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Application of natural bioactive compounds in animal nutrition

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Application of natural bioactive compounds in animal nutrition

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Editorial: Application of natural bioactive compounds in animal nutrition

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Editorial on the Research Topic
Application of natural bioactive compounds in animal nutrition

Introduction

The current decade is facing an array of challenges in animal production such as global warming-induced heat stress, cost production in rearing and transportation, antibiotic-free meat production, encompassing the weanling time period, and mycotoxin contamination-stored animal feeds. Diminutive lacunae in the above domains can lead to serious animal health issues, which can cause acute physiological disorders and deteriorate the entire animal production percentage. They are also associated with health issues in consumers, which reflect greatly in the overall health status of stakeholders. Hence, there is a pressing need to address these problems and provide strategies to advance the animal production sector toward sustainability. The focus of this special issue inclined toward inviting ideas on the identification and utilization of natural bioactive compounds from microorganisms, plants, algae, and sea weeds, etc., for enhancing animal nutrition. Multi-drug antibiotic resistance has been an alarming issue in treating the diseases; however, the adoption of these bioactive metabolites can provide cues for disease treatment and tolerance in animals. A very low quantity of these bioactive metabolites has been reported to offer copious nutrient benefits and pharmacological effects. The intangible reasons of utilizing these compounds are due to lacunae in the scaling-up process and the inadequacy of insights in the mechanism of their action. Further, several bioactive compounds are still unexplored and elusive; thus, it is crucial to explore the interplay of these compounds in the health and production of farm animals. In addition, a targeted delivery of these compounds through inter-disciplinary aspects such as nanotechnology will bring about technological intervention in animal feed production and management strategies. Twenty articles based on the augmentation of bioactive compounds for the betterment of gut function, reduction of oxidative stress, decontamination agents of mycotoxin, and nano-encapsulation-based approaches for improvement in animal nutrition were received.

Role of plant-based metabolites in improving the health status of poultry

Chronic exposure to heat stress (HS)-induced disorders in behavioral, immunological, metabolic, physiological, hormonal, and biochemical mechanisms lead to a decline in the average daily feed intake (ADFI), production of egg, efficiency of feed, and quality of eggs in poultry. Further, HS directly aggravates oxidative stress and disturbs the homeostasis of the antioxidant system. This oxidative stress impacts the internal organs, GI tract, liver, and production of egg yolk or albumen and blood. The dietary augmentation of curcumin and plant extract blend of *Scutellaria baicalensis* in the form of a phytonutrient solution (PHYTO) improved the quality of the young layers, improved the thermotolerance, and upregulated the antioxidant defense system in young layers (25–32 weeks of age) grown in naturally higher temperature circumstances. This enhanced the intestinal villosity and liver performance. Moreover, it induced a translational effect in egg production, with better oxidative stability and eggshell breaking force (Giannenas et al.). However, further studies are needed to ascertain the synergism with the GI-associated microbiota. Curcumin has also been utilized for improving the growth indices and controlling insect pests of livestock (Sureshbabu et al.). In a similar study, the efficacy of *Artemisia ordosica* alcohol extract (AOAE) in augmenting the growth, immune, and inflammatory response in diets was studied with lipopolysaccharide (LPS)-challenged broilers. In other words, dietary supplementation of AOAE enhanced the spleen index value, decreased the bursa index, and alleviated the levels of IL-1β, IL-2, IL-6, IgG, IgM, and IL-4 in serum and associated immune response organs in LPS-challenged broilers. The upregulation and mRNA expression of TLR4, MyD88, TRAF6, NF-κB p65, NF-κB p50, IL-1β, and IL-6 genes were noted. The downregulation of IκBα and PPARγ demonstrated improved immunity and constrained inflammation (Shi et al.). The potential antioxidative and antiinflammatory activities of *Antrodia cinnamomea* polysaccharide (ACP) were evaluated in slow-growing broilers stimulated by LPS. The ACP diet accelerated the population of beneficial cecal microbiota such as *Lactobacillus*, *Faecali bacterium,* and *Christensenellaceae,* which curbed liver damage and brought about the synergistic effects of the antioxidants in the restoration of beneficial microbiota (Ye et al.). The mulberry leaf extract (MLE)-supplemented feed enhanced aspartate transaminase (AST) activity, increased the levels of glutathione peroxidase (GSH-Px), intensified the color of the egg yolk, elevated high-density lipoprotein (HDL-C) and superoxide dismutase (SOD) activity in the serum, and boosted the strength of eggshell. Further, the upregulation of PPARα and SIRT1 and decreased expression of FASN and PPARγ were evident (Zhang B. et al). Feed formulation using honeysuckle extract (HE) was experimentally studied using geese models, in which the growth index, biochemical parameters, immune responses, and gut microbiota were evaluated. The abundance of Bacteroidetes was increased, while the Firmicutes population decreased in HE-fed diets; however, *Bacteroides barnesiae*, *Subdoligranulum variabile*, *Bacteroides plebeius,* and *Faecalibacterium prausnitzii* were notably dominant. Therefore, the addition of honeysuckle extract to the diet impacted the intestinal function and immune tolerance by intervening in the gut microbial composition (Li G. et al.). The effects of Chinese yam polysaccharides (CYP)-fortified diets on the immune function of broilers were also studied. The treated group exhibited a higher thymus index and increased levels of serum IgA, complement C3, C4, IGF-I, T3, T4, INS, GH, IL-2, IL-4, IL-6, and TNF-α in the CYP-fed group. An improved spleen index and serum IgM and IgG concentrations corresponded to elevated immune response in broilers (Deng et al.). Long-term exposure to mycotoxins exerts oxidative stress; poultry in particular are very sensitive to aflatoxins (AFB1) that are common in animal feed commodities. AFB1 is reported to cause stunned growth, immunosuppression, and hepatotoxicity; however, dietary supplementation of plant-based polyphenolic compounds such as Chinese gallnut tannic acid (TA) was reported to catalyze the antioxidant enzyme activity and lower the malondialdehyde content in poultry. Moreover, it prevented the decrease of villus height/crypt depth ratio and liver enlargement in AFB1-treated broilers (Xi et al.).

Quercetagetin (QG) as a dietary supplementation for broilers improved the nutrient digestibility, intestinal morphology, immunity, and antioxidant capacity. It increased the levels of IgG and C4 levels in the blood and elevated the total antioxidant capacity (T-AOC) in serum, jejunum mucosa, and ileum mucosa through the *Nrf2*/antioxidant response element (ARE) signaling pathway mediated by Keap1 (Wu et al.). Supplementation of dandelion tannins or isoflavones of soy bean positively impacted the growth index, serum biochemical indexes, and antioxidant rates and accelerated the cecal microbiota abundance and overall intestinal health of female Wenchang chickens (Li X. et al.). Apart from the plant sources, bioactive compounds from yeast derivatives-supplemented feeds have the potential to be used as antibiotics, immunogenic factors, pathogen inhibitors, and intestinal health and growth promoters in livestock and poultry (Patterson et al.). Physical attributes such as thermal manipulation (TM) during incubation promoted alterations in embryonic development, hepatic amino acid metabolism, and hatching results in layer-type chickens (Han et al.).

Herbal supplementation to improve the overall growth and immune response in livestock and *in vivo* models

Tannic acid-chelated zinc (TAZ) supplementation in the diet ameliorated PEDV-induced changes in new-born piglets. Malondialdehyde levels in the plasma duodenum, jejunum, and colon notably decreased; in parallel, TAZ relieved the oxidative stress and PEDV-induced damage in intestinal mucosa. Further, absorptive function and growth in piglets was improved, which suggested that TAZ can be a potential feed supplement for neonatal and weaning piglets (Zhang Z. et al.). The high-fat diet induced obesity and affected the liver function, and the chronic HF diet also induced megalohepatia, steatosis, inflammation, and hepatocyte apoptosis in rats. The chronic effects of grape seed proanthocyanidin (GSPE) in the liver function and lipid

metabolic parameters were evaluated in rat models. The results advocated that GSPE stimulated the expression of the Wnt3a/β-catenin signaling pathway, which prevented endoplasmic stress and apoptosis in hepatocytes. In addition, microRNA-103 mediated a critical role in the signal transduction pathways. Therefore, it was suggestive that GSPE demonstrated a protective outcome on the liver and offered cues for value addition in animal feed. Further, GSPE downregulated the expression of the genes responsible for lipid synthesis, which prevented fat deposition in the liver (Sun et al.). The microbiota in the GI tract of livestock play a significant role in maintaining the intestinal health and the associated digestive processes. The hydrogen sequestration capacity of the archaea has been utilized in feed formulations to reduce methane emission and energy loss. Supplementation of green additive *Scutellaria baicalensis* and *Lonicera japonica* mixed extracts (SLE) in wheat bran improved the lactation, immunity, and production performance of piglets through a transmission effect. Elevated levels of glucose (GLU), triglyceride (TG), total cholesterol (TC), prolactin (PRL), and interleukin-10 (IL-10) were noted. Fat, IgA, and IgG in colostrum significantly increased post-supplementation in SLE diets (Wang L. et al.). *Lactobacillus plantarum* and *Pediococcus acidilactici* fermented feeds along with the basal diet enhanced weight gain, fecal acetate, and butyrate; moreover, it increased the abundance of short-chain fatty acid (SCFA)-producing microbiota in the gut of nursery pigs (Yang et al.).

Role of metabolomics and molecular signatures in profiling of GI microbiota

The fortification of pet feeds with polyphenol-rich pre-biotic fiber blend improved the microbial saccharolytic and post-biotic metabolism, improved the stool quality, and decreased digestion-related disorders in pet dogs. Further, it was found to be effective in the early stages of the feed routine and to aid in the management of gastroenteritis (Fritsch et al.). The elaborative microbiome and metabolomic profiling threw light on the contribution of dietary fiber in maintaining the adult dog gastrointestinal health status.

Archaea species such as *Methano massiliicoccales* in beef cattle, *Methano brevibacter* in sheep, and *Methanobrevibacter smithii* have been reported to be involved in feed utilization. In context with the above cues, the composition and expression activity of the gut archaea were analyzed on a metagenomic and transcriptomic platform. The archaeal population was reported to be relatively low in the digestive tract; however, their transcript levels were extremely high. Hence, it was suggestive that the archaea were functional and active in regulating the GI health in monogastric animals (Peng et al.). The liver, the largest solid organ, performs vital metabolic functions and serves as a hub of several pathophysiological mechanisms. Understanding the non-coding RNAs (lncRNAs) and their roles in the lipid metabolism during growth and development was studied using rabbit models. The variations of lncRNA and mRNA transcriptomes in different stages of rabbit, from birth to somatic maturity, was evaluated using the RNA-seq bioinformatics platform. As a result, 38 differentially expressed mRNAs such as ACSS2 and 215 lncRNAs such as MSTRG.30424.1 were identified, which can be used as biomarkers to understand the vital roles of the lipid metabolism (Wang G. et al.). The impact of fucoidan on the intestinal health, colon morphology, and cecal contents of weaned lambs was investigated. Apparently, fucoidan improved the levels of propionic acid and butyric acid and elevated the antioxidant levels and immunogenicity, while 16S rDNA screening showed increased levels of beneficial intestinal bacterial strains. Further, fucoidan decreased the diarrhea rate, which relaxed the weaning stress. Moreover, fucoidan as milk replacer supplementation improved the large intestinal health of weaned lambs (Guo et al.). One of the hallmarks of plant-based compounds is that they relieve oxidative stress due to the presence of tannins, phenolics, and many other compounds. These can be well utilized as a cofactor in augmenting diet-based formulations for pets and laboratory model poultry and livestock . However, to attain a clear understanding and investigation of their antioxidant mechanism, clinical and experimental trials must be thoroughly performed. The scaling-up of these dietary supplements with industry–institution connections, technology transfer, patentable ideas, and commercialization would rightly benefit stakeholders and promote sustainable livestock production.

Author contributions

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

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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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Archaea: An under-estimated kingdom in livestock animals

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Archaea are considered an essential group of gut microorganisms in both humans and animals. However, they have been neglected in previous studies, especially those involving non-ruminants. In this study, we re-analyzed published metagenomic and metatranscriptomic data sequenced from matched samples to explore the composition and the expression activity of gut archaea in ruminants (cattle and sheep) and monogastric animals (pig and chicken). Our results showed that the alpha and beta diversity of each host species, especially cattle and chickens, calculated from metagenomic and metatranscriptomic data were significantly different, suggesting that metatranscriptomic data better represent the functional status of archaea. We detected that the relative abundance of 17 (cattle), 7 (sheep), 20 (pig), and 2 (chicken) archaeal species were identified in the top 100 archaeal taxa when analyzing the metagenomic datasets, and these species were classified as the "active archaeal species" for each host species by comparison with corresponding metatranscriptomic data. For example, The expressive abundance in metatranscriptomic dataset of *Methanosphaera cuniculi* and *Methanosphaera stadtmanae* were 30- and 27-fold higher than that in metagenomic abundance, indicating their potentially important function in the pig gut. Here we aim to show the potential importance of archaea in the livestock digestive tract and encourage future research in this area, especially on the gut archaea of monogastric animals.

KEYWORDS

metatranscriptome, metagenome, ruminant, pig, chicken, monogastric animals, archaea

Introduction

Although archaea is a relatively new domain, they are considered one of the oldest organisms on Earth. Since being described as an independent domain by Woese and Fox (1), archaea have been detected in various extreme environments, including marine environments (i.e., oceans and freshwater) and various locations in the mammalian body (2, 3).

According to 16S rRNA sequences in the SILVA database, it is estimated that 20,000 archaeal species from about 30 phyla exist worldwide (4). In the Genome Taxonomy Database (GTDB) (5, 6), a relatively complete taxonomy database of archaea and bacteria, 2339 archaeal species clusters belonging to 19 phyla have been collected from different environments based on single genomes or metagenome-assembled genomes. However, over 70% have yet to be cultured (6).

The gastrointestinal microbial community is the largest and most important ecosystem contributing to the maintenance of intestinal health in mammals, and archaea constitute an essential part of the gut microbiota of mammals. In humans, which have attracted more research attention than other mammals, archaea were detected in almost all ecological niches (7). Chibani et al. (8) assembled 1,167 archaeal genomes using globally available metagenomic datasets from the human gut, including three genera and 15 species, and suggested continued research on human gut archaea is necessary. Recently, Youngblut et al. (2) investigated gut archaeal diversity in vertebrates using archaea-targeting 16S primers and successfully amplified and sequenced eligible archaeal reads from 110 vertebrate species. Youngblut et al. (2) identified six phyla and ten archaeal classes, including four new host-associated taxa. More than 60% of the ASVs (Amplicon Sequence Variants) derived from these taxa had no matched cultured representative (similarity ≥97%), and five classes had no reference sequence with a sequence similarity of 85%, suggesting that a large knowledge gap in vertebrate gut archaea exists. Their results further revealed that host phylogeny had a stronger influence on gut archaeal diversity than diet, and some specific taxa were associated with body temperature (*Methanothermobacter*) and feeding habits (*Methanomethylophilus*). This study by Youngblut and colleagues opens new research horizons on gut archaea in vertebrates.

In ruminants, methanogens, an important subgroup of archaea, are generally considered harmful due to methane production and host energy loss. However, we believe archaea's hydrogen-consuming abilities may benefit the host, as hydrogen inhibits rumen/gut fermentation. Results from Li and Guan (9) showed that *Methanomassiliicoccales* (an archaeal genus) was significantly enriched in the rumen of beef cattle with higher feed utilization. Additionally, in another study, they identified a positive relationship between several archaeal species (such as *Methanobrevibacter smithii*) and feed utilization (10). In sheep, McLoughlin et al. (11) identified three species belonging to *Methanobrevibacter* that were significantly associated with increased feed utilization. Our recent study showed that gut archaea have the functional potential to reduce hydrogen and are involved in carbohydrate metabolism by expressing CAZyme genes in pigs (12). Therefore, the function of archaea in the digestive system of livestock is likely important and should not be overlooked. In this study, we re-analyzed published metagenomic and metatranscriptomic data sequenced from matched individuals to explore the composition and expression activity of gut archaea, providing vital information over gut archaea in livestock. We hope to promote discussion on digestive archaea and encourage future research in this area.

Materials and methods

Data collection and pre-processing

A total of four datasets, containing both metagenomic and metatranscriptomic sequencing reads from the rumen of cattle ($n = 14$) (10) and sheep ($n = 10$) (13) and feces from chickens ($n = 6$) (14) and pigs ($n = 6$) (14), were collected from published articles. Raw reads quality control and host-contamination filtering were performed on these collected metagenomic and metatranscriptomic data using the Kneaddata pipeline v0.7.2 (https://bitbucket.org/biobakery/kneaddata). In brief, first, raw reads were trimmed with Trimmomatic v0.39 (15). Then, host-contamination reads were identified and removed by mapping raw reads to their corresponding host reference genomes [Accession number: GCF_002263795 (Cattle), GCF_002742125 (Sheep), GCF_016699485 (Chicken) and GCF_000003025 (Pig)] with the Bowtie2 software (16). Considering the higher expression of ribosomal RNA in metatranscriptomic dataset, SortMeRNA software (v4.3.2) (17) and SMR v4.3 sensitive database were used to remove potential ribosomal RNA sequences from both metagenomic and metatranscriptomic data, to reduce the interference in quantifying expression of archaeal taxa. Clean reads were acquired for further analysis after the abovementioned raw reads processing steps.

