n = 5,297$).

Breeds[a]	Dogs with otitis within each breed, % (*n*)	Total dogs[b]/ breed, (*n*)
Chinese Shar pei	91.67 (11)	12
Weimaraner	70.00 (7)	10
Pug	68.18 (15)	22
Petit basset griffon vendeen	66.67 (8)	12
Irish glen of imaal Terrier	60.00 (6)	10
Spinone Italiano	60.00 (6)	10
Dogo Argentino	59.09 (13)	22
Welsh Springer Spaniel	58.62 (34)	58
Leonberger	57.14 (28)	49
French Bulldog	56.25 (45)	80
Bullmastiff	55.88 (19)	34
Romagna Water Dog	54.00 (27)	50
Rhodesian Ridgeback	52.94 (18)	34
Miniature poodle	52.00 (39)	75
English bulldog	51.02 (25)	49
Dalmatian	50.98 (26)	51
Irish Setter	48.65 (18)	37
Cocker spaniel	48.19 (40)	83
Border Terrier	47.27 (26)	55
Labrador Retriever	45.83 (154)	336
English Springer Spaniel	45.45 (25)	55
Spanish Water Dog	44.71 (38)	85
Newfoundland	44.07 (26)	59
Hovawart	42.98 (49)	114
Staffordshire Bull Terrier	42.08 (77)	183
Golden retriever	41.52 (93)	224
Nova Scotia Duck Tolling Retriever	39.81 (43)	108
Rottweiler	39.18 (76)	194
German Shepherd	38.08 (214)	562
Mixed breed	23.60 (283)	1,199
Finnish Lapponian Dog	18.14 (39)	215
Belgian Shepherd dog	17.16 (23)	134
Australian Shepherd	15.38 (14)	91
Lapponian Herder	15.38 (16)	104
Shetland Sheepdog (Sheltie)	10.97 (17)	155
Cardigan Welsh Corgi	10.64 (5)	47
Alaskan Malamute	8.82 (3)	34
Schipperke	8.57 (3)	35
Norwegian Elkhound Grey	8.33 (2)	24
Italian Greyhound	8.11 (3)	37
Icelandic Sheepdog	7.89 (3)	38
Pembroke Welsh Corgi	6.52 (3)	46
Siberian Husky	5.45 (3)	55
German Spitz Mittel	5.26 (2)	38
Border Collie	5.22 (7)	134
Samoyed	4.48 (3)	67
Japanese Spitz	4.17 (1)	24
Whippet	3.45 (2)	58
Australian Cattle Dog	0.00 (0)	12
Finnish Spitz	0.00 (0)	22
lancashire heeler	0.00 (0)	21
Peruvian hairless dog	0.00 (0)	14
Pharaoh Hound	0.00 (0)	10
Skye Terrier	0.00 (0)	10
Total	30.92 (1638)	5,297

[a]Breeds with a significant difference between dogs with otitis and dogs without otitis at $p < 0.05$.

[b]Breeds with at least 10 dogs per breed were only included in the analysis.

prevalence of otitis was significantly increased when consuming >75% of their diets as UPCD (Figure 4).

Discussion

Key findings

From the perspective of preventive and supportive medicine, the main novelty of the current study is presenting the early diet as a modifiable risk factor for otitis in dogs. We found that pre- and postnatal dietary patterns have a significant impact, either negative or positive, on the development of otitis in dogs later in life (Figure 3). Our findings agree with the DOHaD hypothesis which assumes that the early life diet can program the immune system of the individual through several proposed mechanisms, providing either protection or susceptibility to diseases later in life (25–27).

Non-modifiable risk/protective factors significantly associated with owner-reported otitis incidence in dogs at over 1 year of age

Findings from the non-modifiable genetic and background-related factors in model I showed that the maternal history of otitis remained in the model as the strongest predictor of otitis incidence in dogs (Figure 3). Maternal history of otitis was positively associated with otitis incidence in dogs later in life in the current study, where the risk increased 8.4-fold in subjects with a maternal history of otitis versus subjects without a maternal history of otitis. As far as we know, the association between the maternal history of otitis in dogs and the disease incidence in the next generation has not been explicitly investigated before. However, a recent study (7) reported the importance to exclude the parental phenotypes which are highly predisposed for otitis in designer breeding in order to reduce risk in the following