Archaeal taxonomy profiling and diversity calculation

For taxonomic classification of both metagenomic and metatranscriptomic data, Kraken2 version 2.1.2 (18) was used to assign clean reads to archaeal reference genomes from the Genome Taxonomy Database release 202 (GTDB 202) (19), which contained 4,316 archaeal genomes representing 2,339 archaeal species, and 245,090 bacterial genomes representing 45,555 bacterial species (Access date: October 13, 2021). The downloaded GTDB database was pre-built using the Struo2 pipeline (20). Subsequently, the metagenomic and metatranscriptomic clean reads were classified based on the GTDB database using Kraken2.

The quantitative table of archaeal and bacterial species in each sample were furtherly processed using QIIME2 platform version 2021.4 (21) to rarefy and calculate the relative abundance of bacteria and archaea in samples. A species-level rarefied archaeal reads count table was re-imported into QIIME2 to

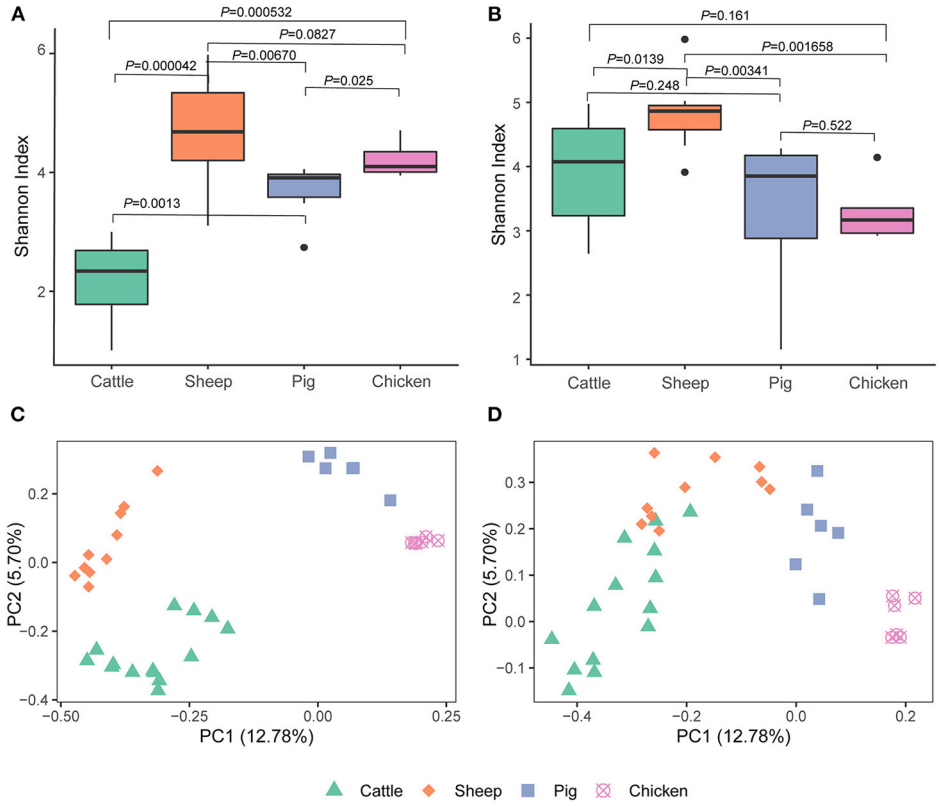

FIGURE 1
Alpha [Shannon index, **(A,B)**] and beta [Bray-Curtis, **(C,D)**] diversity of gut archaea based on the metagenome **(A,C)** and metatranscriptome **(B,D)**.

calculate archaea alpha diversity (Shannon Index) and beta diversity (Bray-Curtis).

Kruskal-Wallis test and Analysis of Similarities (ANOSIM) were performed using the QIIME2 platform. For all analyses, statistical significance was determined at $P \leq 0.05$. All figures were generated using the R package, ggplot2 (22).

Results and discussion

Alpha and beta diversity

We detected differences in alpha diversity (Shannon index) among the four livestock species. Based on the metagenomic analysis, the Shannon index for cattle was significantly lower than that of sheep (cattle vs. sheep, $P = 0.000042$), pigs (cattle vs. pig, $P = 0.0013$), and chickens (cattle vs. chicken, $P = 0.00053$), while pairwise comparisons among sheep, pigs, and chickens revealed that all pairwise comparisons were at $P > 0.01$ (sheep vs. pig, $P = 0.013$; sheep vs. chicken, $P = 0.083$; chicken vs. pig, $P = 0.025$; Figure 1A). This was not the expected result, as differential alpha diversity was observed between the ruminants, and the sheep alpha diversity was more similar to that of pigs and chickens. Further analysis based on metatranscriptomic data revealed that the Shannon index of cattle almost reached that of sheep (cattle vs. sheep, $P = 0.014$). The Shannon index reflects both archaeal species richness and evenness. The higher Shannon index observed in the cattle metatranscriptome compared to the metagenome indicated that the archaeal species present had high expression activity in the cattle rumen. Additionally, no significant difference was observed between pigs ($P = 0.248$) and chickens ($P = 0.161$; Figure 1B). Moreover, the Shannon index of sheep was significantly higher than that of pigs ($P = 0.0034$) and chickens ($P = 0.0017$). No difference between chicken and pigs ($P = 0.522$) was observed.

A metagenome- and metatranscriptome- based comparison among three different cattle breeds by Li et al. (10), the results showed that a greater difference was observed between breeds at the metatranscriptomic level compared to the metagenomic level. Here, we compared the archaeal structure in livestock using both of metagenomic and metatranscriptomic sequencing data to explore the effects of the sequencing technologies on the archaeal structure. The Bray-Curtis-based PCoA plot produced using metagenomic data showed that the four livestock species were separated from each other (ANOSIM, $P < 0.05$; Figure 1C, Supplementary Table 1). Cattle and sheep were close to each other but still separate ($P < 0.05$) and were further separated

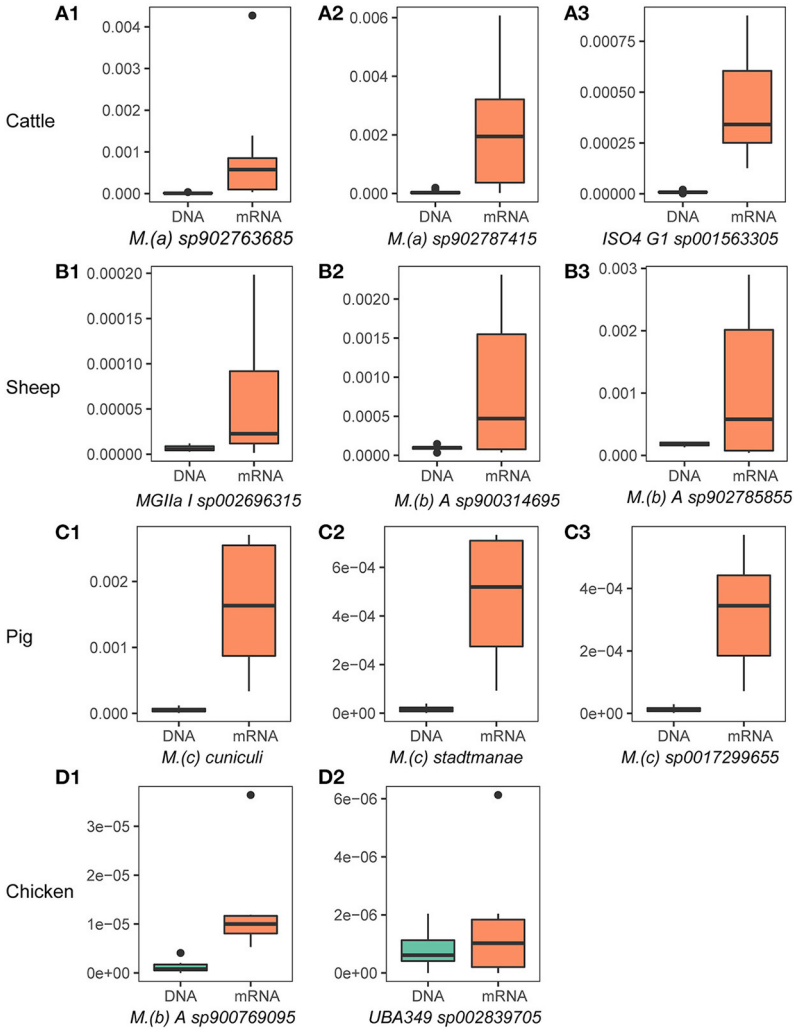

FIGURE 2
Highly active archaeal species in the rumen/gut of cattle **(A1–A3)**, sheep **(B1–B3)**, pigs **(C1–C3)**, and chickens **(D1,D2)**. The y-axis represents species' relative abundance in archaea and bacteria, and labels of DNA and mRNA on the x-axis label represent the relative abundance from metagenome and metatranscriptome, respectively. The x-axis titles are the name of archaeal species, name abbreviations of genus showed in the figure are as follows: *M.(a)*: *Methanomethylophilus*, *M.(b)*: *Methanobrevibacter*, *M.(c)*: *Methanosphaera*. ISO4 G1, MGIIa I, and *UBA349* are genus names or ids defined in the GTDB.

from the chicken and pig clusters in the PCoA plot using metatranscriptomic dataset (Figure 1D, Supplementary Table 2).

Disproportionally expressed archaeal species in the four livestock animals

We further determined the "active archaeal species" by comparing the relative abundance of archaeal species in both the metagenome and metatranscriptome. We considered species to be "active archaeal species" if they (1) were observed at least in 80% of samples and (2) the mRNA relative abundance was at least 2-fold higher than the DNA relative abundance. In total, 17, 7, 20, and 2 archaeal species were identified from the top 100 relatively abundant archaeal taxa in the metagenomic dataset as "active archaeal species" for cattle, sheep, pig, and chicken, respectively (Supplementary Table 3). Figure 2 shows the highly active species identified in each host species. Of them, 6 of the 17 "active archaeal species" in cattle belong to the genus *Methanomethylophilus*, 4 of the 7 "active archaeal species" in sheep belong to genus *Methanobrevibacter*, 12 of the 20 "active archaeal species" in pig belong to the genus *Methanosphaera*, and for chicken, only two "active archaeal species" annotated as *Methanobrevibacter A sp900769095* and *UBA349 sp002839705*, suggesting archaeal taxa-specific expression activity signatures for different host species. To our surprise, archaeal species in the pig showed high expression activity. For instance, both *Methanosphaera cuniculi*

(Figure 2C1) and *Methanosphaera stadtmanae* (Figure 2C2) are known as hydrogen-consuming archaea and are commonly detected in the pig gut (23), and to the best of our knowledge, no study reports solid evidence of their function. Higher expression activity (30-fold expression for *Methanosphaera cuniculi* and 27-fold expression for *Methanosphaera stadtmanae*) may indicate their important function in swine. However, they have been widely ignored in past studies due to the lower abundance of archaea.

It is thought that some archaeal taxa, especially methane-producing archaea, are key species that may affect the composition and function of the microbiota in complex host and non-host environments (24–26). In humans, some archaea, such as trimethylamine N-oxide (TMAO)-reducing archaeal species, have been believed to be potential probiotic candidates (27), while others have been linked to human diseases, such as cancer and obesity (28, 29). Taken together, accumulative evidence suggests that archaea may play important roles in host health and disease. However, the function of archaea is still greatly unknown in livestock. Some studies have focused on the negative effects of methane-producing archaea in the rumen. In addition, others have focused on the association between archaeal taxa and animal growth, identifying a link between archaeal taxa in the rumen and feed utilization (10, 11). Our recent study showed that gut archaea might promote the energy harvest in pigs due to their involvement in gut fermentation, indicating archaea may affect swine growth (12).

Samuel et al. (30) found that methanogenic archaea could reduce hydrogen, increasing host energy harvest and fat deposition in the mouse model; however, it did not seem to entirely explain the effects of methanogenic archaea on the host. Our previous study based on shotgun metagenomic data from pigs suggested that gut archaea may be directly involved in gut fermentation by expressing CAZyme genes, indicating another potential function of archaea in the gut. Archaea coexist with bacteria and fungi in different complex microbiomes. However, archaea-bacteria and archaea-fungi interactions are still poorly understood and could be another important function of archaea (31).

Until now, we have little evidence to speculate on the potential links between archaea in the digestive system and livestock growth performance. Therefore, more studies are needed to investigate the roles that archaea play in animal production.

Conclusion

Archaea are an important component of the complex microbial ecosystem in the digestive tract of humans and animals. This study compared the effects of the metagenome and metatranscriptome on archaeal diversity and composition and revealed the difference between archaeal composition (metagenome) and expression (metatranscriptome). In addition, although the abundance of archaea in the digestive tract is relatively low, we found that the transcripts of several archaeal species are extremely high (≥2-fold), suggesting these archaea are very active and functioning in animals especially in the less-studied monogastric animals.

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 authors.

Author contributions

YP and FD contributed to analysis, interpretation, and drafted the manuscript. YP, TX, and JC contributed to data analysis. ZW, WZ, and TZ contributed to data collection. YP, SH, JZ, and YL contributed to critically revised the manuscript. JZ and YL contributed to conception. All authors gave final approval and agreed to be accountable for all aspects of the work.

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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.2022.973508/full#supplementary-material

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The association of Curcuma and Scutellaria plant extracts improves laying hen thermal tolerance and egg oxidative stability and quality under heat stress conditions

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Chronic exposure to high ambient temperatures is detrimental to laying hen performance and egg quality. Plant secondary metabolites may alleviate effects, partly due to their antioxidant activities. Herein, we investigated the effects of dietary supplementation with a phytonutrient solution (PHYTO) consisting of a plant extract combination of *Scutellaria baicalensis* and *Curcuma longa* on young layers (25–32 wk of age) raised under naturally elevated temperature conditions. Four hundred, 24-wk-old Lohmann hens were allocated in 50 cages and, after a week of adaptation, were offered a diet either containing 2 g/kg of PHYTO or not, for 8 wk. Hen BW was measured at the beginning and end of the trial, and egg production and feed intake were recorded weekly. At week 32, four eggs per cage were collected to determine egg quality characteristics as well as the rate of lipid and albumen oxidation in fresh eggs. At the end of the trial, two hens per cage were blood sampled for assessment of biochemical markers, one of which was euthanized for histopathological evaluation of the liver and intestine and assessment of intestinal histomorphometry. The herbal mixture supplementation significantly increased egg production rate at weeks 28 and 29 and for the overall production period, and feed efficiency at weeks 26–29. In addition, the degree of liver necrosis and microvascular thrombosis was lower ($P < 0.05$) whereas intestinal villosity was greater in duodenal and jejunal segments ($P < 0.05$) in the PHYTO compared to the control group. Supplementation also reduced ($P < 0.05$) blood concentrations of corticosterone, alanine aminotransferase activity, and TBARS, and a reduction in catalase activity was observed.

Egg quality characteristics were not affected, except for eggshell thickness, egg diameter, and eggshell breaking strength that were superior in the PHYTO group ($P < 0.05$). PHYTO supplementation significantly improved egg lipid oxidation status of fresh eggs. In conclusion, supplementation with PHYTO improved laying hen productivity and egg quality, which was associated with an improvement in laying hen thermotolerance.

KEYWORDS

laying hen, heat stress, Curcuma and Scutellaria, egg quality, liver evaluation

Introduction

In high-temperature areas, the occurrence of heat stress (HS) is one of the most important stressors for poultry, causing extensive economic losses, while challenging their health and welfare (1). Moreover, the effects of HS are expected to become more prominent due to climate change, which drives an increase in global temperatures characterized by heat waves of increasing intensity, duration, and frequency (2). Laying hens are particularly vulnerable to HS as they have a limited capacity to maintain the homeostasis of body temperature due to their lack of sweat glands and their excessive feather coverage (3), while they have a long production cycle (50–70 weeks) and are thus susceptible to its long-term effects (4).

When faced with HS, a culmination of behavioral, hormonal, immunological, physiological, metabolic, and biochemical changes leads to reductions in average daily feed intake (ADFI), egg production, feed efficiency, and egg quality (4, 5). A meta-analysis has shown that ambient temperatures above 24°C hens may impair performance and lead to production of eggs of inferior quality (5). Prevailing temperatures in the Mediterranean basin rest above these estimates for extended periods of time. Furthermore, poultry facilities are often not equipped with modern ventilation technologies to efficiently control in-house temperatures (6, 7).

One of the hallmarks of HS is the occurrence of oxidative stress as it directly increases mitochondrial energy generation causing an imbalance between pro-oxidant and antioxidant systems, defined by the presence of reactive species (RS), in excess of the available antioxidant capacity of animal cells (8). Increased production of RS leads to an impairment of mitochondrial function and damages proteins, lipids, and DNA (9, 10) Importantly, the shift of visceral blood flow toward the peripheral circulation during HS to facilitate heat dissipation induces hypoxic conditions in the intestine and renders it particularly susceptible to oxidative stress. Oxidative damage is not limited to the gastrointestinal tract but can also occur in multiple organs (11) and may lead to acute liver injury (12). These effects may be reflected in blood and in egg yolk or albumen by increased concentration of lipid and protein oxidation products.

Certain plant secondary metabolites (PSMs) contained in plant extracts may mitigate the adverse effects of HS on laying hen performance. Recent studies have demonstrated that dietary supplementation with curcuminoids contained in *Curcuma longa* (CUR) improved laying hen thermotolerance by upregulating antioxidant defenses (13), inhibiting the pro-inflammatory cytokine production and improving humoral immunity, while increasing steroidogenesis (14) and exhibiting hepatoprotective effects (13). Furthermore, curcumin supplementation reduced lipid peroxidation levels in egg yolk of laying hens raised in thermoneutral conditions (15). On the contrary, *Scutellaria baicalensis* (SCUT) and its active metabolites baicalin and baicalein have been shown to exhibit potent anti-inflammatory effects in poultry (16) and improve egg oxidative status (17), although research in laying hens exposed to HS is currently lacking. Previously, offering CUR extract alone was not enough to decrease gut inflammation induced by HS in broiler chicks. However, dietary supplementation with a phytonutrient solution (PHYTO) consisting of a mixture of CUR and SCUT plant extracts decreased gut inflammation induced by HS or S. Enteritidis infection (18), pointing toward synergistic effects between their active metabolites.

The aim of this study was to ascertain the effects of dietary supplementation of PHYTO in young laying hens around the peak of lay (25–32 weeks of age) raised under naturally elevated temperature conditions. We hypothesized that supplementation would lead to improved performance due to reduced liver and intestinal stress, as assessed by liver histopathological and intestinal histomorphometrical and histomorphological analysis, respectively, and effects would be associated with improved hen systemic antioxidant status, egg quality, and egg oxidation status.

Materials and methods

Animals, diets, and experimental design

The trial protocol was authorized by the Research Committee of Aristotle University, Thessaloniki, Greece (number 71553, 28.07.2020). Husbandry, euthanasia, experimental procedures, and biosecurity precautions were conducted in accordance with all welfare requirements described by Good Farming Practice Guidelines (Directive 2010/63/EC; Commission recommendation 2007/526/EC) and were approved by the Research Ethics Committee of the Aristotle University of Thessaloniki. Throughout the trial, birds were handled in compliance with local laws and regulations (19) and in accordance with the principles and guidelines for poultry welfare (20). Four hundred Lohmann Brown-Classic laying hens (24-week-old), kept at the poultry farm of the International Hellenic University (IHU, Sindos, Thessaloniki, Greece; 40°39′ N, 22°48′ E), were used in this study. Birds were housed in 50 replicate furnished cages (length 0.8 m; width 0.4 m; height 0.3 m) of eight hens each and were offered a commercial corn and soybean meal-based feed in mash form formulated to meet or exceed the requirements for nutrients and energy content for laying hens (21) (Table 1). From weeks 25 to 32, birds were either maintained in the same feed (Control) or offered the same diet supplemented with a commercial feed additive product (Phyto) consisting of an extract from *C. longa* (CUR) and hydrosoluble flavonoid extract from *S. baicalensis* (SCUT), added at the rate of 0.2 (%) diet replacing an equivalent amount of wheat bran, as described in a previously published study (18). The PHYTO product was pre-mixed with calcium carbonate as a carrier prior to its incorporation to the diets, and constituent plant extracts of CUR and SCUT were included at a 1:1 ratio. The principal bio-active metabolites of the extracts used are baicalin and curcumin for SCUT and CUR, respectively. The dose tested of the plant extract combination was based on the results of a previously published study where supplementation was effectively shown to decrease gut inflammation induced by heat stress or *S. enteritidis* infection, in broiler chicken (18). Experimental diets were formulated to meet or exceed the requirements for nutrients and energy content for laying hens (21). Birds had *ad libitum* access to feed and water. The lighting program was set at 16 h of continuous light per day.

TABLE 1 Ingredients and composition of the control layer diet.

Ingredients	Composition (g/kg)
Maize, grains	552.0
Wheat, grains	50.0
Soybean meal	240.0
Wheat bran	33.0
Soy oil	5.0
Limestone	95.0
Monocalcium phosphate	10.5
DL-Methionine	3.2
Lysine	1.3
Threonine	0.5
Valine	1.0
Sodium chloride, iodized	2.3
Sodium bicarbonate	2.2
Vitamin premix[a]	1.5
Trace-mineral premix[b]	1.5
Total	1000.0
Calculated analysis[c]	**(g/kg)**
Dry matter	883.9
Crude protein	167
Ether extract	27
Crude fiber	33
Ash	84.1
Calcium	36.5
Phosphorus (total)	6.5
Metabolizable energy (MJ/kg)	11.6

[a]Supplying per kg feed: 4.82 mg all-trans retinol acetate, 62.5 μg cholecalciferol, 30 mg α-tocopheryl acetate, 2 mg menadione sodium bisulfite, 2 mg thiamine hydrochloride, 3 mg riboflavin, 4 mg pyridoxine hydrochloride, 0.02 mg cyanocobalamin, 20 mg niacin, 10 mg pantothenic acid, 1.0 mg folic acid, 0.07 mg biotin, 50 mg ascorbic acid, 300 mg choline chloride, and 40 mg carotenoids.
[b]Supplying per kg feed: 80 mg Zn, 40 mg Mn, 160 mg Fe, 70 mg Cu, 0.25 mg Co, 1 mg I, and 0.2 mg Se.
[c]According to NRC (21).

Ambient conditions

The experiment was conducted between mid-July and mid-September, when the temperature is substantially high during typical Mediterranean summer temperate conditions, in a poultry house without modern ventilation systems. In-house temperatures were recorded during the morning (9:00), local noon (13:00), and evening hours (17:00). Regional temperature data are presented for the period of the trial, and total hours per day with in-house ambient temperatures exceeding 28°C are presented in Figures 1, 2, respectively.

Determination of the total phenolic content of diets, additives, and egg yolks

PHYTO supplemented diets were analyzed for their total phenolic content (TPC) according to the method of Singleton et al. (22) and expressed as gallic acid equivalents (GAE) mg/g, as determined by using the Folin–Ciocalteu assay. Extraction of phenolics from egg yolks and determination of the total phenolic content were carried out according to the protocol of Shang et al. (23). For the extraction of phenolic compounds,

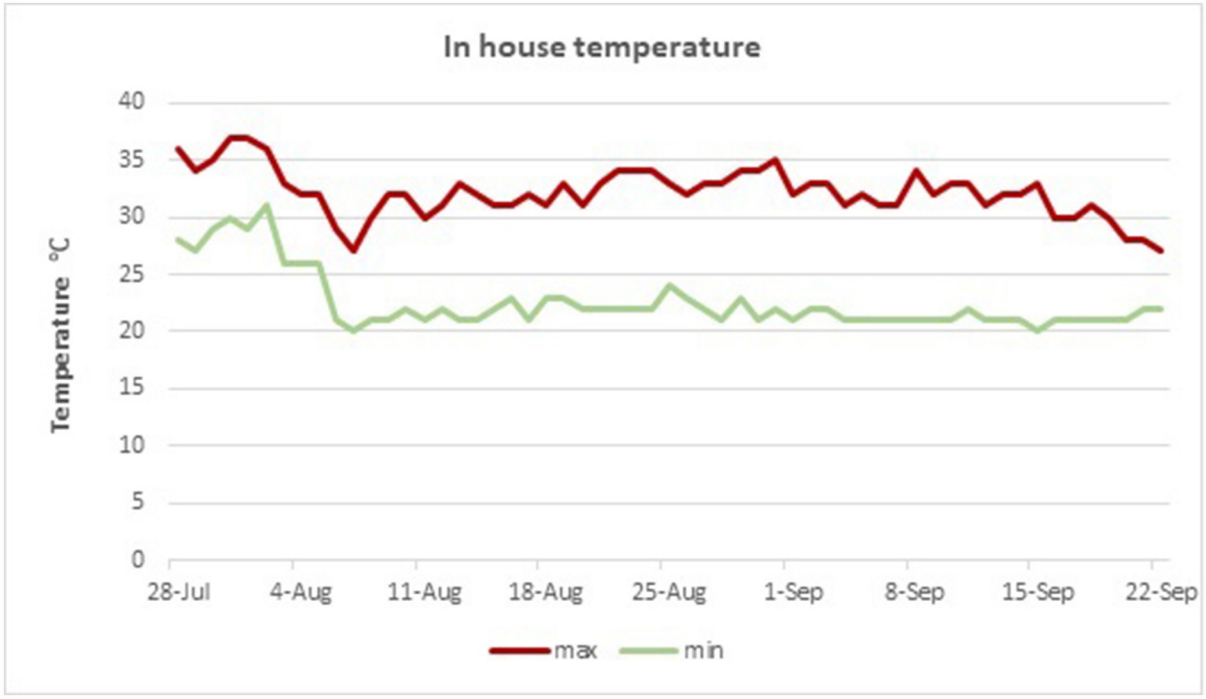

FIGURE 1
Measured in house minimum and maximum temperatures during the experimental period.

the yolks were separated from the albumen and homogenized. About 8 mL of 50% aqueous methanol [MeOH(aq) 50%] was added to 2 g of homogenized egg yolk, and the mixture was vortexed and then centrifuged at room temperature (3,000 g, 20 min). The supernatant was collected, and 4 mL of it was added to 400 μl TCA 10% w/v. After a second centrifugation (3,000 g, 20 min), the supernatant was once again collected. For the determination of the total phenolic content, 125 μl of egg yolk extract was added to test tubes containing 125 μl of Folin–Ciocalteu reagent and 2.25 ml of Na_2CO3 7%. The samples were left in the dark for 30 min, at room temperature, and their absorbance was measured afterward, at $\lambda = 725$ nm. The results were expressed as μg of gallic acid equivalents/ mL of extract (μg GAE/mL extract).

Hen performance

Hens were weighed prior to offering the experimental diets and at the end of the trial. Egg production and feed intake were recorded weekly. To provide an overall estimation of feed efficiency during the experimental period, we calculated the average of the egg mass produced by dividing the sum of egg mass at the start and at the end of the experimental period. Then, we calculated the biweekly feed: egg weight ratio for each cage using weekly measured feed intake data. The estimated feed: egg weight ratio is expressed as g of feed per g of eggs produced according to Papadopoulos et al. (24).

Blood sampling and biochemical analysis

Two hens per cage were randomly selected at the end of the experimental period for blood sampling *via* the brachial vein in tubes without anticoagulant (BD Vacutainer® Plymouth, UK) to obtain serum. The samples were allowed to clot for 4–5 h at 4°C, which were centrifuged at 3,000 RPM, for 15 min (HERMLE, Wehingen, Germany), and the sera were collected and stored in −20°C pending analysis photometrically (Siemens ADVIA 1800 Chemistry System, Erlangen, Germany). Serum samples were tested for antioxidant enzymes, namely alkaline phosphatase (ALP), alanine transaminase (ALT), aspartate transaminase (AST), gamma-glutamyltransferase (γ-GT), and corticosterone (CORT) to estimate the effect of PHYTO supplementation on liver function. Antioxidant enzyme production was assessed by measuring superoxide dismutase (SOD), glutathione peroxidase (GPx), and catalase (CAT) to assess endogenous antioxidant enzyme production by ELISA Kits (Siemens Healthcare GmbH, Erlangen, Germany). Thiobarbituric acid-reactive substance (TBARS) was assessed as a marker of hen oxidative status and corticosterone as a general marker of heat stress by the methodology of Ahn et al. (25).

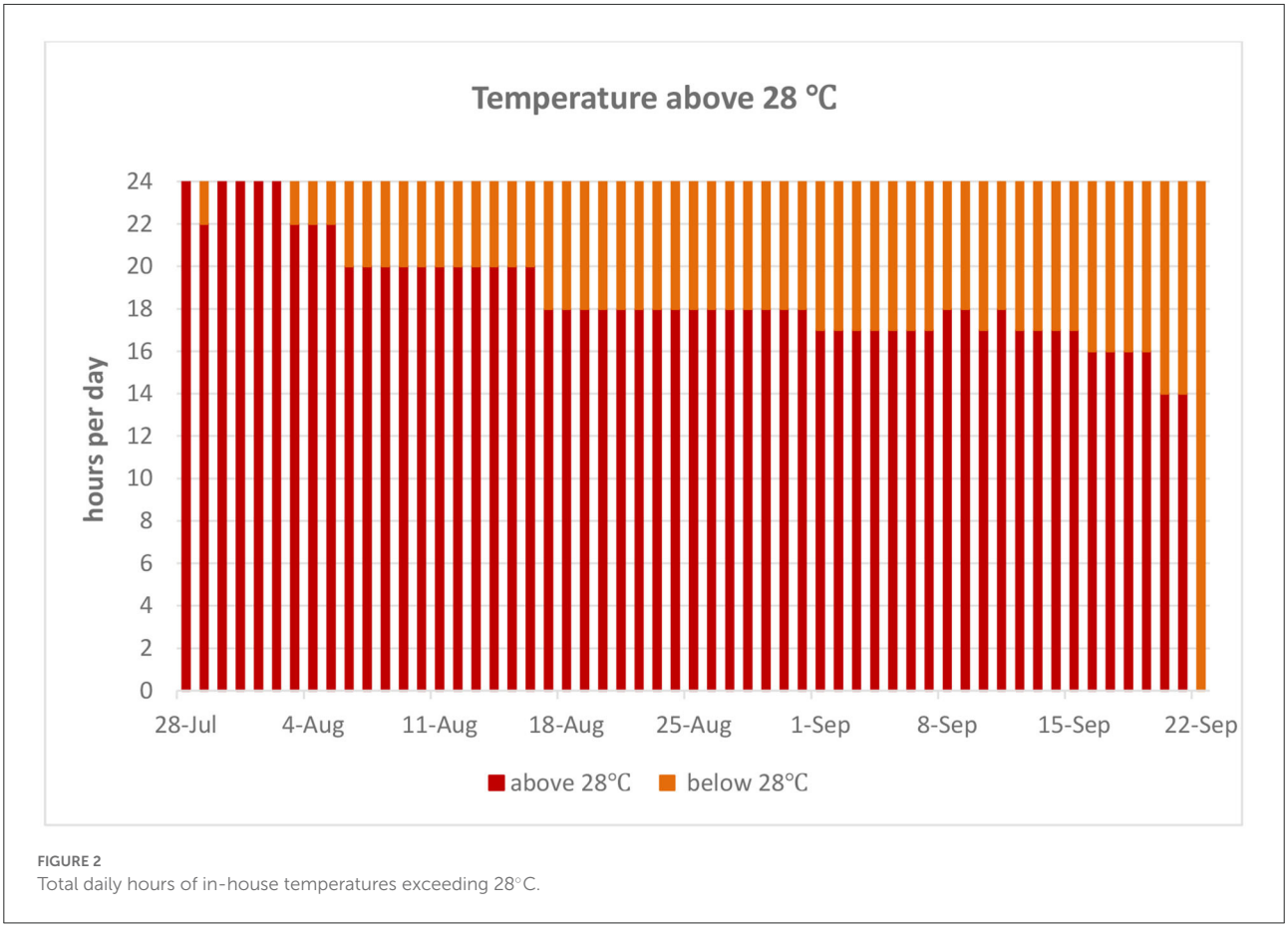

FIGURE 2
Total daily hours of in-house temperatures exceeding 28°C.

Evaluation of liver histopathology and small intestine histomorphometry and histomorphology

One out of two selected hens per cage selected for blood sampling was euthanized at the end of the trial for evaluating liver macroscopic and microscopic observations of HS-related lesions and intestinal histomorphometrical alterations. The liver was dissected and was directly evaluated macroscopically. Then, samples were collected from each liver, fixed in a 10% formalin solution, and embedded in paraffin. Section 4 μm were taken and stained with hematoxylin and eosin (H&E) for histopathological analysis. They were also stained with Martius Scarlet Blue trichrome staining (MSB) for fibrin visualization. At a microscopic level, liver samples were examined for alterations caused by thermal stress, which are related to the presence of hemorrhagic necrosis, microvascular thrombosis, and hepatocellular necrosis (26, 27). Closed intestinal samples were obtained from the duodenum, jejunum (before Meckel's diverticulum), and ileum, submitted to the hemicylindrical section, and were fixed in 10% formaldehyde. From each sample, section 4 μm were taken, routinely processed, and stained with H&E for morphological and morphometrical analysis, using light microscopy, according to the criteria of Gava et al. (28). Only intact villi were measured for villus height (VH) and crypt depth (CD). The measurements were performed with a Nikon microscope (Nikon Eclipse 200, Tokyo, Japan) coupled with a computer-assisted digital image analysis software (Image-Pro Plus, 2017). For the small intestine's morphological evaluation, the Chiu/Park scale was applied (29).

Egg quality

To determine egg quality indices, four eggs from each replicate (100 eggs per group) were collected during the first and last day of the trial (8th week). Egg quality indices were assessed at the beginning of the trial as a proxy to certify that there were no potential biases related to the level of oxidative stress experienced by the hens prior to their allocation to the treatments. All eggs were weighted using a balance (Navigator TM, N2B110, OHAUS Corporation, city, country). Egg length and width were measured with a digital caliper (EMC, LTD) of 0.01 mm accuracy, while egg shape index was calculated using the formula: shape index = (width/length) × 100. Eggshell color was measured with a reflectometer (EQ Reflectometer, York

Electronics Centre), while egg-specific gravity was calculated using the method based on Archimedes' principle. Eggshell deformation was determined by performing a compression test with Texture Analyzer (TA.HD. plus Texture Analyzer, Stable Micro Systems Ltd, Surrey, UK). Eggs were placed horizontally and compressed on the equator under a force of 500 g for 10 s. The distance by which the eggshell deformed was recorded. Eggshell breaking force was also determined with Texture Analyzer (TA.HD. plus Texture Analyzer, Stable Micro Systems Ltd, Surrey, UK) on the equatorial region with a compression platen. The peak force ($kg{\cdot}m/s^2$) on the force–time graph was recorded as the breaking force. Haugh units were measured by using designated equipment by the EQM York Electronics Centre (Egg Quality Microprocessor, Technical Services & Supplies Ltd., Dunnington, York, UK). Egg yolk was separated from the albumen and weighted on a balance (Navigator TM, N2B110, OHAUS Corporation, Nanikon, Switzerland). Yolk color was scored visually by using the Yolk Color Fan® scale and measured instrumentally with Chroma Meter CR-410 (Konica Minolta, Osaka, Japan) using the L*a*b* color space. Subsequently, the eggshell was washed to remove the adhering albumen and air-dried. The thickness of the eggshell with the membranes was measured with a caliper (AMES, Waltham, MA, USA, accuracy 0.001 in), while its weight was measured using a balance (Navigator TM, N2B110, OHAUS Corporation, Parsippany, NJ, USA). Albumen weight was calculated by subtracting the weights of egg yolk and shell from the weight of the egg.