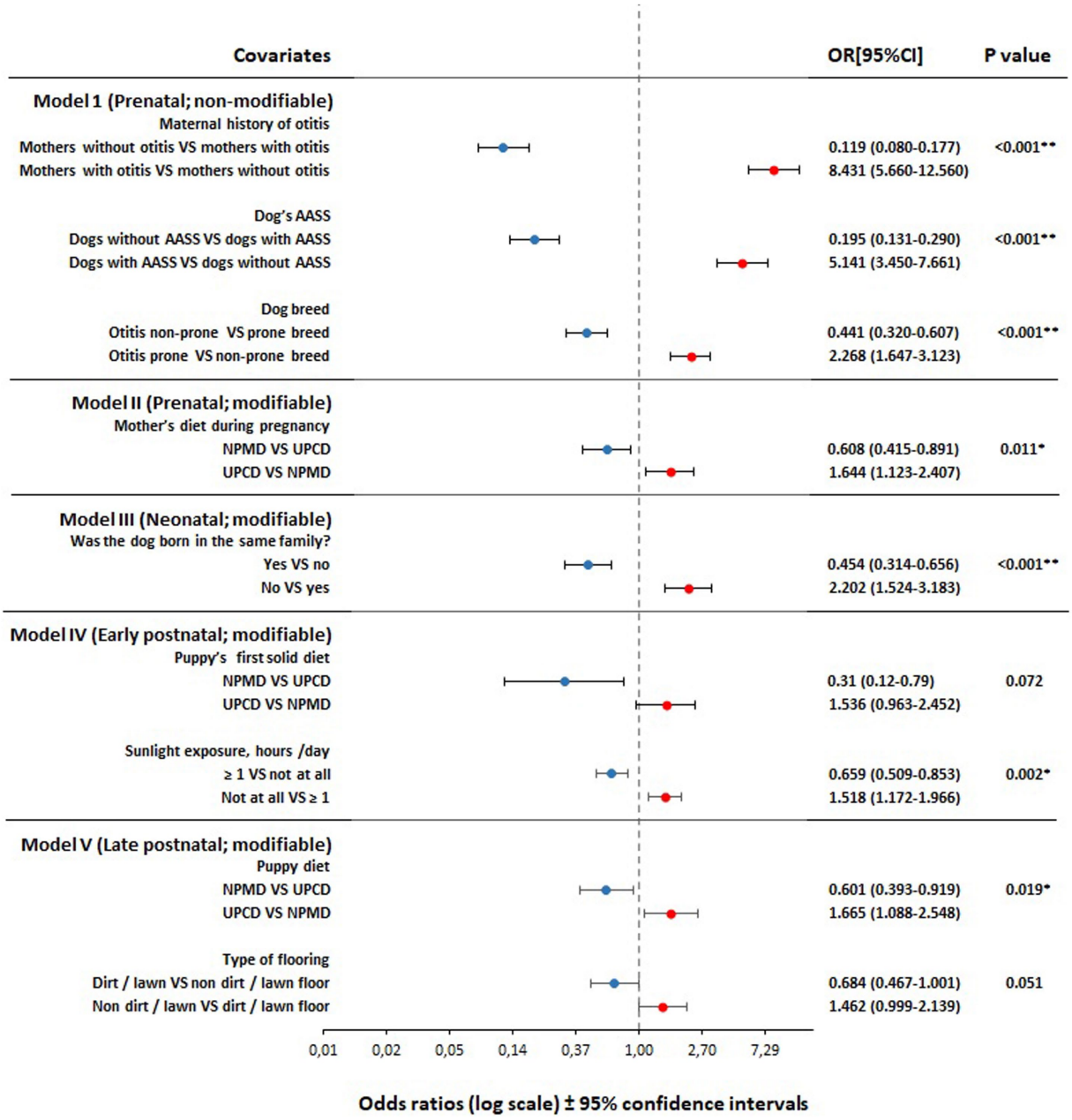

FIGURE 3
Forest plot of adjusted odds ratios for the association between pre-, neo-, early post-, and late postnatal period variables and otitis incidence in adult dogs (n = 3,064), based on backward stepwise multivariate logistic regression analyses. Models adjusted for age and sex. Dogs included in the analyses; Model I (n = 939), model II (n = 1,824), model III (n = 1,706), model IV (n = 1,003), and model V (n = 1,119). VS, versus; OR, odds ratio; CI, confidence interval; AASS, atopy/allergy skin symptoms; NPMD, non-processed meat based diet; UPCD, ultra-processed carbohydrate based diet; *, significant at $p < 0.05$; **, significant at $p < 0.001$.

generation (28). This finding is similar to our previous findings, where we found that the maternal history of canine atopic dermatitis and inflammatory bowel disease were directly associated with the same disease incidence in their offspring later in life (19, 20). From human research, the family history of otitis media and its association with otitis incidence in their children later has been suggested by several studies (29–34). This finding supports the notion that either predisposing factors (7) or genetic components (35) can be passed on to the next generation. It is also noteworthy that a newborn and up to 2 months old puppy share a common environment with the mother, including diet. This external factor could be as strong, or stronger, than a genetic or epigenetic factor and it is not possible to separate these factors in an epidemiological study of this type.

In the present study canine atopic dermatitis / atopy / allergy skin symptoms (CAD / AASS) was demonstrated to be a prominent risk factor for otitis (Figure 3). We observed a five-fold risk in dogs with AASS versus dogs without symptoms of atopy/allergy. Canine atopic dermatitis has been reported to be one of the primary causes of otitis (13). The association between CAD and otitis in dogs has

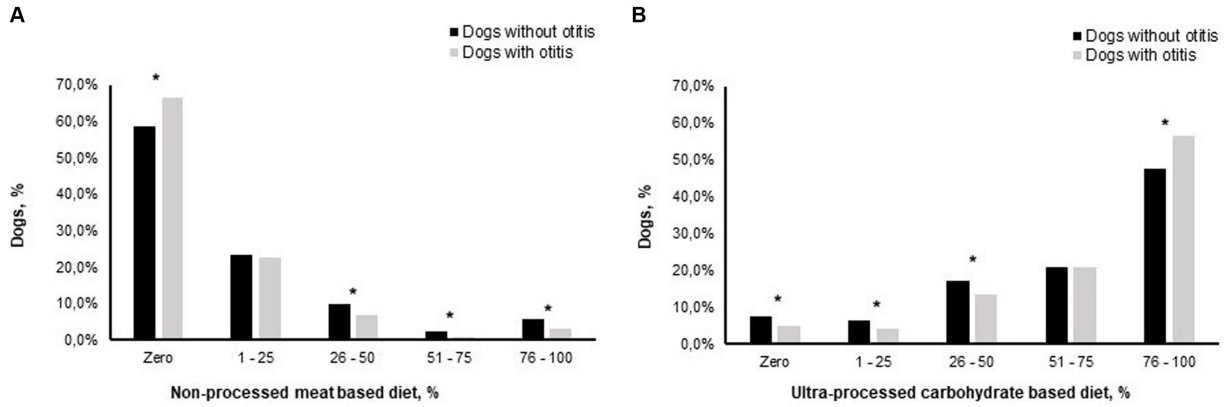

FIGURE 4
Otitis prevalence later in life is associated with feeding patterns of puppies of 2 to 6 months of age (n = 5,477), consuming different proportions of a non-processed meat based diet **(A)**, and an ultra-processed carbohydrate based diet **(B)**, of their total diets. *, the difference between the percentages of dogs with otitis and dogs without otitis is significant at $p < 0.05$.

been confirmed in several studies, either by the prevalence of otitis symptoms within atopic dogs (5, 12, 36–38) and/or by the concurrence of the age of the symptom's onset (39–41). In a canine study, otitis was seen in 83% of dogs with CAD, where otitis was the initial sign in 25% of reported cases (42). Another study showed that 68% of dogs with CAD developed otitis (12). Furthermore, a study found that the age of otitis onset coincides with the age of CAD onset in dogs (39), and another study further showed that 50% of otitis cases developed at the age of 1–5 years, and 30% of otitis cases initiated before the age of 1 year (12). Moreover, a connection between the skin microbiota and the ear canal microbiota has been suggested (43). Ngo et al. (44) found that there was a difference in the composition of the ear canal microbiota between healthy and atopic dogs without symptoms of otitis, indicating that CAD is a predisposing factor for dysbiosis in the ear canal microbiota and may manifest later as otitis externa (43). Our findings agree with the wide body of literature suggesting that CAD is the underlying disease for otitis in dogs (5, 36–38, 42, 45, 46).

In the present study, dog breeds were associated significantly with otitis development. The study showed that genetically prone breeds developed otitis 2.27-fold more often than non-prone breeds. From the FFQ data, several dog breeds have been found to be prone to develop otitis (Table 2; Figure 3). The later findings are supported by other authors' observations as shown in Supplementary Table S1. However, the wide variations in the breed predisposition of otitis are subject to several factors such as geographical location (9, 11), allergy predisposition (12, 40), lifestyle-related factors such as outdoor activity and swimming frequency (11), ear conformation (7), the presence of hair follicles within the ear canal (10), and any other predisposing factor for ear infections such as foreign bodies, excessive grooming and bathing, and systemic debilitation (7, 10). Moreover, in the current study, other predisposing factors were detected as well, such as breeds with specific ear shape or breeds with hair in the ear canal. However, although these traits were significantly associated with otitis incidence in dogs in the univariate regression analysis, they did not reach significance in the final models (Table 1).

Modifiable risk/protective factors significantly associated with owner-reported otitis incidence in dogs at over 1 year of age

Early life diets were associated with the later development of otitis in dogs during three of the four perinatal life periods: prenatal, early postnatal, and late postnatal (Figure 3).

In the current study, consumption of NPMD by pregnant dams and by puppies during puppyhood from 2 to 6 months of age were significantly associated with a reduced risk of later otitis incidence when compared to the consumption of UPCD, while the consumption of UPCD during the same periods was significantly associated with a higher risk of otitis later in life. Although the association between the puppies' first solid diet and otitis incidence later in life did not reach significance ($p = 0.072$), it showed a tendency towards a lower risk. Our present findings concerning early diets are consistent with our previous findings where we found that the early life diets were associated significantly with the incidence of AASS and inflammatory bowel disease/canine chronic enteropathy in dogs later in life (19–22).

NPMDs are raw non-processed meat-based diets consisting mainly of raw meats, raw organs, raw meaty bones, raw fish, raw eggs etc. NPMDs are high in protein and fats with low carbohydrate content. Additionally, the NPMDs contain raw vegetables, fruits, and berries. According to the average calculated from the NPMDs available in the Finnish market, it consists of 43.7% crude protein, 44% fat, 4.8% total carbohydrates, and 7.5% ash on dry matter basis (unpublished data). The UPCDs are commercial dry dog foods (kibbles) that contain a high amount of ultra-processed carbohydrates such as cereal grains (e.g., wheat, corn, oats, barley, rice, etc.) or potato starch, with a relatively low amount of animal-derived proteins when compared to the amount of fresh meat and bones included in the NPMDs. In addition to processed animal derived protein sources, the UPCD often contains processed plant-based proteins. The average composition of the UPCDs in Finland is as follows: 28.1% crude protein, 15.5% fat, 50% total carbohydrates, and 6.4% ash on a dry matter basis (unpublished data).