Egg yolk oxidative stability of fresh eggs

Eggs collected at the end of the trial were delivered fresh to the Laboratory of Nutrition, School of Veterinary Medicine, Aristotle University, in a cool box at 4°C immediately after collection and tested upon arrival. To determine the oxidative stability of egg yolk, TBARS was measured based on malondialdehyde content, a secondary lipid oxidation product formed by hydrolysis of lipid hydroperoxides, according to the method of Ahn et al. (25). Absorbance was read at 530 nm against a blank sample using an UV-Visible spectrophotometer (UV-1700 PharmaSpec, Shimadzu, Japan). Protein carbonyl determination was Image-Pro Plus, Rockville, USA. Briefly, 50 μL of 20% TCA was added to 50 μL of egg albumen homogenate (diluted 1:2), and this mixture was incubated in an ice bath for 15 min and centrifuged at 15,000 g for 5 min at 4°C. The supernatant was discarded, and 500 μL of 10 mmol/L 2,4-dinitrophenylhydrazine (DNPH; in 2.5 N HCL) for the sample (500 μL of 2.5 N HCL for the blank) was added in the pellet. Calculation of protein carbonyl concentration was based on the molar extinction coefficient of DNPH (22×103 M^{-1} cm^{-1}).

Statistical analysis

Data were analyzed using the IBM SPSS Statistics Ver.25 software package (SPSS 25.0 Version, Chicago, IL, USA). Cage was considered as the experimental unit for all data. Statistical significance was considered at $P < 0.05$ and tendency at $0.05 < P < 0.1$. The results are presented as mean ± standard error of the mean (pooled SEM) unless stated otherwise. Daily performance data were used to calculate ADFI and egg-laying production biweekly and for the overall production period. The estimated feed: egg weight ratio was calculated for the same intervals. Performance data were analyzed with one-way ANOVA of the general linear model. Egg quality data obtained at the start and at the end of the experimental period (1st and 8th week) and serum biochemical measurements were analyzed with one-way ANOVA of the general linear models. Before statistical analysis, Levene's test was applied to test the homogeneity of the variances. Histomorphometrical measurements were compared between treatments using the Mann–Whitney test with the GraphPad Prism software (version 9.1.2 for Windows®, GraphPad Software, San Diego, CA, USA). Chi-square test was also performed to investigate the effects on the degree of hepatocellular necrosis, hemorrhagic lesions, and microvascular thrombosis.

Results

Determination of the total phenolic content in diets and eggs

The results of the TPC analysis showed that the diet of the control group contained 4,305 mg GAE/g dry mass. Accordingly, the PHYTO diet contained 16.384 mg GAE/g dry mass. The PHYTO product contained 181.2 mg TCP as far as the TPC of egg yolk is concerned, and samples from treated hens showed a substantial increase in the PHYTO group vs. the control group (123.077 μg GAE/mL extract vs. 75.846 ± 9.321 μg GAE/mL extract).

Hen performance

The effects of the dietary supplementation with PHYTO on laying hen performance parameters are shown in Table 2. Egg production (%) was significantly increased ($P < 0.05$) by PHYTO supplementation during weeks 28–29 and for the overall experimental period and remained within expected levels for the birds' age. ADFI of the PHYTO group was significantly decreased during weeks 26–27 and 28–29 of the trial ($P = 0.003$ and $P = 0.010$, respectively), while it tended to be lower during the overall period ($P = 0.061$). PHYTO supplementation

TABLE 2 Effect of dietary supplementation with PHYTO on laying hen performance.

	Groups[1]			
Egg production, %	**Control**	**PHYTO**	**SEM[2]**	***P***
Week 24 (adaptation)	86.4	87.0	0.734	0.680
Weeks 25–26	91.3	92.7	0.601	0.225
Weeks 27–28	86.4[a]	91.5[b]	0.796	**<0.001**
Weeks 29–30	86.6	89.4	0.916	0.145
Weeks 31–32	81.0	84.0	1.500	0.161
Total (25–32 w)	86.8[a]	89.0[b]	0.343	**<0.001**
Average daily feed intake, g				
Week 24 (adaptation)	107.46	106.36	0.349	0.118
Weeks 25–26	112.48[b]	110.34[a]	0.377	**0.003**
Weeks 27–28	114.81[b]	112.66[a]	0.426	**0.010**
Weeks 29–30	115.64	115.26	0.300	0.530
Weeks 31–32	118.26	118.08	0.333	0.791
Total (25–32 w)	113.73[x]	112.54[y]	0.160	0.061
Feed: Egg weight ratio				
Week 24 (adaptation)	2.00	1.93	0.020	0.087
Weeks 25–26	2.10	2.00	0.020	**0.024**
Weeks 27–28	2.14	2.05	0.023	**0.043**
Weeks 29–30	2.16	2.10	0.021	0.167
Weeks 31–32	2.20	2.15	0.022	0.197
Total (25–32 w)	2.10	2.04	0.020	0.120
BW (kg)				
Week 25	1.865	1.864	4.846	0.866
Week 32	1.978	1.981	7.775	0.851

[a,b]Values in the same row with different superscripts differ significantly ($P \leq 0.05$).
[x,y]Values in the same row with different superscripts tend to differ ($0.05 < P \leq 0.10$).
[1]Groups of layer hens fed the control or the supplemented diet; [2]SEM, standard error of the mean.

resulted in a numerically lower estimated feed: egg weight ratio, at weeks 26–27 ($P = 0.024$) and 28–29 ($P = 0.043$), compared to the control treatment. Hen BW did not differ significantly, either at the start or at the end of the trial between treatment groups.

Blood markers

The effects of the dietary supplementation with PHYTO are presented in Table 3. The level of TBARS, CAT, and ALT was significantly lower in the PHYTO supplemented group than the control one ($P = 0.021$, $P < 0.001$, and $P = 0.014$, respectively). Furthermore, PHYTO supplemented hens showed significantly lower circulating corticosterone levels ($P < 0.001$). On the contrary, the levels of SOD, GPx, AST, and gGT were similar between groups ($P > 0.05$).

TABLE 3 Effect of dietary supplementation with PHYTO on serum biochemical parameters measured in two hens per replicate cage ($n = 50$ per group) at the end of the experimental period (week 32).

	Groups[1]			
Parameter	**Control**	**Phyto**	**SEM[2]**	***P***
ALP (IU/L)	271.7	344.8	51.58	0.182
ALT (IU/L)	10.6[b]	7.8[a]	0.52	**0.014**
AST (IU/L)	486.1	368.6	47.41	0.366
γ-GT (IU/L)	15.6	15.1	0.886	0.683
TBARS (nmol/ml)	20.77[b]	13.49[a]	1.59	**0.021**
CAT (U/ml)	1.17[b]	0.54[a]	0.077	**<0.001**
SOD (U/ml)	1.36	1.32	0.040	0.645
GPx (nmol/ml)	0.191	0.197	0.015	0.847
Corticosterone μg/dL	0.97[b]	0.75[a]	0.031	**0.001**

ALB, albumin; ALP, alkaline phosphatase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; γ-GT, gamma-glutamyltransferase; TBARS, thiobarbituric acid-reactive substances; CAT, catalase; SOD, superoxide dismutase; GPx, glutathione peroxidase (GPx).
[a,b]Values in the same row with different superscripts differ significantly ($P \leq 0.05$).
[1]Groups of layer hens fed the control or the supplemented diet; [2]SEM, standard error of the mean.

Liver histopathology and small intestine histomorphometry and histomorphology

Liver evaluation (Figure 3) showed that both the incidence and severity of hepatocellular necrosis and microvascular thrombosis were reduced in the PHYTO group in comparison with the control group ($P = 0.034$ and $P = 0.015$, respectively; Table 4). The effects of PHYTO supplementation on intestinal histomorphometrical parameters are presented in Figure 4. VH was significantly higher in the duodenum, jejunum, and ileum in the group supplemented with the phytogenic additive compared to the control one ($P < 0.0001$, $P < 0.0001$, and $P < 0.01$, respectively). CD was also higher in the PHYTO group than the control, however, only in the duodenal part ($P < 0.01$). Mucosal damage was noticed in the duodenum of the control group compared to the PHYTO group. Based on the Chiu/Park scale, the damage was in the subepithelial space with moderate (degree 2) to the massive lifting of villi and partial tip denudation (degree 3). The intestinal segments of the jejunum and ileum were normal or with limited minor injuries (degree 1), and there were no differences among the groups (29, 30).

Egg quality and oxidative stability

Egg quality parameters measured in eggs collected at the start and at the end of the trial are presented in Tables 5A,B, respectively. There were no differences between groups on egg quality parameters prior to offering the experimental diets. A tendency for increased eggshell thickness ($P = 0.07$) and a

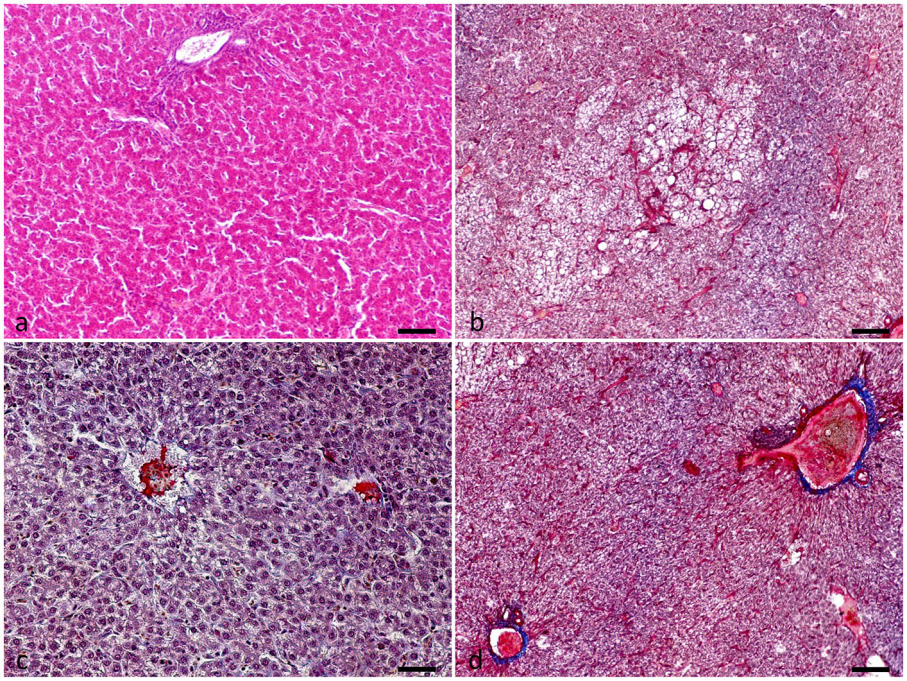

FIGURE 3
Liver evaluation of hens at the end of the experimental period (week 32). **(a)** Normal liver tissue, **(b–d)** Presence of intravascular organized fibrin consistent with the formation of thrombi, degeneration, and necrosis of hepatic cells with micro-hemorrhages. Hematoxylin and Eosin **(a)**. Martius-Scarlet-Blue **(b–d)**. Scale bar: 250 μm.

TABLE 4 Effect of dietary supplementation with PHYTO on hen liver evaluation at the 8th week of the trial.

	Groups*			
Liver evaluation	**Control**	**PHYTO**	**SEM**	**P**
Hepatocellular necrosis	1.48[b]	1.36[a]	0.107	**0.034**
Hemorrhagic lesions	0.96	0.60	0.144	0.370
Microvascular thrombosis	1.60[b]	0.84[a]	0.154	**0.015**

*N = one liver sample per replication (25 liver samples per group).
[a,b]Values in the same row with different superscripts differ significantly (P ≤ 0.05).
[1]Groups of layer hens fed the control or the supplemented diet; [2]SEM, standard error of the mean.

significant increase in resistance to breaking force ($P = 0.022$) and egg diameter ($P = 0.036$) were found for the PHYTO group, compared to the control group, whereas all other parameters remained unaffected ($P > 0.1$). As far as egg oxidative stability is concerned, egg white (albumen) oxidation expressed as protein carbonyls at the 8th week of the trial showed no difference between groups ($P > 0.1$). The egg yolk oxidation test showed that the eggs of the PHYTO group had lower TBARS values ($P = 0.001$) at the end of the experimental period ($P = 0.027$), whereas no differences were noted for eggs collected on the first day of experimentation ($P > 0.1$).

Discussion

In the present trial, we investigated the effects of supplementing phytonutrient solution consisting of a plant extract combination of Curcuma and Scutellaria in young laying hens raised in typical high ambient temperature conditions prevailing during the summer period in the Mediterranean basin. The temperature threshold at which HS occurs may vary as hen thermotolerance is affected by bird characteristics such as age, genotype, and level of productivity. In addition, HS parameters such as intensity, duration, whether it is cyclic or constant, and relative humidity levels define the magnitude of the effects on laying hen performance. Regardless, measured in-house temperatures exceeded 28°C which persisted for more than 8 h per day for the overall trial period; therefore, it is reasonable to state that hens were experiencing HS (31). Since the trial started in July, hens were already exposed to elevated temperatures typical of the Mediterranean climate prior to supplementation, although in-house temperatures were not recorded during that period. It has been clearly demonstrated that exposure to heat stress at peak production either cyclic or constant may lead to decreases in BW gain (32). Since layers did not lose BW between the start and the end of the trial, one can assume that they partially adapted to it (4), and/or the heat stress was of mild intensity (4, 32).

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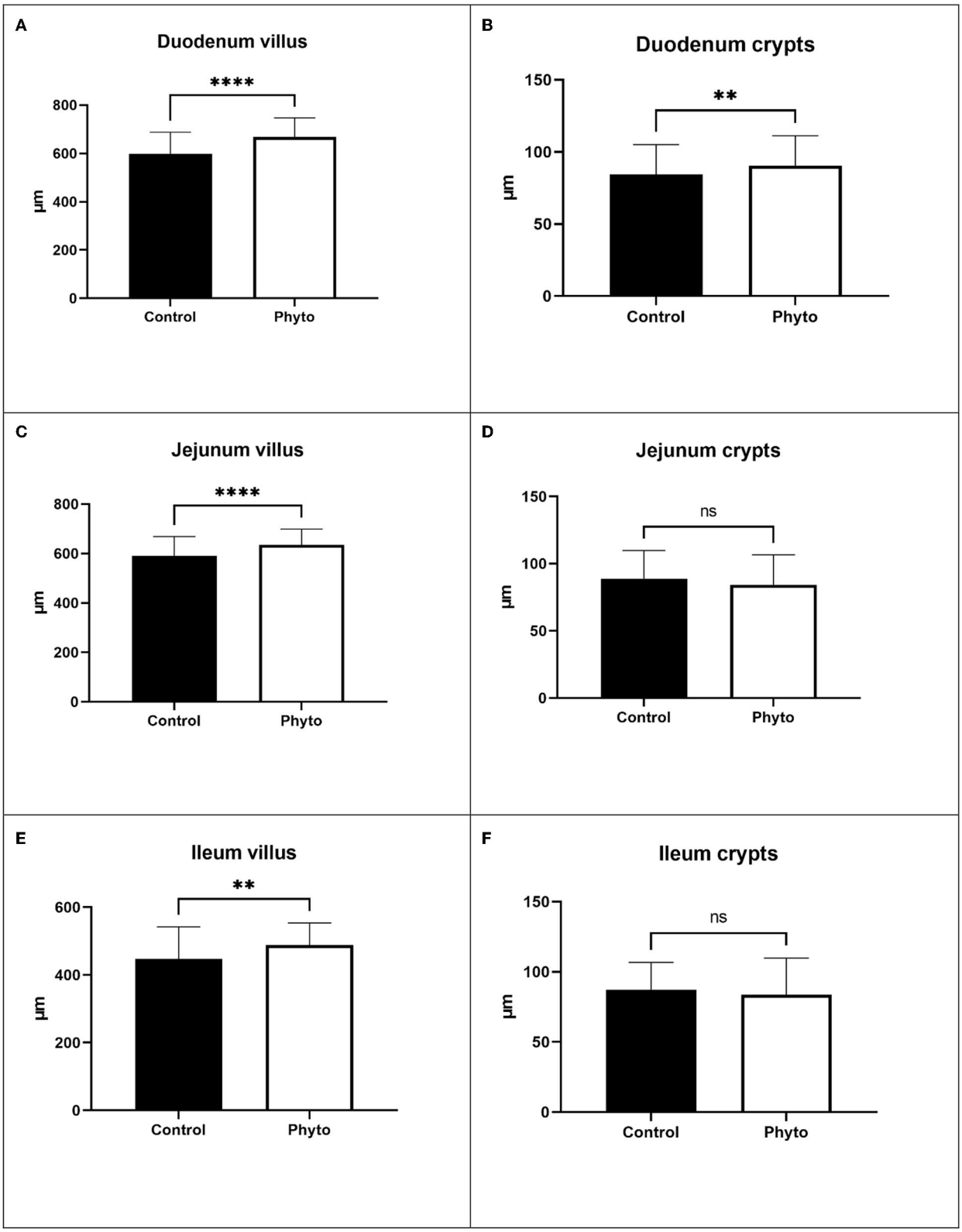

FIGURE 4
Effect of dietary supplementation with PHYTO on duodenal, jejunal, and ileal villus height and crypt depth of laying hens at the end of the trial (1 hen per replicate cage; $n = 25$ per treatment). **(A)** Duodenum villus; **(B)** Duodenum crypts; **(C)** Jejunum villus; **(D)** Jejunum crypts; **(E)** Ileum villus; **(F)** Ileum crypts. Bars represent mean values and error bars standard deviation of values within each experimental group. $^{****}P < 0.0001$; $^{**}P < 0.01$; ns, not significant.

TABLE 5A Effect of dietary supplementation with PHYTO product on egg quality parameters at the 1st week of the trial.

Egg quality parameter	Groups*, 1 Control	PHYTO	SEM[2]	*P*
Egg weight (g)	60.82	60.72	0.700	0.944
Yolk weight (g)	16.20	16.03	0.192	0.660
Egg white weight (g)	39.01	39.25	0.664	0.860
Egg weight in water (g)	4.35	4.69	0.102	0.101
Egg weight, specific (g/cm^3)	1.068	1.065	0.0018	0.101
Haugh unit	77.92	73.56	1.481	0.143
Egg diameter (cm)	43.44	43.36	0.200	0.836
Egg lateral index (cm)	59.07	59.42	0.452	0.705
Egg shape index	73.64	73.16	0.477	0.625
Egg shell thickness (mm)	0.45	0.43	0.006	0.606
Egg shell weight (g)	5.61	5.45	0.104	0.433
Egg yolk color	14.08	13.69	0.116	0.136
Egg shell color	33.14	27.03	1.997	0.127
Egg strength				
Eggshell breaking force (N/m^2)	3750.2	3706.9	0.160	0.606
Eggshell deformation (N/m^2)	0.029^x	0.024^y	0.001	0.084
Egg Yolk TBARS				
TBARS, nmol/mL	1.709	1.871	0.038	0.973

*N = 25 (pens), four eggs per replication.
^{x,y}Values in the same row with different superscripts tend to differ (0.05 < P ≤ 0.10).

TABLE 5B Effect of dietary supplementation with PHYTO on egg quality parameters at the 8th week of the trial.

Egg quality parameter	Groups*, 1 Control	PHYTO	SEM[2]	*P*
Egg weight (g)	65.48	67.12	0.625	0.194
Yolk weight (g)	16.37	16.78	0.156	0.194
Egg white weight (g)	43.41	44.50	0.414	0.194
Egg weight in water (g)	4.93	5.11	0.079	0.261
Egg weight, specific (g/cm^3)	1.082	1.083	0.001	0.791
Haugh unit	85.15	89.29	1.363	0.131
Egg diameter (cm)	44.00^a	44.78^b	0.187	**0.036**
Egg lateral index (cm)	60.24	60.02	0.431	0.788
Egg shape index	73.21	74.73	0.557	0.174
Egg shell thickness (mm)	0.45^x	0.48^y	0.007	0.070
Egg shell weight (g)	5.69	5.83	0.054	0.194
Egg yolk color	13.60	13.68	0.156	0.800
Egg shell color	27.71	25.58	1.275	0.409
Egg Strength				
Eggshell breaking force (N/m^2)	3706.96^a	4250.16^b	119.83	**0.022**
Eggshell deformation (N/m^2)	0.030	0.031	0.0015	0.783
Egg Yolk TBARS				
TBARS, nmol/mL	3.003^b	2.089^a	0.101	**0.001**
Egg protein carbonyls				
Albumen protein carbonyls, nmol/mL (day 1)	56.33	53.19	2.705	0.567

*N = 25 (pens), four eggs per replication.
^{a,b}Values in the same row with different superscripts differ significantly (P ≤ 0.05).
^{x,y}Values in the same row with different superscripts tend to differ (0.05 < P ≤ 0.10).
[1]Groups of layer hens fed the control or the supplemented diet; [2]SEM, standard error of the mean.

It was expected that under such challenging environmental conditions, mitigating oxidative stress and ensuing inflammation would ameliorate performance. Indeed, in response to the PHYTO supplementation, egg production was increased, and this was particularly evident between 28 and 29 weeks of age. Supplemented hens maintained an overall higher rate of egg production which was accompanied by a decreased ADFI over the period 26–29 weeks and consequently improved the estimated feed: egg weight ratio. Although ADFI reduction is the primary driver for the impaired performance observed under HS conditions in laying hens (4, 32), it is apparent that this plant extract association acted mainly on egg production efficiency. An absence of effect on ADFI is in agreement with the results of studies investigating curcumin supplementation in heat-stressed laying hens (14) and SCUT supplementation in hens raised in normal ambient conditions (17).

We hypothesized that positive effects of the plant extract supplementation on performance will be reflected on markers of systemic oxidative stress, but also on heat stress sensitive tissues such as the liver (33, 34) and the intestine (12, 35), and on animal products such as the egg (36). Positive effects of dietary supplementation with CUR extracts and its principal metabolite curcumin have been previously observed in poultry species in the absence of HS (37), but also specifically in heat-stressed laying hens (13, 14). On the contrary, there are no studies investigating the effects of offering SCUT, or its active metabolites in heat-stressed laying hens although positive effects on laying hens, broilers, and other livestock species have been previously observed (16 for a recent review). Although dietary supplementation of a combination of CUR and SCUT, or their PSMs show synergistic effects in comparison with their isolated supplementation (18, 38), the underlying mechanisms have not yet been fully elucidated.

The combination of morphological and morphometrical analyses allows a reliable evaluation of subtle differences in intestine and the effects of dietary intervention strategies under HS conditions (30). It is well-established that HS can negatively impact intestinal histomorphometric features, leading to impaired nutrient digestion and absorption (39). Recent studies have shown that HS reduces nutrient digestibility (3) and absorption capacity as indicated by decreased VL and increased CD in laying hens (40) and causes a downregulation of nutrient transporters in the intestinal tract of broiler chicken (41). In our study, the effects on egg production were

accompanied by increased villosity across the gastrointestinal tract. The morphological analysis indicated that the duodenum was mainly affected by HS. Jejunum and ileum morphology were unaffected. In a previous study, duodenum and jejunum morphology showed alterations upon HS exposure, whereas the ileum remained undamaged by HS (30). Amelioration of intestinal indices was also observed in laying hens offered CUR powder and raised in thermoneutral conditions (42), which were accompanied by decreased ileal *Escherichia coli* populations. A decreased microbial content in the cecum has also been found in response to SCUT supplementation in laying hens (17). Upon oral intake, curcumin is effectively taken up by the intestinal epithelial cells, where they initiate or modulate several signaling pathways that ultimately lead to downregulation of inflammatory pathways preventing the disruption of intestinal barrier function (43, 44). It has been proposed that the resulting attenuation of luminal bacteria or bacterial products such as LPS underlies the observed beneficial effects on intestinal function (45). As far as baicalin is concerned, which is the principal active component of SCUT extracts, a downregulation of intestinal inflammation and associated oxidative stress in deoxynivalenol (DON)-treated piglets, in response to its dietary supplementation, has been observed (46). Both plant extract principal constituents have been shown to inhibit NF-κB and to increase mTOR signaling to modulate downstream inflammatory and oxidative responses in the intestine (43, 46). Importantly, although offering CUR extract alone was not enough to decrease gut inflammation induced by HS, a mixture of CUR and SCUT extracts decreased gut inflammation induced by heat, or *S. Enteritidis* infection in broiler chicks (18). Since, microbiota composition is modulated by dietary intake of PSMs contained in both extracts (44, 47) and given that HS alters fecal volatile fatty acid production and nutrient digestibility in laying hens (3), further research is required to disassociate their effects on laying hen microbiota composition.

Liver evaluation for hens kept under HS showed that the plant extract constituents positively affected the integrity of hepatic and endothelial cells, resulting in milder hepatocellular necrosis and microvascular thrombosis. In general, the pathogenesis of HS on liver tissue injury is multifactorial (34). To some degree, it may be affected by increased bacterial translocation and their toxins from the intestinal tract which consequently induces an inflammatory response (48). It is suggested that HS affects initially the endothelial cells, which leads to diffuse vascular damage and activation of the coagulation cascade, resulting in hypercoagulability, the formation of multiple microthrombi, and, subsequently, diffuse microvascular thrombosis (49). Main lesions observed are the multifocal to diffuse bleeding, hemorrhagic necrosis, and widespread microthrombi due to indirect endothelial damage and direct hyperthermic hepatocellular damage (50). In agreement with liver histological findings, the reduced degree of liver injury was accompanied by a significant reduction in ALT in PHYTO supplemented hens. An increase in ALT has been previously attributed to cellular leakage and loss of the functional integrity of hepatic cell membrane induced by HS, resulting in its release from the cytoplasm (51). Both CUR and SCUT and their metabolites possess hepatoprotective effects. Recently, curcumin supplementation reduced inflammatory cell infiltration around the central veins, dilation of sinusoidal capillaries, dilation of central veins, reduced the size of hepatocytes, and necrosis in the hepatic lobules in heat-stressed laying hens. This was associated with a downregulation of pro-inflammatory cytokine gene expression levels and protein expression of NF-κB in the liver. These effects may be related to the reduced translocation of intestinal endotoxin LPS in the circulation, which activates toll-like receptor signaling pathways to induce Nf-kB inflammatory pathways in the liver (52). On the contrary, recent studies highlight the effects of baicalin in attenuating inflammatory pathways under conditions which challenge liver health and function (53, 54). Interestingly, the combination of curcumin and baicalin has been recently shown to exert superior hepatoprotective effects in ethanol-challenged rats, in comparison with monotherapy with the respective metabolites (38), set aside already demonstrated the effects on the intestine (18). A synergistic activity in the liver has been loosely attributed to their combined effects on multiple signaling pathways regulating the pro-inflammatory response and transcription factors, although further research is required (38).

In line with an improvement of gut morphology and liver integrity, a reduction in corticosterone was observed, which is typically elevated in response to stressors, among HS. Briefly, HS exposure leads to the activation of the neuroendocrine system, which stimulates the hypothalamic–pituitary–adrenal axis (HPA) to increase plasma corticosterone (55). Both baicalin and curcumin are viewed as potent anti-stress agents, capable of modulating the HPA axis (56, 57). Offering curcumin has been recently shown to reduce laying hen corticosterone levels in heat stress conditions (14), while SCUT extract has been shown to modify the thermoregulatory behavior of broilers raised in moderate HS conditions (58). Corticosterone has pro-oxidative functions leading to increased mitochondrial metabolism (59), increases energy deposition at the expense of reproductive function (60), and may lead to decreased steroidogenesis and as a result impaired ovarian development and function (61, 62). Therefore, positive effects observed on egg production of supplemented birds may be associated with reduced corticosterone production.

Laying hens supplemented with the plant extract combination experienced systemic oxidative stress to a smaller degree as indicated by blood TBARS values, according to expectations, however, that occurred without a change in systemic levels of serum antioxidant enzymes, apart from CAT, which was in fact reduced in supplemented bird's contrary to conventional expectations. Antioxidant enzyme activities

are believed to be compromised by heat stress conditions corroborated with a reduction in Nrf2 expression, although differences in antioxidant enzyme activities might largely depend on the HS conditions, species, tissue, and sampling in relation to the induction of HS (8, 10). On the contrary, the effects of PSMs are partially attributed to their ability to upregulate transcription factor Nrf2-mediated antioxidant enzymes such as CAT, GPx, and SOD (9). Previous studies in heat-stressed layers offered curcumin have illustrated that CAT concentration was increased following 3 and 6 weeks (13) and 6 weeks of HS exposure (14) in comparison with non-supplemented birds. Since in this study supplemented hens were experiencing oxidative stress to a smaller degree, a lower requirement for CAT production may be implied. Future trials should focus on laying hen oxidative responses over time under varying environmental conditions in response to plant extract supplementation.

As far as egg quality is concerned, egg weight, eggshell thickness, eggshell percent, and eggshell density are negatively affected by high ambient temperature (63). Other egg quality parameters, like eggshell color, are mostly affected by genetic factors and to a lesser extent by other factors such as age, diseases and stress, electrolyte balance, and nutrient levels. In our study, the eggshell and egg yolk color were not substantially influenced PHYTO supplementation. Supplementation with CUR or curcumin has been previously shown to improve eggshell thickness, eggshell strength, and albumen height (14) and parameters, such as specific gravity and yolk color (64). Importantly, these effects may be dose- and time-dependent in relation to sampling following supplementation (42). There is a scarcity of studies on the effects of SCUT supplementation on egg quality. Although there was no effect on albumen height in the present trial, it has been previously shown that eggs produced by SCUT supplemented hens showed a tendency to have greater Haugh units after 2-week storage (17).

Our results showed that enrichment of the diet with a PHYTO product bearing a substantial TCP content for 56 days improved substantially yolk oxidative stability in line with an improved hen oxidative status, while albumen protein oxidation was not affected. It has been previously shown that the dietary supplementation with curcumin-laying hens reduced the levels of TBARS in the yolk of fresh and stored eggs (15). Similarly, the MDA contents in stored eggs were significantly lowered by feeding SCUT extract (17). These results collectively show that SCUT and CUR and/or its metabolites may minimize lipid peroxidation in stored eggs. It has been reported that the total phenolic content of the diet is well-correlated with oxidative status of the layer hens and the produced eggs (65) or broiler chickens and breast and leg filets (66, 67). However, published information on the beneficial effects of phytonutrients on layers and egg characteristics is considerably less abundant (68). It is well-known that the fat-soluble vitamin content of egg yolk, such as tocopherol or xanthophyll, can be affected by manipulating their dietary levels (69). The literature is prolific with evidence of *in vitro* antioxidant activity of medicinal plants and their extracts, based on their ability to donate a hydrogen or electron, as well as to delocalize the unpaired electron within the aromatic structure protecting biological molecules against oxidation (70), which is well-correlated with their total phenolic content (71). Many published studies have shown an increased postprandial antioxidant capacity of phenolic compounds from various feedstuffs (72, 73); however, when biomarkers of the redox status are measured after phenolic compound consumption, the results obtained are often contradictory (74, 75). In the case of curcumin upon oral intake, it undergoes rapid metabolic redaction and conjugation to glucuronides, resulting in limited systemic bioavailability after oral administration (76). Following an oral dose of 0.1 g/kg administered to mice, a yield of a peak plasma-free curcumin concentration was only 2.25 μg/mL (77). Moreover, it has been previously shown that curcumin is not deposited inside the eggs (15). Similarly, pharmacokinetics of SCUT metabolites, after entering the body, are mostly metabolized in glucuronidase and sulfatase forms (77). Therefore, observed effects may be related to direct or indirect transference of antioxidant compounds to the yolk, since overall oxidative stress experienced by the hens was reduced in this study. Probably, these antioxidants are involved on the reduction in yolk lipid peroxidation, since the TBARS values were reduced.

Conclusion

In conclusion, offering an additive containing SCUT and CUR extracts increased egg production in laying hens raised in high ambient conditions. The improved performance was accompanied by increased intestinal villosity and reduced liver damage, the effects thought to be mediated by a reduction in oxidative stress and an improvement of intestinal and liver function. Importantly, these effects translated into the production of eggs with improved oxidative stability and eggshell breaking force. Thus, the dietary supplementation of the current PHYTO mixture could be considered as a useful natural alternative to help sustain egg production of layers raised under HS. Future studies should investigate the efficacy of this plant extract combination over time and explore microbiota-related effects.

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 trial protocol was authorized by the Research Committee of Aristotle University, Thessaloniki, Greece (number 71553, 28.07.2020). Husbandry, euthanasia, experimental procedures, and biosecurity precautions were conducted in accordance with all welfare requirements described by Good Farming Practice Guidelines (Directive 2010/63/EC; Commission recommendation 2007/526/EC) and were approved by the Research Ethics Committee of the Aristotle University of Thessaloniki.

Author contributions

IG, PS, and GAP contributed to conception and design of the study. SD and IS organized the database. GAP performed the statistical analysis. IG and PS wrote the first draft of the manuscript. IS, GAP, SD, and TP performed the analysis. IM, VT, IG, and SD performed the experimental trial. All authors wrote sections of the manuscript and contributed to manuscript revision, read, and approved the submitted version.

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Conflict of interest

The use of Scutellaria extract in animal feed is a subject of French patent application FR 14/51501 and PCT application PCT/FR2015/050450.

Authors PS, MP, and FR were employed by CCPA Group.