Maternal diet during pregnancy and postnatal diet both have a programming effect on the fetal immune system during these critical periods of developmental plasticity, thus influencing the long-term health of the offspring (47). Moreover, the dog microbiome's sensibility to environmental exposures including the diet is time-dependent, where it is more sentient earlier in life and sensibility declines as the dog ages (48). Hence, this relatively short yet critical time-period from conception to 6 months of age is important for developing risk or protection.

The ontogeny of the immune system in dogs begins *in utero* (48–50) and is primarily reliant on appropriate nutrition (51, 52). One of the proposed mechanisms underlying fetal programming by diet is the direct impact of the maternal diet on fetal cytogenesis and organogenesis (17). The entire fetal growth and embryonic organ development, including the thymus, are mainly dependent on nutrients received from the mother (18). This can, in turn, result in permanent developmental changes in organs, tissues, and consequently physiological functions in the future (53). Therefore, providing pregnant dams with a species-appropriate diet such as NPMD is very crucial (54–56). The NPMD is a high-protein, high-fat diet, that secures proper protein and fat quantity and quality, as well as energy requirements that are important for immune system integrity. It has been reported that sufficient protein during pregnancy provides an ideal medium for the fetal thymus development and hence sustains better immune competence later in life (18). On the contrary, UPCD often lacks good quality animal-derived proteins, and instead has a high carbohydrate content, which is not an essential nutrient for dogs (57–59). A study on pregnant rats showed that there was a massive reduction in the level of the thymus and spleen proliferation in offspring exposed to a diet with a relatively low protein content compared to those exposed to a diet with adequate protein (60). A relatively low protein diet during pregnancy was also associated with several detrimental effects in mature rodent offspring, including impaired immunity and reduced density of cerebral cortex capillaries (61).

Early life environmental factors including pre- and postnatal diet, and the microbial composition, establish epigenetic changes that can affect developmental programming (16). Evidence suggests that the epigenetic changes resulting from early diet and microbiome interaction can be trans-generationally inherited, therefore having a substantial effect on evolution and an individual's long-term health outcomes through modulating the immune response and the inflammatory molecular pathways (16, 62). This advocates for the importance of the early microbiome in driving the gut functions of the newborn for the rest of its life (51) and programming the immune system (63). The prenatal intestinal colonization is mainly shaped by the maternal gut, placental, and amniotic fluid microbiome (51, 64–66). A recent study reviewed the maternal diet-related changes in the immunity and microbiome of the offspring, both in humans and animals (52). Mirpuri reported that the maternal diet can alter the maternal microbiome, and this results in alternation in the offspring colonization either *in utero* and/or by vertical transmission via skin or the vaginal canal at birth. The maternal diet can also modify dietary metabolites and other dietary Toll-like receptors (TLR), which the fetus then will be exposed to in the uterus (52). Furthermore, the maternal diet has been found to alter cytokines, immunoglobulins, and other microbial products which also can alter TLR signaling in the embryonic gut and accordingly modulate the innate and adaptive newborn's immune system (52, 67). Additionally, the presence of maternal diet-mediated cytokines in the amniotic fluid together with other growth factors have been found to stimulate the fetal gut immunity (68). However, transit dysbiosis in the maternal intestinal and intrauterine microbiota during pregnancy was correlated with metabolic and immune-mediated disorders in human offspring (69). Other pathways through which the maternal diet can mediate epigenetic modifications are via physiologic and metabolic changes that are accompanied by increased or decreased predisposition to the later development of diseases (70).

Owing to the above, the appropriate selection of the maternal diet during pregnancy has a profound effect on the health and immunity of the offspring. Many studies have revealed that dogs fed NPMD had a difference in the composition of the gut microbiome and metabolism versus those fed UPCD, where they found that dogs fed a NPMD showed a high gut and fecal microbial diversity compared to those fed dry kibbles (71–74). Schmidt et al. (75) reported that dogs fed a BARF (=Biologically Appropriate Raw Food, similar to the NPMD in the current study) diet showed a higher diversity of *Lactobacillales*, *Fusobacterium*, *Enterobacteriaceae*, and *Clostridium* while dogs fed an ultra-processed commercial diet (=kibble) showed a higher abundance of *Clostridiaceae, Ruminococcaceae, Erysipelotrichaceae,* and *Lachnospiraceae*. By contrast, the consumption of high-carbohydrate diets has been found to increase gut dysbiosis, inflammation, and gut permeability in mouse and calve studies (76–78). Interestingly, human studies have found that a leaky gut caused by gut dysbiosis can generate a chronic systemic inflammatory state, which can extend to extra-intestinal organs such as the skin and ears (79–81). Moreover, a study in humans reported that gut microbial dysbiosis was associated with loss of hearing as it stimulated cochlear inflammation (79). Another human study from Finland stated that gut microbiota diversity was inversely associated with the severity of atopic eczema in infants at 6 months of age (82). According to studies, dogs are expected to have the same associations as in humans and other mammals between the gut microbiome and the skin immune defense (43, 81). In addition, in a previous study we have demonstrated that the hereditary basis of a disease can be modified by environmental factors like the diet (83). Anturaniemi et al. (83) found that the gene transcription profile of the raw fed atopic dogs is compatible with an improvement of the innate immunity and reduction in the oxidative stress that can prevent hypersensitivities and disturbed immunity.

The role of the early diet on programming the immune system is not restricted to the maternal diet, also the postnatal diet has an effect (16, 17, 63). The immune system maturation in dogs begins at birth and becomes fully mature approximately at 6 months of age (48). The postnatal diet has a role in shaping the postnatal gut microbiome, and this results in epigenetic signatures that can act on the properties of the gut mucosal barriers and their defensive role in opposition to later insults, therefore possibly prompting or restraining the later development of inflammatory diseases (16, 63). The postnatal diet provides the required nutrients for the newborns' growth and for their organ development (18). Besides the benefits and risks of the NPMD and UPCD mentioned above, a study reported that adequate high quality protein intake is essential for the proliferation of gut mucosal goblet lymphocytes, which have a role in eliminating infections that can generate disease (84). Moreover, a human study found that there was a positive association between inadequate protein intake and an increased risk of different diseases in children (85). Furthermore, some nutritional factors have been found to be partially responsible

for hearing loss in humans, where they found that a higher intake of carbohydrates and a lower intake of protein was associated with bad hearing status (86).

Another modifiable domestic risk factor that was observed in the current study is the question of being born in the same family that the dog now lives with, or not. We found that dogs that were born and continued to live in the same family were exposed to a lower risk to develop otitis later in life, while having been born in a different family, was associated with an increased 2.2-fold risk. These findings agree with our previous findings, where we found the same associations with CAD (19, 24). Research showed that daily in-house contact between the dog, its dam and siblings, home environment, and also between the puppy and its owner, reflected immune system adaptation to the same environmental stimuli through sharing the same microbiome (87–89). An opposing theory also exists: Garrigues et al. (51) found that puppies moving into big cities after leaving the birth kennel, showed a higher bacterial diversity compared to dogs living in small cities. This might be due to the exposure to a wide range of environmental influences and microbial exchange with other dogs and people during leash walking which means that the microbial development is affected by the geographic localization (51, 90).

An important environmental risk factor in the current study was that dogs that were exposed to sunlight for at least 1 hour daily during their early postnatal life (= from 1 to 2 months of age) showed a lower risk of developing otitis later in life versus dogs that were totally deprived of sunlight exposure. This result is similar to our previous findings in dogs suffering from CAD (19). The same has been observed in early childhood, where regardless of vitamin D status, they found that the exposure to direct ultraviolet rays decreased AD development in young children (91). Additionally, when a dog is outdoors for an hour or more daily, it also guarantees exposure to different environmental allergens. This, in turn, will stimulate the dogs' immune system (51).

The type of flooring the dogs were brought up on during puppyhood and up to 6 months of age was also associated with otitis later in life, although it did not reach significance (p=0.051). We found that dogs that had been raised on a dirt floor (earth) or lawn had a 0.7-fold lower incidence of otitis later versus dogs using other floor types. Also, this is akin to our previous findings (19, 21). The positive role of soil microbiome on the immune system stimulation is well accepted and has been elucidated in several studies (20, 92).

Our analysis Figure 4 shows that the prevalence of otitis was significantly higher within dogs that were not consuming NPMD at all during puppyhood, while the prevalence of otitis was significantly reduced within the groups of dogs that were consuming from 26 to 50%, 51–75%, and from 76 to 100% of their diet as non-processed foods. On the other hand, eating 76–100% of the diet during puppyhood as kibble significantly increased the prevalence of otitis later in their life. These findings agree with our previous findings regarding AASS (21), where we found that consumption of at least 20% of a NPMD reduced the prevalence of AASS for dogs in their later life, while the consumption of 80% or more of UPCD of the dog's diet significantly increased the prevalence of AASS later in life. This implies that consuming only a small ratio of the diet as NPMD (less than 26%) might not be enough to exert a beneficial impact on the immune system, whereas supplying the puppy with a sufficient quantity of NPMD provides the required nutrients from high-quality sources, as discussed above.

Strengths and limitations

The main strength of the current study is that we have tested a wide range of heterogeneous variables over four early-life periods starting from conception up to puppyhood. The research provides an epidemiological paradigm that also can be used in human research. Moreover, the study accounted for reverse causality by setting cut points for the age of the cases and the controls included in the study, based on a general age of onset (Figure 1). Another strength is that the data from the FFQ has been validated, securing its reliability (23).

The current study also has some limitations. Our measure of otitis in dogs is based on owners' reports and not on a veterinarian's confirmed clinical diagnosis. However, we handled this weakness by posing a set of additional questions related to the targeted disease, otitis. For instance, we asked how often the dog had suffered from otitis? At which age the dog suffered from otitis for the first time? If the dog still suffers from otitis or not? etc. These related questions helped to validate the owners' responses. The owners' responses were also confirmed by sending the owners emails to re-answer the survey (manuscript under preparation). As the study was cross-sectional, this might have led to recall bias. However, in addition to the data validation, the questions used in the FFQ were multiple-choice questions, helping to overcome the recall bias.

Conclusion

In conclusion, the current study showed that the early life diet and some environmental exposures were associated significantly with the incidence of otitis in dogs over 1 year of age. We conclude that the consumption of a NPMD during pregnancy, early and late postnatal life, was associated with a reduced risk of otitis at over 1 year of age. On the contrary, the consumption of an UPCD during the same periods was associated with a higher risk of otitis development later in life. The study recommended that the consumption of NPMD should be >25% of the dog's whole diet while the consumption of UPCD should be <75% of the dog's diet. Moreover, being born in the current family was associated with a lower incidence of otitis at over 1 year of age when compared to those puppies that moved to new families. Also, daily sunlight exposure for at least 1 hour was associated with a lower incidence of otitis in dogs at over 1 year of age compared to subjects not exposed to sunlight at all. Furthermore, the current study identified a lower risk for otitis development in dogs raised on a dirt floor or lawn during late puppyhood versus dogs raised on other kinds of floors. The study also identified the maternal history of otitis, CAD, and dog breed as significant risk factors for otitis development.

This study provides new insights into otitis in dogs that can inspire researchers and veterinarians to apply a primary preventive strategy for otitis in dogs. These findings suggest causality but do not prove it. Diet interventions should be conducted to confirm our observations, with a special focus on early 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 studies involving animals were reviewed and approved by the Ethical Board of Viikki Campus, University of Helsinki (29.4.2016). Written informed consent from the owners was not required to participate in this study in accordance with the national legislation and the institutional requirements.

Author contributions

MH and AH-B planned, designed, drafted the study, performed the data extraction, and did the statistical analysis. AH-B organized the database. MH, KV, NB, RM, SR, JA, AE-L, and AH-B wrote sections of the manuscript and edited it. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare 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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Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2023.1186131/full#supplementary-material

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Nutritional management of a dog with hepatic enzymopathy suspected to be secondary to copper-associated hepatitis: a case report

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A 4-year-old, female-spayed American Bulldog presented to the Ontario Veterinary College's Health Sciences Center's Clinical Nutrition Service for nutritional management of hepatic enzymopathy and suspected copper-associated hepatitis. Medical history revealed a 3-month history of gradually increasing serum ALT. Additional diagnostics included negative *Leptospira* titters, normal bile acids, and laparoscopic liver biopsy. Histopathology findings were consistent with diffuse moderate vacuole hepatocellular degeneration, mild positive copper staining, mild chronic lymphoplasmacytic hepatitis both portal and central, and mild biliary hyperplasia. Hepatic copper quantification results were above normal ranges (630 μg/g dry tissue), but below those seen in familial copper-associated hepatitis (>800–1,000 μg/g dry tissue). The patient was prescribed ursodeoxycholic acid, recommended to be fed a homemade diet (HMD), and referred for a nutrition consult. Two days before the nutrition consult, serum ALT fell within the normal range. The body condition score was 5/9, with a good muscle condition score and the dog's appetite and body weight remained stable. Energy intake was appropriate for maintenance. Key nutrient levels of all diets reported were compared to industry standards (AAFCO, NRC, and FEDIAF). Diet history included a commercially available raw meat-based diet (RMBD), of unknown copper content; a high energy commercial dry food (HEC), with copper content higher than the maximum recommended by FEDIAF and immediately prior to nutrition consult the patient had been eating an unbalanced homemade diet (HMD1) for 4 weeks. HMD1 was low in copper and deficient in the hepatoprotectant nutrients vitamin E and zinc. As per the owner's preference and to accommodate the patient's unique nutritional needs, a homemade diet addressing key nutrients for liver disease was formulated (HMD2), with copper content just above the recommended minimum. The new diet was found palatable and the patient's body weight, body, and muscle condition scores remained unchanged. Two months after starting HMD2, all bloodwork values remained within the normal range, including ALT. The reduction of dietary copper content likely reduced serum ALT. However, unbalanced diets cause a risk of nutrient deficiencies and excess. This dog was maintained with no known adverse effects on a complete and balanced HMD diet with a moderately low copper content, moderate protein, and inclusion of hepatoprotective nutrients.