The remaining authors 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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Dietary Grape Seed Proanthocyanidin Alleviates the Liver Injury Induced by Long-Term High-Fat Diets in Sprague Dawley Rats

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In mammals, the liver is the most important organ that plays a vital function in lipid metabolism. Grape seed proanthocyanidin (GSPE) is a kind of natural polyphenolic compound primarily obtained from grape skin and seeds. Recent research found it had high bioavailability in defending against obesity, hyperlipidemia, inflammatory, oxidative stress, and targeting liver tissue. However, the mechanism of GSPE in regulating obesity induced by dietary high-fat (HF) was not fully understood, particularly the influences on liver functions. Therefore, this study aimed to investigate the effects of GSPE supplementation on the liver function and lipid metabolic parameters in rats fed HF diets long-term. A total of 40 healthy female Sprague Dawley rats were selected. After 8 weeks of obesity model feeding, the rats were randomly divided into four treatments: NC, standard diet; NC + GSPE, standard diet + 500 mg/kg body weight GSPE; HF, high-fat diet; HG + GSPE, high fat diet + 500 mg/kg body weight GSPE. Results indicated that long-term HF feeding caused severe liver problems including megalohepatia, steatosis, inflammation, and hepatocyte apoptosis. The supplementation of GSPE alleviated these symptoms. The results of the current experiment confirmed that GSPE addition up-regulated the expression of the Wnt3a/β-catenin signaling pathway, thereby restraining the liver cell endoplasmic reticulum stress and hepatocyte apoptosis. Furthermore, the microRNA-103 may play a role in this signal-regulated pathway. In summary, GSPE had a protective effect on the liver and the current experiment provided a reference for the application of GSPE in animal nutrition as a kind of natural feed additive.

Keywords: liver injury, grape seed proanthocyanidin, Wnt3a/β-catenin signaling pathway, endoplasmic reticulum stress, microRNA, lipid metabolism

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INTRODUCTION

In the last few decades, non-alcoholic fatty liver disease (NAFLD), a chronic liver disease, has become a global public health problem that impacts about a quarter of adults worldwide (1). As it develops, a series of pathological changes occur in liver tissues and cells such as high lipid accumulation, abnormal triglyceride cycle, inflammation, mitochondrial dysfunction, cell apoptosis, and endoplasmic reticulum stress (ERS) (2). It not only increases greatly the probability of liver-related diseases, but also causes extrahepatic medical conditions such as type 2 diabetes, cardiovascular, and cerebrovascular diseases (3). Although the exact pathogenesis of NAFLD is still not fully understood, it is associated with some metabolic abnormalities including lipid metabolism.

Grape seed proanthocyanidin (GSPE), a natural polyphenolic compound enriched in phenolic hydroxyl groups, is primarily found in grape skin and grape seeds. Its role in defending organs and tissues against obesity, hyperlipidemia, inflammatory, and oxidative stress is widely acknowledged (4). Margalef et al. (5) suggested the bioavailability of GSPE in targeting liver tissue; with dietary supplementation of 375 mg/kg of GSPE, the rats' liver had comparatively higher concentrations of GSPE flavonoid metabolites including catechin, epicatechin, and glucuronidation of methyl. Moreover, it was considered that microRNAs (miRNAs) may be a key factor in altering lipid metabolism in NAFLD (6). *In vivo* study confirmed that polyphenols treatment repressed fatty liver disease in hyperlipidemic mice, and the positive effects were relevant to the expression of lipid metabolism-related miRNAs (7). A recent study demonstrated that taking orally GSPE decreases the size of the liver and alleviates the level of steatosis in patients with NAFLD (8).

The endoplasmic reticulum, an essential site for secretory protein and lipid synthesis has a notable capacity to maintain steady lipid metabolisms in the liver and plasma. But, excessive accumulation of triglycerides in non-adipocytes can lead to ERS and activate stress signaling pathways thereby resulting in the dysregulation of lipid metabolism (9). ERS has been considered to play an important role in the initiation of NAFLD progression (10). Moreover, ERS can intervene in the functional expression of the proapoptotic BCL2-family members, which may eventually induce hepatocyte apoptosis (11). It was reported that NAFLD correlates with the severity of hepatic cell apoptosis (12). Hence, anti-apoptotic therapy may be one of the therapeutic methods worth trying. On the other hand, regulations on the lipid metabolism pathway are also considered to be an alternative therapeutic solution. Wnt is a secretory protein and plays an important role in cellular development and proliferation (13). Recently, researchers found that the Wnt family and signaling also had a relevant effect on lipid metabolism (14). For instance, it was found that Wnt signaling led to repression of liver steatosis and triglyceride concentrations in zebrafish, thereby regulating lipid metabolism (15). In European NAFLD cases, gene expression analysis showed that Wnt signaling pathways may be pivotal in NAFLD pathogenesis (16).

A high-fat diet causes obesity and fatty liver is one of the possible factors leading to NAFLD. How exactly GSPE alleviates the liver affected by HFD-induced obesity has not yet been studied. Our study, therefore, hypothesized that the inclusion of GSPE in high-fat diets may reverse liver injury by alleviating the cell apoptosis from ERS and restraining lipometabolism. This experiment was carried out to investigate the effects of dietary GSPE on the liver function and lipid metabolic parameters of rats fed with or without high-fat diets. It is anticipated that the data obtained from this study will provide basic support for the functional uses of GSPE.

Abbreviations: NAFLD, non-alcoholic fatty liver disease; GSPE, grape seed proanthocyanidin; ERS, endoplasmic reticulum stress; NC, standard diet; HF, high-fat diet; PPARγ, peroxisome proliferators activate the receptor γ; FAS, fatty acid synthetase; ATF6, activating transcription factor 6; CHOP, CCAAT/enhancer-binding protein homologous protein; TNF-α, tumor necrosis factor α; IL-1β, interleukin-1β; IL-6, interleukin-6.

MATERIALS AND METHODS

Female Sprague Dawley (SD) rats were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd (Beijing, China). Grape seed proanthocyanidin (GSPE) was purchased from Tianjin Jianfeng Natural Product R&D CO., Ltd. (Tianjin, China). GSPE in this study contained >95% proanthocyanidins as per the manufacturer's instructions. The protocols were performed in accordance with the guidelines for the care and use of laboratory animals approved by the Institutional Animal Care and Use Committee of Northeast Agricultural University.

Animals and Feeding

A total of 40 healthy female SD rats about five to seven-week-old were individually housed under a standard room (21± 4°C) with a 12 h light/dark cycle. All rats consumed food and water *ad libitum*. After 1 week of acclimation, rats were randomly distributed into two treatments (n=20): (1) one group fed with a standard diet (12.95% kcal from fat, 24.02% kcal from proteins, and 63.03% kcal from carbohydrates) supplied by Beijing Keao Xieli Feed CO., Ltd. (Beijing, China); (2) another group fed with high-fat diet (52.64% kcal from fat, 17.07% kcal from proteins, and 30.29% kcal from carbohydrates). After 8 weeks of feeding, an obesity model was set up based on the data shared in our previous study (17). The standard diet group and high-fat diet group rats were further divided randomly into 2 groups respectively (n=10): including (1) the NC (standard diet), (2) the GSPE (GSPE based on standard diet), (3) the HF (high-fat diet), and (4) the HF+GSPE (GSPE based on high-fat diet) treatments. The GSPE group and the HF+GSPE group were given daily gavage of 500 mg/kg body weight GSPE by gavage administration. The GSPE was dissolved in saline. The body weight and feed intake of rats were weighed weekly. These treatments lasted for 4 weeks. The rats were anesthetized after 12 h fasting. Liver tissues were removed swiftly and weighed. The collected tissues were frozen at −80 °C until analysis.

Histopathology

The liver sample was fixed with a 10% neutral-buffered formalin solution for at least 24 h. The fixed specimens were dehydrated through a series of ethanol, cleared in xylene, and embedded in paraffin. The liver sections were cut into 4-μm sections and rehydrated through a series of incubations in xylene and ethanol solutions and then stained with hematoxylin and eosin (H&E). Sections were analyzed under light microscopy.

Wnt3a/β-Catenin Pathway

Wnt3a and β-catenin levels in renal tissues were assessed using Rat Protein Wnt3a ELISA Kit (mlbio, Shang Hai, China) and Rat beta-catenin ELISA Kit (mlbio, Shang Hai,

China), respectively. The manufacturer's instructions were followed exactly.

Quantitative Real-Time PCR

TRIzol reagent was used to isolate Total RNA including miRNA from liver tissues, and RNA samples were purified and frozen at −80 °C until assay. cDNA was synthesized by reverse transcription of 5 μl total RNA using Prime Script RT reagent Kit, with gDNA Eraser according to the manufacturer's protocol as described. Primer sequences of target genes were designed from published GenBank and synthesized by Sangon (Shanghai, China) (**Table 1**). In this study, β-actin was used to normalize the expression of target gene transcripts. The sample was centrifuged momentarily and carried out on the Applied biosystem 7500 Real-Time PCR thermal cycler apparatus at the matched program (40 cycles of 95 °C for 5 s, 60 °C for 34 s). All PCR reactions were performed in triplicate and mRNA was evaluated by the 2-ΔΔct method as described previously.

Western Blot Analysis

The liver tissues were stored at −80°C. Tissue samples were homogenized in ice-cold RIPA lysis buffer (Millipore, Billerica, MA, USA) for protein extraction. Tissue debris was removed by centrifugation, and the resulting supernatants were collected and analyzed for protein concentration by the BCA protein assay kit. The protein was separated on a 10% SDS polyacrylamide gel and then transferred to nitrocellulose membranes (Beyotime Biotech, Shanghai, China). The membranes were incubated with specific primary antibodies overnight at 4°C. The primary antibodies included anti-mouse β-actin (Beyotime Biotech, Shanghai, China), anti-rabbit ATF6, anti-rabbit CHOP, anti-rabbit Wnt3a, and anti-rabbit β-catenin (Company ABclonal, Inc., Wu Han, China). After washing, the membranes were allowed to react with diluted horseradish peroxidase-conjugated secondary antibodies, including goat anti-rabbit IgG antibody and goat anti-mouse IgG (Beyotime Biotech, Shanghai, China) at room temperature for 2 h. An enhanced chemiluminescence system (Beyotime Biotech, Shanghai, China) was used to visualize antibody-antigen complexes.

Statistical Analysis

All data were analyzed based on a 2 × 2 factorial arrangement using the general linear model procedure of the SAS software (SAS Inst. Inc., Cary, NC). The individual rats were considered as the experimental unit. The statistical model included the main effects of the GSPE and dietary fat, as well as the interaction between GSPE and dietary fat. Means were separated using Tukey's Test. GraphPad Prism 5.0 software was used for data plotting. Variability in the data was expressed as the pooled standard error of the means (SEM). A $P < 0.05$ was considered to denote statistical significance.

RESULTS

Effects of GSPE on Liver Performance in Rats Fed Long-Term High-Fat Diet

In this experiment, the liver indexes (the ratio of liver weight and body weight) and cell morphological sections were considered as the liver performance. As shown in **Figure 1**, all rats fed with HF diets had higher ($p < 0.05$) liver indexes than those fed with NC diets. Compared with these non-supplementation treatments, the addition of GSPE observed lower liver indexes. Significant interactions were noted between dietary fat level and GSPE supplementation ($p < 0.05$). Rats in the HF group had the highest ($p < 0.05$) liver index than those in the other three groups. Liver index in HF + GSPE was also significantly ($p < 0.05$) higher than NC and NC + GSPE treatments. The liver morphological sections are shown in **Figure 2**. Compared with NC groups, hepatocytes in rats from HF diets observed adipose and injury. Furthermore, the most serious ones occurred in the HF treatment group, which showed circular lipid droplets filled in the cytoplasm, some of the cells were vacuolated and the nucleus had disappeared, and whole cells were filled with fat along with inflammatory infiltration. Protective responses were observed

TABLE 1 | Primer sets for qPCR.

Gene	Forward primer (5′-3′)	Reverse primer (5′-3′)
miR-103	ACACTCCAGCTGGGGGCTTCTTTACAGTGCTG	TGTCGTGGAGTCGGCAATTC
PPARγ	GCCCTTTGGTGACTTTATGGAG	GCAGCAGGTTGTCTTGGATGT
FAS	ACCTCATCACTAGAAGCCACCAG	GTGGTACTTGGCCTTGGGTTTA
ATF6	GGATTTGATGCCTTGGGAGTCAGAC	ATTTTTTTCTTTGGAGTCAGTCCAT
CHOP	CCTTCACTACTCTTGACCCTG	CACCACTCTGTTTCCGTTTC
Bax	GAGGATGATTGCTGATGTG	AGTTGAAGTTGCCGTCTG
Bcl-2	GTGGCCTTCTTTGAGTTCGGT	CATCCCAGCCTCCGTTATCC
TNF-α	GATCGGTCCCAACAAGGAGG	GTGAGGAGCACATAGTCGGG
IL-6	AGCGATGATGCACTGTCAGA	TAGCACACTAGGTTTGCCGA
IL-1β	GACTTCA CC ATGGAACCCGT	GGAGACTGCCCATTCTCGAC
β-actin	GCAGAAGGAGATTACTGCCCT	GCTGATCCACATCTGCTGGAA

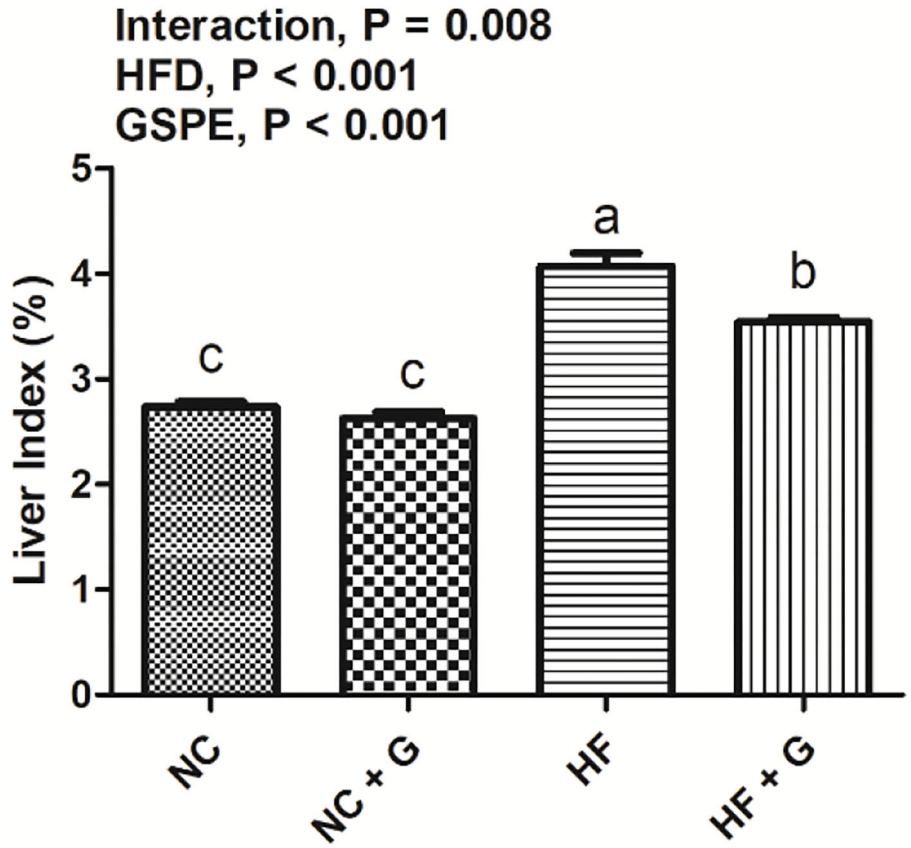

FIGURE 1 | Effects of GSPE on liver index in rats fed long-term high-fat diet. Data were expressed as the mean ± SEM. Different letters means with different superscripts differ ($P < 0.05$). NC, standard diet; NC + G, standard diet + 500 mg/kg body weight GSPE; HF, high fat diet; HF + G, high fat diet + 500 mg/kg body weight GSPE.

in the hepatocytes from HF + GSPE-fed rats. The fatty degeneration was alleviated, part cell morphology returned, the lipid droplets reduced or disappeared, and no inflammatory cells were observed.

Effects of GSPE on Lipid Metabolism Genes' Relative Expression in the Liver of Rats Fed Long-Term High-Fat Diet

Peroxisome proliferators activate the receptor γ (PPARγ) and fatty acid synthetase (FAS) are two fat synthesis-related genes. As shown in **Figure 3A**, rats in HF groups had higher ($p < 0.05$) expression on mRNA of PPARγ compared with the rats in NC groups, but no effects on FAS expression (**Figure 3B**). Also, significantly ($p < 0.05$) higher PPARγ mRNA expression was observed in the GSPE supplementation treatment group than that in these non-addition treatment groups. Moreover, significant interactions were shown between dietary fat and GSPE for the PPARγ mRNA expressions. HF group had the highest ($p < 0.05$) expression among the treatments. However, there were no significant influences on FAS mRNA expression among the treatments.