KEYWORDS

clinical nutrition, dietary copper, liver disease, homemade diet, alanine transaminase, hepatoprotective nutrients, liver biopsy, canine

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Background

Copper (Cu) is considered an essential dietary nutrient by all published pet food industry standards, National Research Council (NRC), American Association of Food Control Officers (AAFCO), and the European Pet Food Industry Federation (FEDIAF) (1–3). It is necessary for multiple metabolic processes as a cofactor for multiple enzymatic reactions, including mitochondrial respiration, erythropoiesis, protection against free radicals, neurotransmitter synthesis, pigmentation, and iron metabolism (4–6). Excessive accumulation of Cu could result in an increase in oxidative stress in the hepatocytes, consequently leading to cell damage and inflammation (7). Hepatic accumulation of Cu was first described in pure-breed dogs, such as Bedlington Terriers (8) and Labradors (9) among other dog breeds (10). The pathophysiological origin of this mineral accumulation was attributed to a genetic impairment in the Cu excretion from the hepatocytes (7). Without a genetic component, Cu accumulation could be secondary to cholestatic liver disease (11). According to the 2019 consensus statement of the American College of Internal Medicine on the diagnosis and treatment of chronic hepatitis in dogs, Cu is considered the most common toxic injury causing chronic hepatitis (12). Recent reports have questioned this finding and called for a revision of the current guidelines for Cu content in pet foods, suggesting an association with an increased incidence of Cu-associated hepatitis in dogs (12, 13). Regulatory officials recently responded by stating that Cu guidelines would remain unchanged while agreeing to a lack of evidence linking Cu content in foods with Cu-associated hepatitis (14). Nutritional management of Cu-associated hepatitis usually includes Cu restriction and supplementation of zinc and vitamin E (9, 11).

This case report presents a patient with elevated serum alanine transaminase (ALT) activity suspected to be secondary to Cu-associated hepatitis, who was previously fed a commercial raw meat-based diet (RMBD),[1] a high-energy commercial extruded diet (HEC),[2] and an unbalanced homemade diet (HMD1). The goal of the nutritional management was to prevent further liver damage while providing a complete and balanced diet. This case diet history includes one diet in which the Cu content was higher than the legal limit established by FEDIAF and one raw meat-based diet that had no nutritional adequacy statement and lacked a nutrient analysis, potentially exposing the dog to nutritional deficiency or excess. Lastly, the patient was eating a homemade cooked diet, which presented multiple nutritional deficiencies, including key nutrients that are commonly used for the management of liver disease (9, 11).

1 Beef meal – K9 Kitchen, Brantford, Ontario, Canada.

2 Inukshuk 32/32—Inukshuk Professional Dog Food, Fredericton, New Brunswick, Canada.

Abbreviations: AAFCO, American Association of Food Control Officers; ALT, Alanine transaminase; BW, Body weight; BCS, Body condition score; MCS, Muscle condition score; Cu, Copper; DHA, Docosahexaenoic acid; EPA, Eicosapentaenoic acid; FEDIAF, European Pet Food Industry Federation; HEC, High energy commercial dry food; HMD, Homemade diet; NRC, National Research Council; OVC, Ontario Veterinary College; RMBD, Raw meat-based diet; WSAVA, World Small Animal Veterinary Association.

Case presentation

A 4-year-old, female spayed American Bulldog presented to the Clinical Nutrition Service at the Ontario Veterinary College's Health Sciences Center for a homemade diet (HMD) formulation for the nutritional management of hepatic enzymopathy and suspected Cu-associated hepatitis. A case timeline is provided in Figure 1. Medical history obtained from the referring internal medicine specialist revealed a 3-month history of gradual increase in serum ALT activity (Figure 2). On routine bloodwork 3 months before the nutrition consultation, serum ALT activity was found to be increased (132 IU/L; reference range 18–121 IU/L). The following month, a repeated serum biochemistry showed a further increase (166 IU/L). Serum ALT continued to rise over the next month (259 IU/L). No other significant findings or clinical signs were noted during any of these visits. The dog's owner reported no other ongoing medical concerns for the patient.

To further explore the cause of the increased serum ALT activity, additional diagnostics included: *Leptospira* titers (negative result), bile acids (under normal ranges), and laparoscopic biopsy samples sent for bacterial culture, and Cu staining and quantification. Histopathology of liver samples was consistent with diffuse moderate vacuole hepatocellular degeneration, mild positive Cu staining, mild chronic lymphoplasmacytic hepatitis portal and central, and mild biliary hyperplasia. Cu quantification in liver tissue revealed a slightly higher value (630 µg/g dry tissue) than normal (< 500 µg/g dry tissue) (15). Following biopsy results, the referring internal medicine specialist prescribed ursodeoxycholic acid 125 mg PO BID and temporarily transitioned onto a low-Cu homemade cooked diet (HMD1). The patient was referred to a clinical nutrition consultation for long-term dietary management.

Bloodwork performed 2 days before the nutrition consultation showed serum ALT activity within normal ranges (75 IU/L). The patient ate HMD1 for an 8-week period including a 2-week transition period, 2 weeks as a sole diet, and 4 weeks during the transition onto HMD2. During this time, the patient maintained a stable body weight (BW; 28.2 kg), body condition score (BCS) 5/9 (16), and normal muscle condition score (MCS) (17). A nutritional assessment was performed, and nutrition risk factors were identified using the WSAVA Nutritional Assessment Checklist (18). The risk factors found included multiple pets in the household, previous or ongoing medical conditions, current administration of medications and an unconventional diet. An extended dietary evaluation was pursued using a standard diet history form and a directed inquiry during the consultation.

The patient had been eating a RMBD for more than 1 year before presenting to the internal medicine specialist. Three months before the nutrition consult, at the time of the first blood analyses revealing high serum ALT concentrations, the patient's diet was mainly RMBD (see text footnote 1) and occasionally HEC (see text footnote 2). When the second blood sample was taken 1 month later, the patient was fed mostly HEC (see text footnote 2); by the third blood sample, 1 month prior to the nutrition consult, the patient was transitioning from HEC (see text footnote 2) to HMD1. The patient's owner specified that strict sanitary measures were followed for handling and offering RMBD (see text footnote 1) to the patient. After the diagnostics that followed the second blood sample, the patient was recommended a temporary homemade diet (HMD1, Table 1) by the internal medicine specialist

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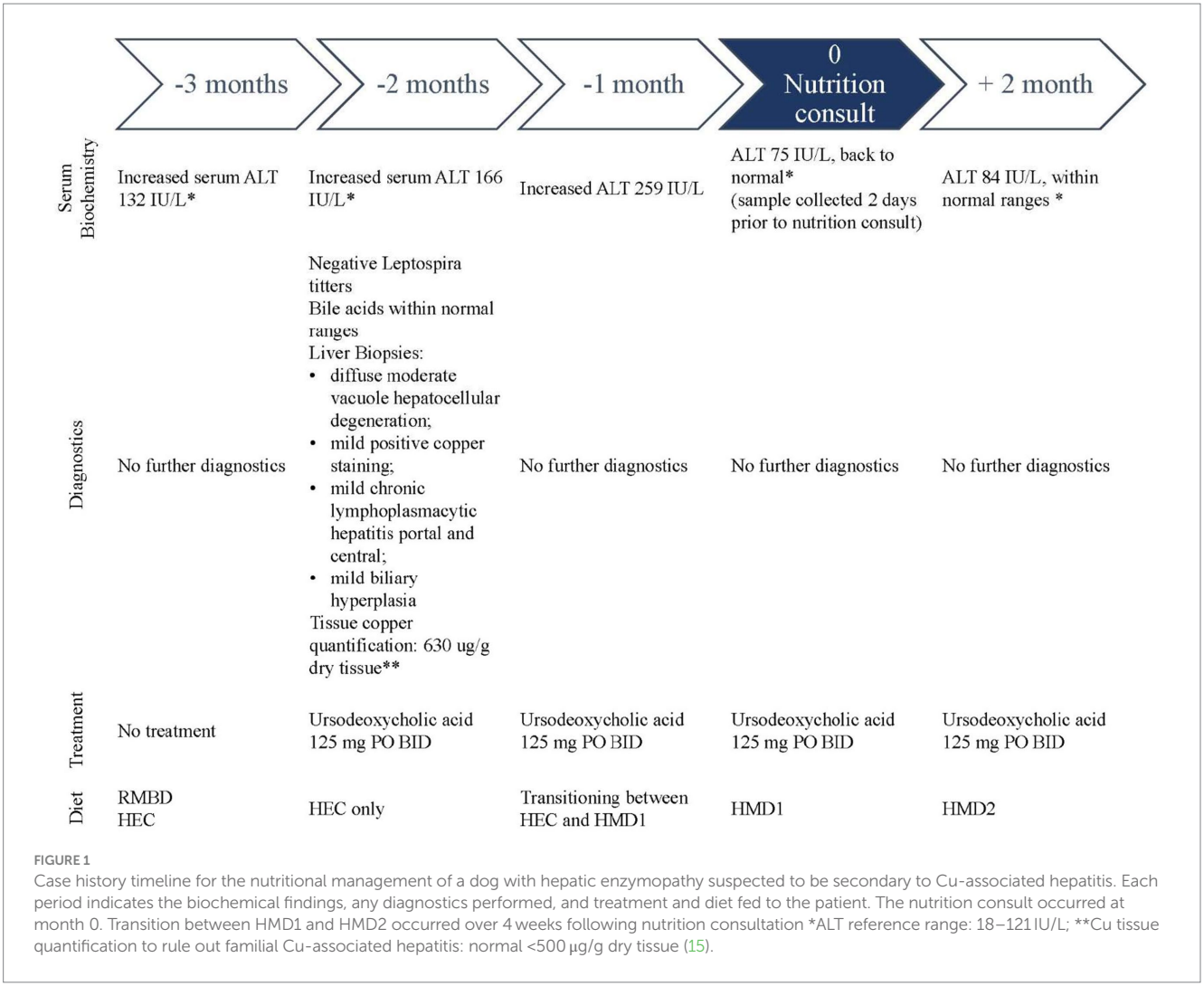

FIGURE 1
Case history timeline for the nutritional management of a dog with hepatic enzymopathy suspected to be secondary to Cu-associated hepatitis. Each period indicates the biochemical findings, any diagnostics performed, and treatment and diet fed to the patient. The nutrition consult occurred at month 0. Transition between HMD1 and HMD2 occurred over 4 weeks following nutrition consultation *ALT reference range: 18–121 IU/L; **Cu tissue quantification to rule out familial Cu-associated hepatitis: normal <500 μg/g dry tissue (15).

while waiting for a nutrition consult. This HMD1 consisted of one full boneless chicken breast (172 g), one cooked steak filet (262 g), and 3/4 cup of potatoes without skin (117 g). Based on this information, the patient's calorie intake coming from the HMD was estimated to be around 1,074 kcal per day (19). Treats consisted of two commercial pill pockets treats[3] (23 kcal/treat) and commercial dried fish-based treats[4] (124 kcal/treat) for a total daily energy intake of around 1,244 kcal.

The patient's energy requirements were calculated based on BW, BCS, and MCS using the equations recommended by the 2021 American Animal Hospital Association guidelines (20). The equation $70*BW^{0.75}$ was used to determine resting energy requirement (RER, 856 kcal/day). Based on an ideal BCS, moderate activity levels, and current energy intake, the initial daily energy requirement (DER) was decided as 1.5 × RER (1,284 kcal).

Using the information provided in the diet history, the Cu levels of the previous diets were assessed and compared to the recommended ranges by NRC, AAFCO, and FEDIAF (1–3). The nutrient analysis was provided by the HEC (see text footnote 2) manufacturer (8.6 mg/1,000 kcal), though the manufacturer of the RMBD (see text footnote 1) failed to provide a nutritional adequacy statement and any nutrient analysis beyond a proximate analysis when this was requested by the Clinical Nutrition Service. Moreover, food samples of this diet were no longer available for nutrient analyses, so the nutrient profile was estimated based on the ingredient list on the product label. Copper level of this diet was estimated using the web-based formulation software BalanceIT® Autobalancer[5] based on the ingredient list order (Table 1). The Cu content for HMD1 was also estimated using the same software based on the ingredients and amounts provided by the dog's owner.

According to the medical and diet history, the patient's problem list consisted of an imbalanced diet and elevated liver enzymes.

3 Greenies pill pockets peanut butter flavor capsule. Mars Canada, Bolton, Ontario, Canada.

4 Icelandic+ Herring Whole Fish Dog Treats, Icelandic PLUS, EHF, Smáratorg, Kópavogur, Iceland.

5 BalanceIT® Autobalancer software. DVM Consulting, Inc., Woodland, CA, United States.

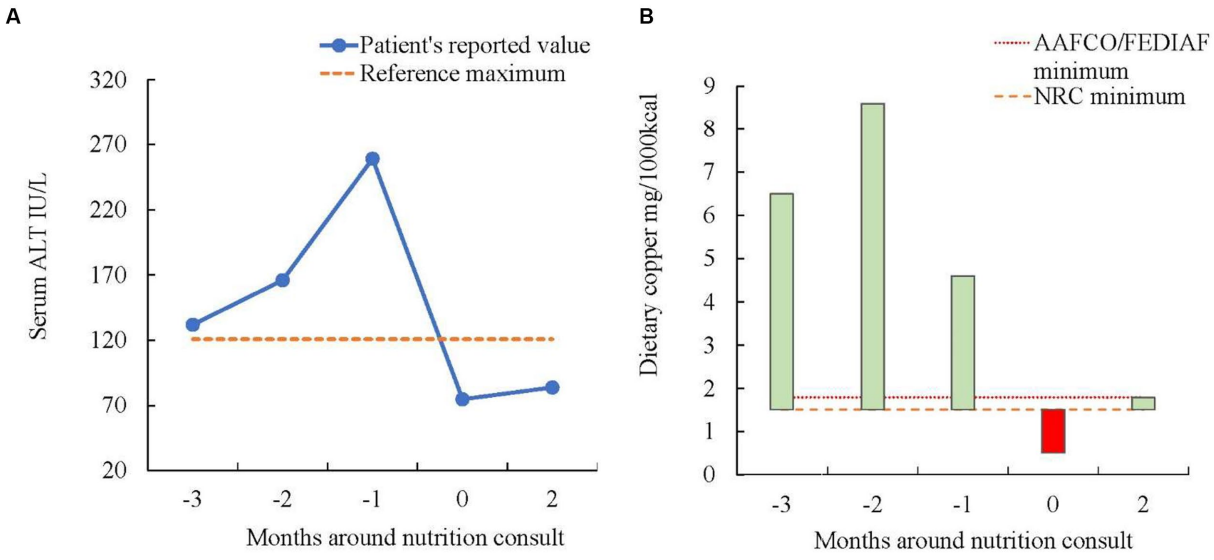

FIGURE 2
(A) Serum ALT concentrations in a 4-year-old, female spayed American Bulldog with hepatic enzymopathy. Nutrition consult occurred at month 0. The patient was eating diets with different copper content. At the time of nutrition consult, the patient was eating an unbalanced diet with copper content below the minimum recommended by NRC, this coincides with the ALT values falling within normal reference ranges. **(B)** This figure exemplifies the dietary Cu content fed to a 4-year-old, female spayed American Bulldog with hepatic enzymopathy compared vs. the minimum requirements by the NRC, AAFCO, and FEDIAF at different time points. The red colored bar indicates when the dietary Cu content was below the minimum recommended by NRC. Nutrition consult occurred at month 0.