Effects of GSPE on the Expression of Endoplasmic Reticulum Stress mRNA and Proteins in the Liver of Rats Fed Long-Term High-Fat Diet

Activating transcription factor 6 (ATF6) and CCAAT/enhancer-binding protein homologous protein (CHOP) are two of the main factors during the ERS signal pathway. As described in **Figures 4A,B**, higher dietary fat resulted in higher ($p < 0.05$) expressions on mRNA expression of ATF6; whereas, it did not affect the mRNA expression of CHOP compared with the samples from normal diets. Supplementation of GSPE observed significant decreases in both the expression of ATF6 and CHOP mRNA than those no-supplement ones. Interactions between GSPE and dietary fat were observed in the expressions of both ATF6 and CHOP. These mRNA expressions were all reduced in the HF + GSPE treatment than in the other treatments. The results of protein expressions on ATF6 and CHOP genes are shown in **Figures 4C,D**. For the protein expression on ATF6, there were no effects among treatments on dietary fat, GSPE, or the interaction. Higher dietary fat had significantly ($p < 0.05$) higher protein expression on CHOP than those fed with normal diets. Also, dietary inclusion of GSPE

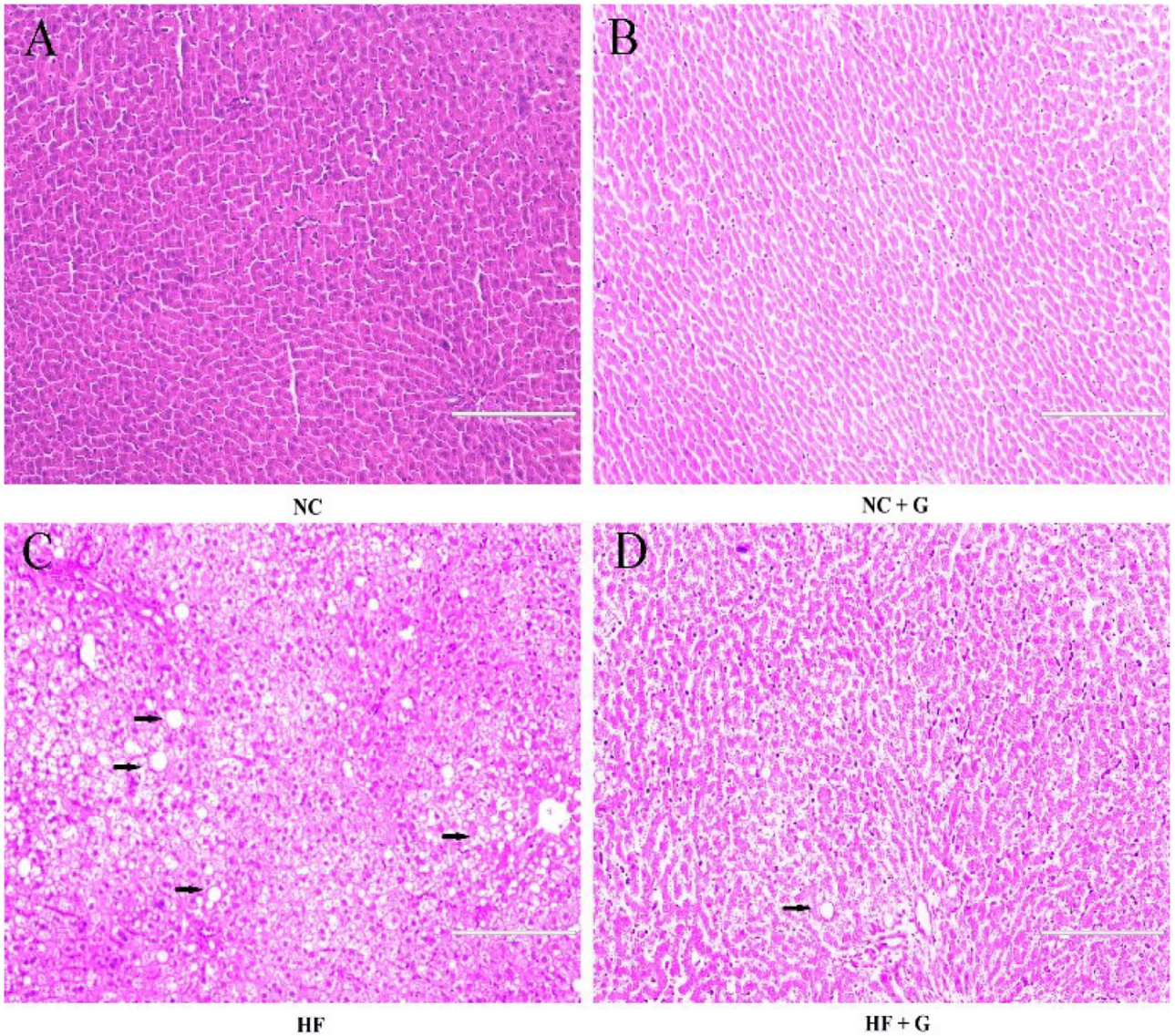

FIGURE 2 | (A) NC, standard diet; **(B)** NC + G, standard diet + 500 mg/kg body weight GSPE; **(C)** HF, high fat diet; **(D)** HF + G, high fat diet + 500 mg/kg body weight GSPE.

significantly ($p < 0.05$) decreased the CHOP protein expression compared with those clean ones. There were no interaction effects on CHOP protein expression between dietary fat and GSPE supplementation.

Effects of GSPE on the Relative Expression of Apoptosis mRNA in the Liver of Rats Fed Long-Term High-Fat Diet

In the current experiment, the genes of Bax, Bcl-2, and Caspase-3 were considered as the genes that regulate apoptosis. As shown in **Figure 5**, no significant effects were observed on the expression of the three mRNA in HF treatments compared with those fed normal diets. Similarly, GSPE supplementation also did not influence the mRNA expression of Bax and Caspase-3. However, the inclusion of GSPE greatly ($p < 0.05$) improved the Bcl-2 mRNA expression in rats' livers. Interactions were noted between dietary fat and GSPE for Bax and Bcl-2 mRNA expressions. The Bax mRNA in HF + GSPE group showed lower ($p < 0.05$) expression than that in NC + GSPE and HG treatments; moreover, the addition of GSPE in HF treatment observed the highest ($p < 0.05$) expression of Bcl-2 mRNA compared with the other three treatments.

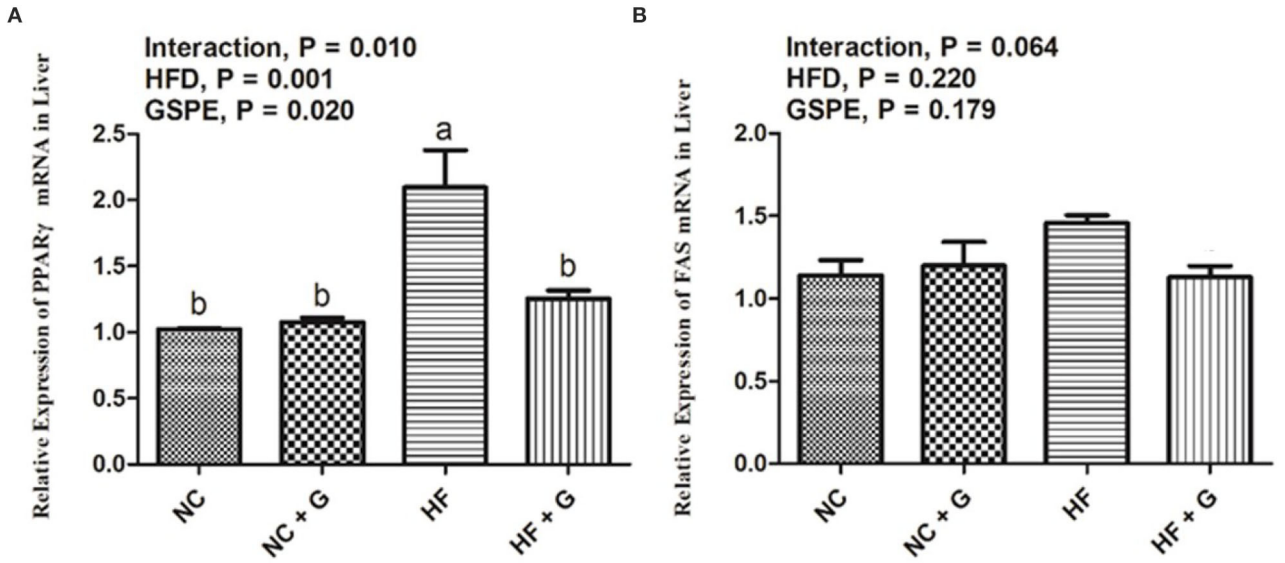

FIGURE 3 | Effects of GSPE on relative expression of lipid metabolism genes of liver in rats fed long-term high-fat diet [**(A)** PPARγ mRNA expression; **(B)** FAS mRNA expression]. Data were expressed as the mean ± SEM. Different letters means with different superscripts differ ($P < 0.05$). NC, standard diet; NC + G, standard diet + 500 mg/kg body weight GSPE; HF, high fat diet; HF + G, high fat diet + 500 mg/kg body weight GSPE.

Effects of GSPE on the Relative Expression of Inflammatory Cytokines mRNA in the Liver of Rats Fed Long-Term High-Fat Diet

The results of relative expression of inflammatory cytokines TNF-α, IL-1β, and IL-6 mRNA in rats' livers are shown in **Figure 6**. The HF diet significantly ($p < 0.05$) increased the expressions of TNF-α, IL-1β, and IL-6 mRNA than those in the basal diets. Also, GSPE supplementation reduced ($p < 0.05$) the TNF-α and IL-1β expressions but had no significant effects on IL-6 expression compared with these non-supplemented ones. Significant interactions were shown in all three parameters among the treatments. Similarly, mRNA expressions of TNF-α and IL-1β in the HF group had the highest ($p < 0.05$) level than the other three treatments. But the IL-6 mRNA expression in the HF diet only showed higher ($p < 0.05$) values than in the NC treatment.

Effects of GSPE on the Relative Expression of MicroRNA-103 in the Liver of Rats Fed Long-Term High-Fat Diet

The relative expression of miRNA-103 is described in **Figure 7**. Rats fed with HF diets observed higher ($p < 0.05$) expression of miRNA-103 than those fed with basal diets. Also, GSPE supplementation significantly ($p < 0.05$) decreased the expression compared with the non-supplemented ones. No interactions were observed in miRNA-103 expression in the liver.

Effects of GSPE on Wnt3a/β-Catenin Pathway Proteins Concentration and Expression in the Liver of Rats Fed Long-Term High-Fat Diet

The concentrations of Wnt3a/β-catenin pathway protein in rats' livers are shown in **Figures 8A,B**. There were no significant influences on GSPE supplementation among the treatments. Higher dietary fat significantly ($p < 0.05$) decreased the Wnt3a protein concentration (8A) in livers but did not affect the β-catenin protein concentration (8B). Significant interactions were observed in the Wnt3a and β-catenin protein concentrations between dietary fat level and GSPE. The HF group had the lowest protein concentrations on both Wnt3a and β-catenin compared with NC and HF + GSPE groups. However, the HF + GSPE group observed a lower ($p < 0.05$) concentration of Wnt3a protein than the NC group. As described in **Figure 8C**, higher dietary fat all resulted in higher ($p < 0.05$) expression of Wnt3a protein than those in normal diets. Similar results were also observed ($p < 0.05$) in GSPE groups compared with those of non-supplemental ones. Also, interactions were observed in the Wnt3a protein expression between dietary fat level and GSPE. The HF treatment had the lowest ($p < 0.05$) expression among the four treatments and the HF + GSPE group received the highest expression ($p < 0.05$) of Wnt3a protein among the other three groups. However, there were no significant effects on the β-catenin protein expression among the experimental treatments (**Figure 8D**).

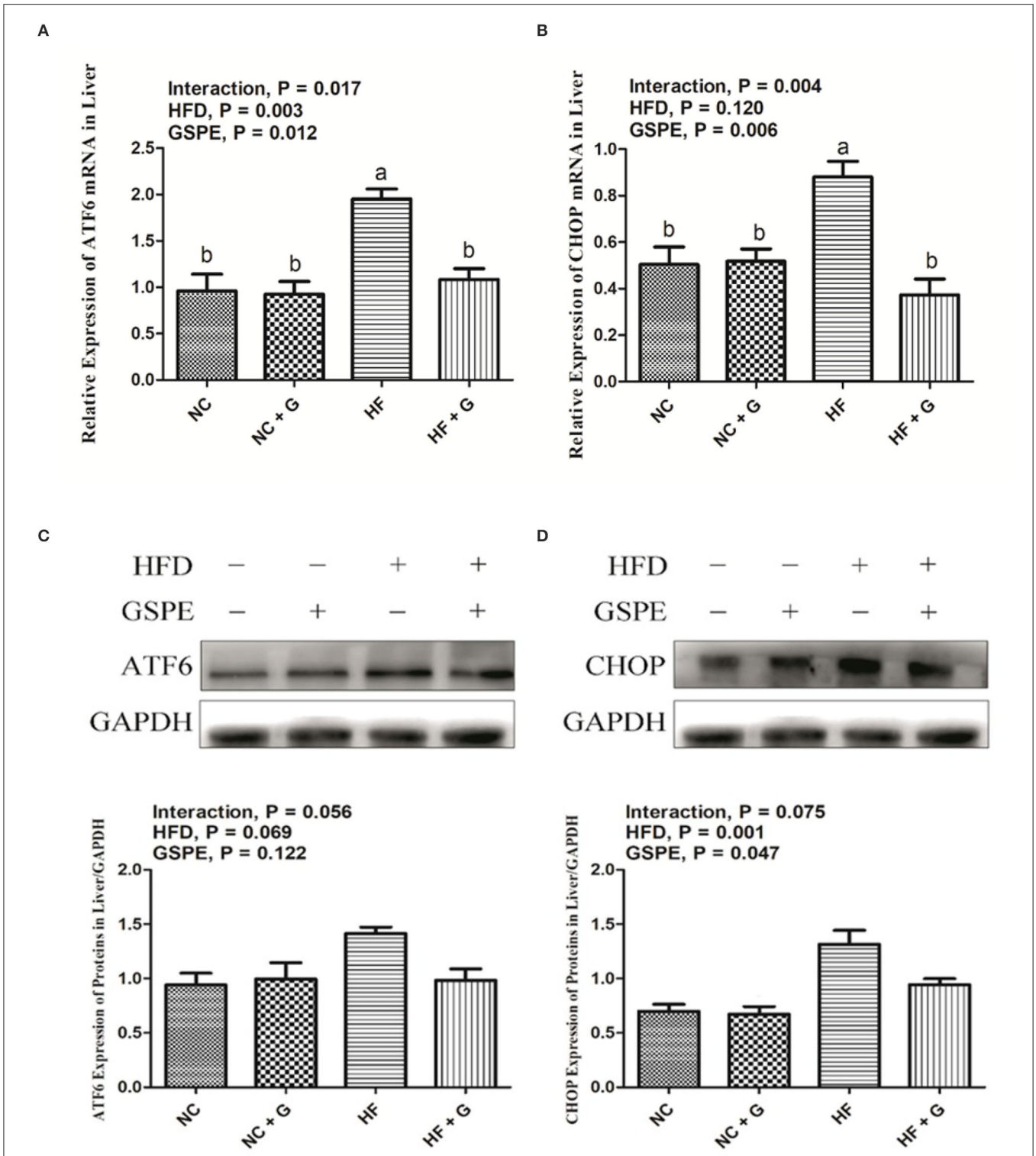

FIGURE 4 | Effects of GSPE on relative expression of endoplasmic reticulum stress of liver in rats fed long-term high-fat diet [**(A)** ATF6 mRNA expression; **(B)** CHOP mRNA expression; **(C)** Protein production on ATF6 gene; **(D)** Protein production on CHOP gene]. Data were expressed as the mean ± SEM. Different letters means with different superscripts differ ($P < 0.05$). NC, standard diet; NC + G, standard diet + 500 mg/kg body weight GSPE; HF, high fat diet; HF + G, high fat diet + 500 mg/kg body weight GSPE.

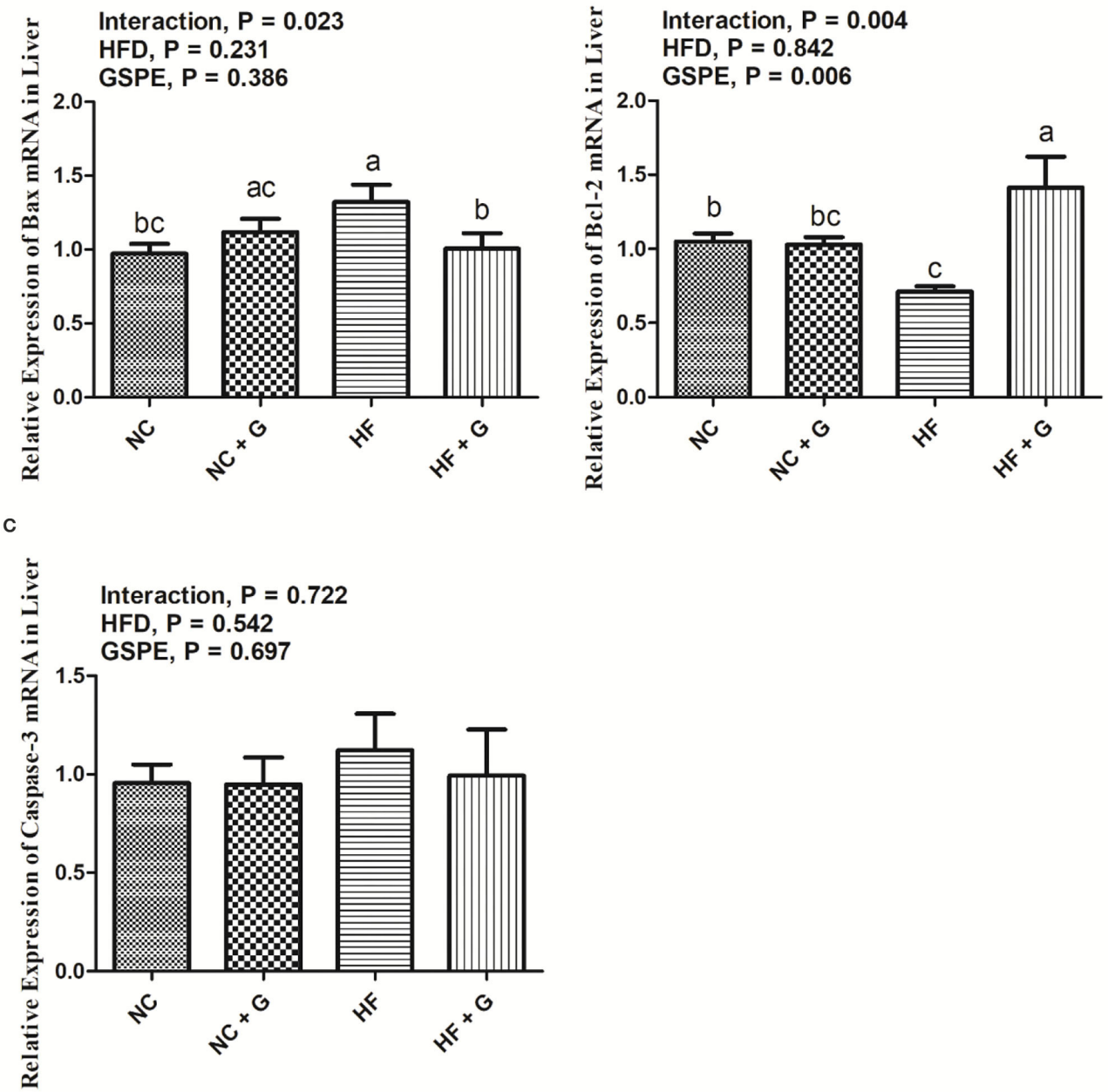

FIGURE 5 | Effects of GSPE on relative expression of apoptosis proteins of liver in rats fed long-term high-fat diet [**(A)** Bax mRNA expression; **(B)** Bcl-2 mRNA expression; **(C)** Caspase-3 mRNA expression]. Data were expressed as the mean ± SEM. Different letters means with different superscripts differ ($P < 0.05$). NC, standard diet; NC + G, standard diet + 500 mg/kg body weight GSPE; HF, high fat diet; HF + G, high fat diet + 500 mg/kg body weight GSPE.