TABLE 1 Key nutrients of diets fed to a dog with hepatic enzymopathy compared to industry standards.

	Protein (g/1,000 kcal)	Fat (g/1,000 kcal)	Vitamin E (IU/1,000 kcal)	Copper (mg/1,000 kcal)	Zinc (mg/1,000 kcal)
FEDIAF minimum (110 kcal $BW^{0.75}$)	45	13.7	9	1.8 (maximum 2.8 mg/100 gDM)	18
NRC minimum	25	13.8	11.2	1.5	30
AAFCO minimum	45	13.8	12.5	1.8	20
Diet 1: RMBD[a]	122	52	1	4.5	23
Diet 2: HEC[b]	71	71	23.5	8.6	50.3
Diet 3: HMD1	117	45.9	0	0.5	15.9
Diet 4: HMD2	79	45	75	1.8	30.2

[a]Raw meat-based diet.
[b]High energy commercial dry food.

Dietary treatment

Nutritional management of this case was to formulate an HMD as per the owner's request. The patient's owner reported having previous experience preparing HMDs for former pets and preferred to feed less processed foods. Following the nutrition consultation, the diet (HMD2) was formulated using the web-based formulation platform, BalanceIT® Autobalancer, which utilizes the USDA database for ingredient nutrient composition. Ingredients were selected in agreement with the owner's preferences, known palatability for the patient, and consistent availability. Formulation details for HMD2 are available in Supplementary material (Supplementary Table 1 for ingredients and full nutrient profile). Calorie intake considered for the HMD formulation was based on 90% of calculated DER (1,150 kcal), allowing for the remaining 10% (~134 kcal) to be given as treats.

The patient's owner was instructed to measure the ingredients on a gram scale and was informed that ingredient substitutions will alter the nutrient profile of the diet, therefore, any intended changes would have to be verified and adjusted by the Clinical Nutrition Service. During the consultation and in conversations prior to the formulation, the owner asked if meat ingredients could be given raw. The risks of bacterial contamination were thoroughly explained to the patient's owner, including the possibility of infection that could lead to further liver damage (21). The dog's owner was given specific cooking instructions and confirmed that they would follow these instructions.

Given that the patient did not present with any gastrointestinal disease signs or previously reported food intolerance, a standard gradual transition over 7 days was recommended for all the ingredients except for the dietary supplements. A calcium supplement[6] was recommended to be introduced over the course of 2 weeks, an

6 Tums Ultra Strength 1000. GlaxoSmithKline, Mississauga, Ontario, Canada.

omega-3 fatty acid source[7] over 3 weeks, and vitamin/mineral premix[8] over 4 weeks.

Monitoring and follow-up

For monitoring, it was suggested to keep a detailed diary to record HMD, treat intake and stool consistency daily, BW and BCS weekly, and inform the Clinical Nutrition Service of any changes. Over the follow-up period, communication with the patient's owner was limited; however the few times they contacted the Clinical Nutrition Service, they were very positive about how the food transition was going, in terms of palatability, absence of gastrointestinal signs, and recipe compliance. The patient's BCS and BW remained stable.

Follow-up bloodwork was performed by the family veterinarian 2 months after Clinical Nutrition consultation. All serum biochemistry values were within the normal laboratory range, including serum ALT concentrations (84 IU/L).

Discussion

This case was referred to the Clinical Nutrition Service with a very comprehensive workup and with absence of clinical signs. Referral to a veterinary nutritionist may be needed for recommendations for Cu-restricted diets given the limited commercial therapeutic diet options with reduced Cu content, which usually involve a moderate protein restriction (12, 22) and was not required for this patient. Furthermore, the patient's owners had a preference for unconventional diets; therefore, a homemade diet was considered and recommended as an effective alternative. For this patient, hepatic Cu levels fell into the "gray zone" (630 µg/g; 600–1,000 µg/g; rhodamine staining technique) (12), meaning that Cu-associated hepatitis could not be definitively diagnosed. Therefore, the indication of a strict dietary Cu restriction remained uncertain. The commonly used ranges for dietary Cu restriction are based on levels for breed-associated Cu accumulation (23), with dietary intake of Cu below minimum recommendations by AAFCO or NRC (1, 2). The breed of the patient has not been previously reported as predisposed to Cu storage hepatitis (7). There is a main limitation.

Achievement of stable blood liver enzymes within normal ranges was noted in this patient. It is impossible to confirm which factors had a stronger effect on preventing further liver damage, the dietary changes themselves or the combination of dietary management and response to an extended period of ursodeoxycholic acid therapy. Ursodeoxycholic acid administration was consistent after initial prescription by the internal medicine specialist, given its potential properties as choleretic and anti-inflammatory (12). Considering that the Cu content of the previously fed commercial diets was higher than the subsequent HMD, limiting dietary Cu intake was suspected to impact the reduction of serum ALT. The way to confirm these assumptions would be to repeat liver biopsies and Cu quantification, which was not a possibility for a healthy client-owned animal. When the serum ALT activity was reported within normal ranges, the patient was eating HMD1, which was a low copper (0.5 mg/1,000 kcal) yet unbalanced diet, over a period of 8 weeks, including diet transitions. HMD1 presented multiple nutrient deficiencies (Table 1), some of which are considered key nutrients when formulating or choosing diets for liver disease, like Vitamin E and zinc (11). As these hepatoprotective nutrients were limited, there is a strong indication that the reduction of dietary Cu content was the main dietary factor to reduce the hepatic damage. ALT activity is considered the earliest indicator of chronic hepatitis and is also treated as the most important monitoring factor for treatment success (12). The patient of this case report presented improvement in the ALT activity after being fed a low copper diet, and therefore a moderately low copper diet was maintained for over 2 months. This is graphically exemplified in Figure 2, where (a) represents the ALT reported values and (b) the dietary Cu content. To the authors' knowledge, this is the first time, a case is reported using a low-Cu homemade diet to manage a canine patient in the "gray zone" (12) of hepatic Cu concentration.

Blood liver enzymes remained stable and within normal range after implementation of a nutritionist-designed diet plan. The food recommended and used in this case report, HMD2, was specifically formulated for mild inflammatory hepatobiliary disease. In addition to limited Cu intake, HMD2 also addressed other key nutritional factors considered as per hepatobiliary disease standard of care (11). Although HMD entails high labor intensity for the pet owners, it also provides the nutritionist with a unique opportunity for customization of the nutrient profile.

Protein in the diet should be of good quality and provided in sufficient amounts to support liver regeneration (24). Patient liver function was not compromised, and they did not seem at the risk of developing hepatic encephalopathy or hyperammonaemia (25, 26); therefore, protein levels were not restricted. However, the patient had been fed a high protein diet for a long time so a reduction in protein intake was elected. The target protein levels of HMD2 were between 75 and 80 g/1,000 kcal, in comparison for patients with compromised liver function or hepatic encephalopathy, for whom the target range is between 37 and 50 g/1,000 kcal (11).

As the pathology report identified an inflammation in the evaluated tissue, when formulating HMD2, an enhanced dose of the omega-3 fatty acids eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) was included due to their anti-inflammatory properties. The target anti-inflammatory dose was 125 mg/kgBW$^{0.75}$ (~1,500 mg/day) of EPA and DHA per day (27–29). The introduction of the concentrated supplement (see text footnote 7) was recommended to start after all other ingredients of the diet were already known to be well tolerated.

Excessive Cu intake was avoided to prevent further accumulation in the liver tissue, which could lead to increased oxidative damage. From the biopsy results and Cu quantification, genetic Cu storage disease was ruled out (15), suggesting that the mild Cu accumulation seen was secondary to hepatobiliary inflammation. Therefore, a strict Cu-restricted diet was not required and only a mild restriction of Cu was considered for the formulation of diet HMD2. A strict Cu-restricted diet is recommended, as for confirmed genetic Cu storage disease, Cu levels should be under 1.25 mg/1,000 kcal (11, 23). The target values for HMD2 of this mineral were just above the low end recommended by AAFCO (1.83 mg/1,000 kcal) (2) and the minimum required by the NRC (1.5 mg/1,000 kcal) (1). In comparison,

7 Nordic Naturals Omega 3 pet liquid. Nordic Naturals Mig., Watsonville, California, United States.

8 Balance IT canine vitamin, mineral and amino acid powder. DVM Consulting, Inc., Woodland, California, United States. Patents US 8,968,806, US 10,194,685 & CA 2,606,109.

one study found that the median concentration in commercial dog maintenance foods was 4.4 mg/1,000 kcal (22, 30), with only the liver-targeted therapeutic diets going below 2.1 mg/1,000 kcal (11, 23). The possibility of adjusting the Cu levels of the diet further by changing to a Cu-restricted vitamin/mineral supplement was discussed with the patient's owner and left open, pending bloodwork results. In this case, Cu levels of the diets varied between 8.6 and 0.5 mg/kcal. The estimated Cu content of the RMBD (see text footnote 1) was 4.5 mg/kcal, which is close to the average values for commercial diets previously reported in the literature (22, 30). However, given that the ingredient list for the RMBD (see text footnote 1) references organ meats like liver and kidney meat, the Cu content could be even higher than estimated, as they are considered ingredients with a high concentration of Cu (31).

Alternatives or additional recommendations to dietary Cu restriction would have been dietary zinc supplementation (5–10 mg/kg BW or > 50 mg/1,000 kcal) (7, 11). Although it has been reported that supplementation of zinc gluconate did not produce any difference for Cu-associated hepatitis in Labradors (9). The prescription of *D*-Penicillamine has also been reported as an option to reduce Cu availability (7, 11). In this case, the dog owner preferred to try a low Cu diet first before the addition of extra medications. Furthermore, the efficacy of this medication without a Cu-restricted diet may be reduced (12).

Vitamin E, as an antioxidant, could potentially help with some forms of acute and chronic liver injuries by reducing the oxidative damage caused by free radicals and reactive oxygen (11). Vitamin E content of HMD2 was formulated at 75 IU/1,000 kcal, greatly exceeding minimum requirements by AAFCO (12.5 IU/1,000 kcal) (2, 3) or NRC (8.9 IU/1,000 kcal) (1). Vitamin E recommended contents for hepatobiliary disease are above 100 IU/1,000 kcal (11). Further supplementation of vitamin E was left as an open option if the initial recommendations failed in the goal of maintaining serum liver enzymes within normal ranges.

The patient was eating four different diets, all of which presented different nutrient profiles and contrasting levels of Cu. Blood ALT values seemed to increase when dietary Cu was higher and returned to normal ranges when the Cu was close or under the minimum requirement established by the NRC (1). This patient was presented to the OVC Clinical Nutrition service for a formulation of an HMD due to difficulty in finding a diet with low Cu levels without restriction of protein content. Furthermore, it is challenging for pet owners and veterinarians to find commercial diets moderate in Cu content, as most greatly exceed the minimum requirements (22, 30). Even gastrointestinal therapeutic diets are not moderate in Cu content, despite gastrointestinal tract health and Cu excretion being so closely related (5, 32). To the author's knowledge, there are no reports of recommended Cu levels for secondary Cu accumulation in the liver. Thus, further studies in which a reduction of Cu intake is the only nutrient intervention are warranted to determine standard ranges for dog maintenance diets.

Conclusion

This case report exemplifies the need for establishing different grades of dietary Cu restriction when mild or subclinical liver disease is present and in the absence of genetic predisposition. Biochemical analysis of liver enzymes was used for monitoring hepatocellular integrity. The elevated ALT values were the trigger for further diagnostics and therapeutic interventions. It also highlights the utility of an HMD and demonstrates the benefits of an individualized approach in the absence of a commercial diet alternative for patients that may benefit from Cu restriction and may not need the remaining limitations of commercial therapeutic diets targeted to liver disease. It is important to mention that this is only one case subject and further study of similar cases is warranted to confirm any clinical inferences.

Further research is warranted to determine a safe upper limit of Cu to be included in the nutritional guidelines for commercially available foods, especially with the rise of pet foods that use organ meat as the main ingredients.

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.

Ethics statement

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

Author contributions

FP was the clinician in charge of management of case. CG was the supervising faculty during management of case. FP, CG, ÉR, and AV participated in collection of data, writing and editing manuscript, and review of final submission. All authors contributed to the article and approved the submitted version.

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Conflict of interest

This case report is published in fulfillment of FP's Doctor Veterinary Science's degree and residency training for the European College of Veterinary and Comparative Nutrition. FP receives a scholarship from Royal Canin Canada for this graduate and residency training. FP and ÉR declare that they have participated in paid engagements with pet food companies within Canada. At the time of patient care, CG held the Nestle Purina

Professorship in Companion Animal Nutrition at the Ontario Veterinary College, was the Clinical Nutrition Service Chief at the Ontario Veterinary College's Health Sciences Center. CG is also the owner of Grant Veterinary Nutrition Services and consults with Simmons Pet Food. AV is the Royal Canin Veterinary Diets Endowed Chair in Canine and Feline Clinical Nutrition and declares that they serve on the Health and Nutrition Advisory Board for Vetdiet and has also received honoraria and research funding from various petfood manufacturers and ingredient suppliers. At the time of patient care, the Ontario Veterinary College received funding from Nestlé Purina Proplan Veterinary Diets to support a Registered Veterinary Technician in Clinical Nutrition, who helped with case management and client communication for this patient.

Publisher's note

Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2023.1215447/full#supplementary-material

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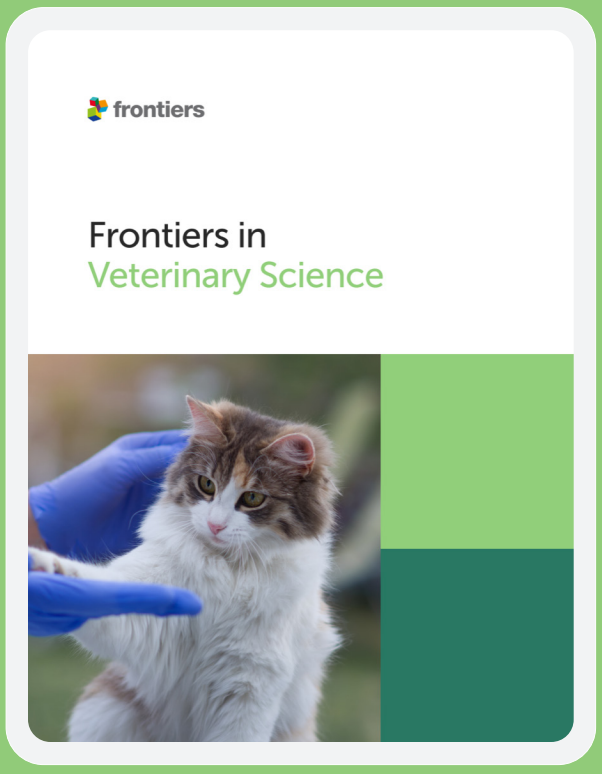

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