DISCUSSION

In mammals, the liver plays a very important role in lipid metabolism. Pathologically, NAFLD mainly causes excessive deposition of liver fat and hepatic cell steatosis, and it was normally caused by a long-term high-fat diet (1, 18). Following the progression of NAFLD, metabolic problems including oxidative stress, inflammatory cytokine release, ERS, and apoptosis accompanied (2). GSPE is a natural polyphenolic compound primarily obtained from grape skin and seeds. It was indicated that GSPE plays an influential role in antioxidant, anti-inflammatory, and anti-apoptosis (19). The current experiment was designed to investigate the effects of GSPE on liver index and morphological, lipid metabolism, ERS, apoptosis, and

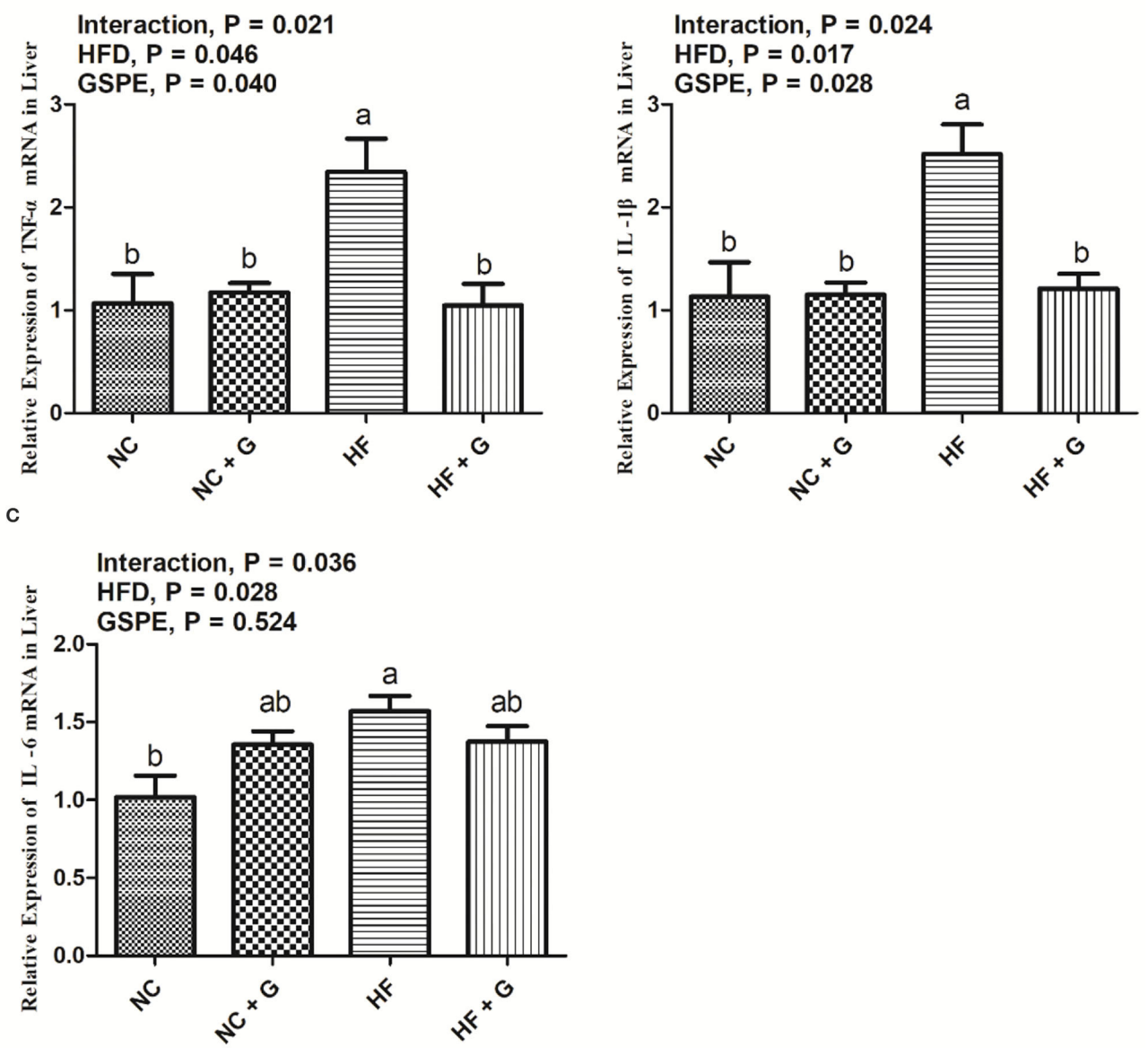

FIGURE 6 | Effects of GSPE on relative expression of inflammatory cytokines of liver in rats fed long-term high-fat diet [**(A)** TNF-α mRNA expression; **(B)** IL-1β mRNA expression; **(C)** IL-6 mRNA expression]. Data were expressed as the mean ± SEM. Different letters means with different superscripts differ ($P < 0.05$). NC, standard diet; NC + G, standard diet + 500 mg/kg body weight GSPE; HF, high fat diet; HF + G, high fat diet + 500 mg/kg body weight GSPE.

Wnt3a/β-catenin pathway in rats fed with high-fat long-term diets, and subsequently provide a reference for the prevention and cure for NAFLD.

Obesity always leads to the adipose degeneration of the liver and it is also one of the main factors which cause NAFLD (20). In this experiment, rats in HF treatment had the highest liver indexes than other groups and their hepatocytes also showed adipose and injury such as circular lipid droplets, filled with fat, and inflammatory infiltration. These results demonstrated that these rats in the HF group had symptoms of NAFLD. At the same time, the addition of GSPE significantly decreased the liver index than those in HF treatment. This result was in accordance with the results from Khoshbaten et al. (8) who reported that oral GSPE reduced the liver volume and the steatosis level in people with NAFLD. Moreover, in the current study, it was observed that the supplementation of GSPE significantly relieved the steatosis of the liver and decreased the inflammatory infiltration. It provides proof of the liver

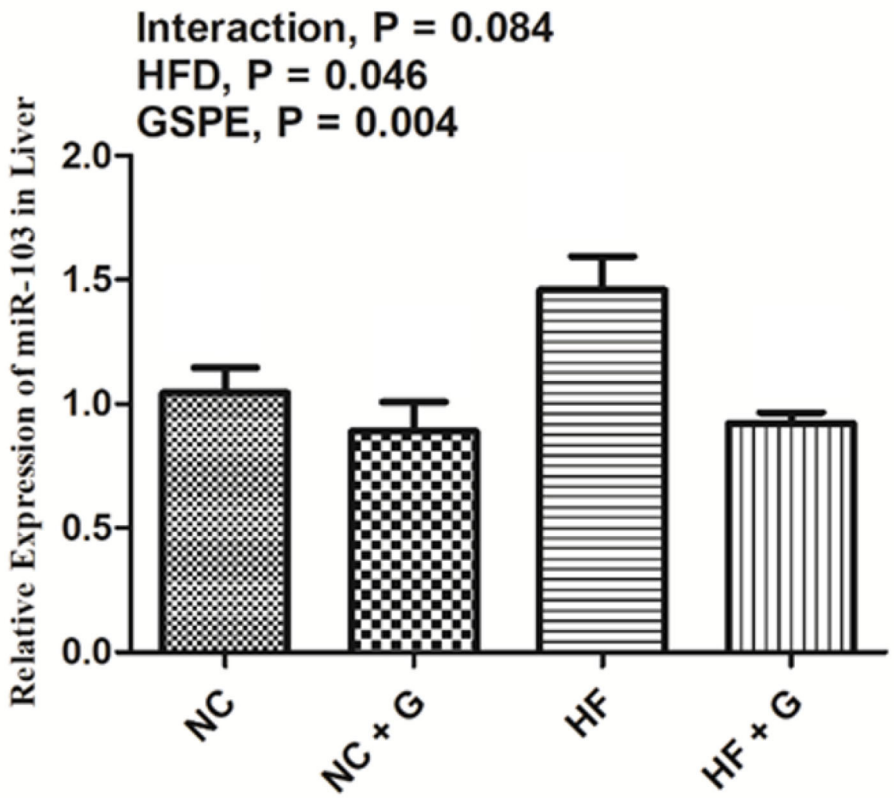

FIGURE 7 | Effects of GSPE on relative expression of micro-RNA-103 of liver in rats fed long-term high-fat diet. Data were expressed as the mean ± SEM. Different letters means with different superscripts differ ($P < 0.05$). NC, standard diet; NC + G, standard diet + 500 mg/kg body weight GSPE; HF, high fat diet; HF + G, high fat diet + 500 mg/kg body weight GSPE.

protective responses triggered by the application of GSPE at the cellular level. In the liver, abnormal lipid deposition was attributed to the out-of-balance between lipid availability and consumption (21). Meanwhile, the metabolic disturbance of lipid and lipoproteins were also considered one of the etiological factors of NAFLD (22). PPARγ belongs to the nuclear receptor superfamily and plays an important role in adipogenesis (23). Horie et al. (24) reported that mice with liver-specific deficiency of PPARγ observed decreases in hepatic steatosis and lipogenic genes FAS. Similarly, the expression of PPARγ in the liver triggers the expression of adipocyte-specific genes and hepatic lipid accumulation (25). On the other hand, it was reported that the addition of GSPE could repress the expression of PPARγ. An *in vitro* experiment indicated that grape skin ethanolic extract treatment significantly reduced the expressions of the adipogenic genes including PPARγ in 3T3-L1 cells (26). A similar result was obtained in this experiment; GSPE addition decreased the PPARγ expression in the liver. It was known that PPARγ may induce the transcription of the fat synthesis gene FAS in steatosis livers (27). Previous studies suggested that GSPE also showed an ability to inhibit FAS activity by combination with β-ketoacyl reductase of FAS; whereas, in this study, no effects on FAS were observed (28). This may be due to the differences in concentrations and experiment models between studies.

MicroRNAs (miRNAs), are non-coding RNAs that are small and regulate gene expression at the post-transcriptional level. They are highly conserved between different species and binding to the 3' untranslated region of the target mRNA thereby controlling multiple signaling pathways at once (29, 30). It was suggested that miRNAs play important roles in the liver fatty acid homeostasis, adipogenesis, and lipid metabolism (31). In the current experiment, the HF diet improved the miR-103 expression and GSPE supplementation decreased the expression. The miR-103 was reported to have the ability to promote fat synthesis, and it was confirmed that it targeted regulating the mRNA expression of FAS (32, 33). However, Park et al. (34) reported that in diet-induced obese mice the expression of miR-103 was decreased. In line with this experiment, Joven et al. (7) indicated that the expression of miR-103 was increased in hyperlipidemic mice caused by diet. Moreover, they also observed that plant-derived polyphenols significantly reduced the miR-103 expression. Also, *in vitro* experiment conducted on the human hepatocellular carcinoma cell line Huh 7 reported that after apigenin treatment, the expression level of mature endogenous miR-103 decreased (35). It was suggested that the structure of polyphenols may determine their activity in fatty acid synthase (28). The inhibition of miR-103 expression in this experiment may be due to this, but further experiments are still needed

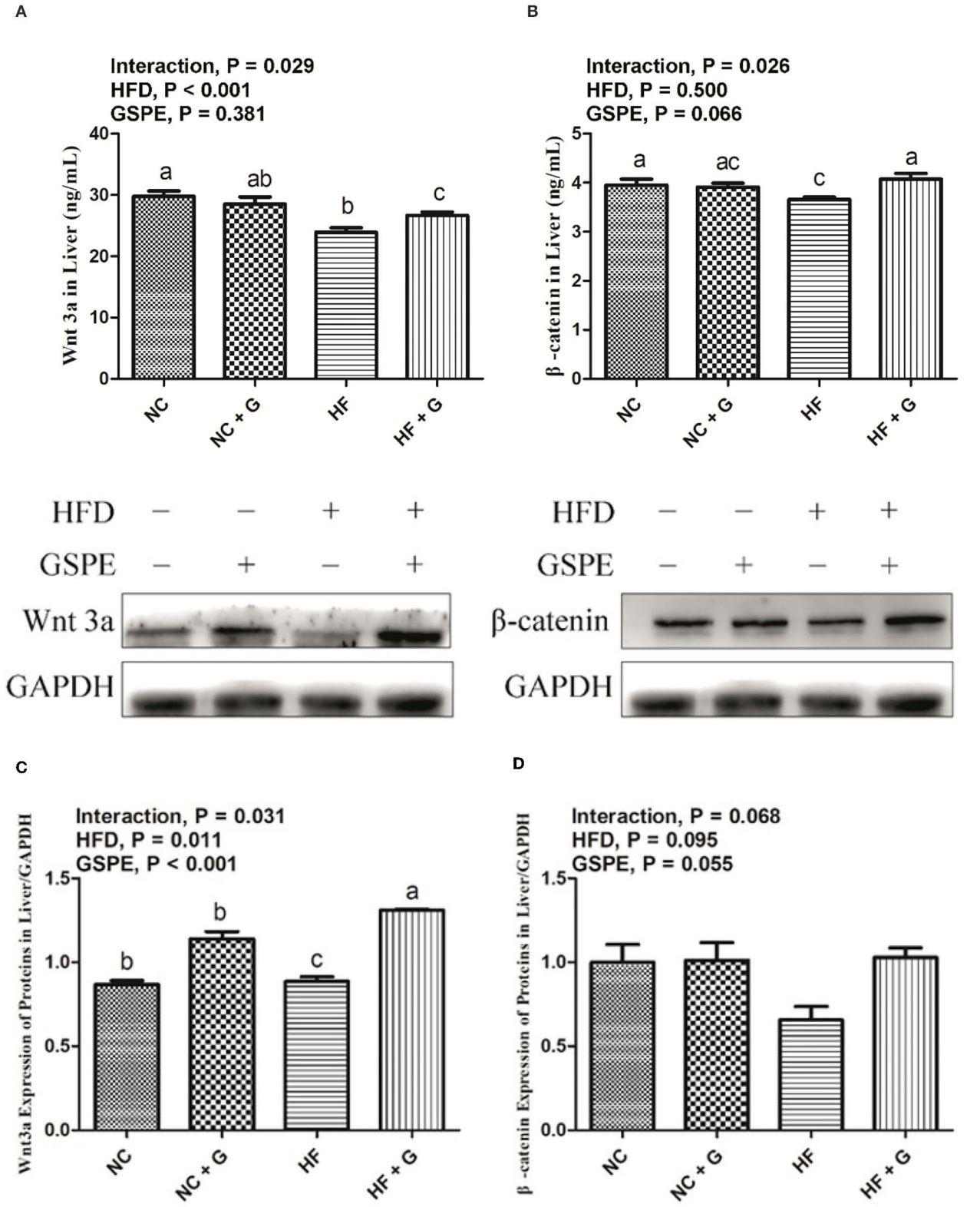

FIGURE 8 | Effects of GSPE on Wnt3a/β-catenin pathway protein concentration and expression of liver in rats fed long-term high-fat diet [**(A)** Wnt3a protein concentration; **(B)** β-catenin protein concentration; **(C)** Protein production on Wnt3a; **(D)** Protein production on β-catenin]. Data were expressed as the mean ± SEM. Different letters means with different superscripts differ ($P < 0.05$). NC, standard diet; NC + G, standard diet + 500 mg/kg body weight GSPE; HF, high fat diet; HF + G, high fat diet + 500 mg/kg body weight GSPE.

to figure out the exact mechanisms of GSPE on the regulation of miRNAs.

The Wnt/β-catenin pathway is also known as the classical Wnt signaling pathway, which depends on the activation of β-catenin. It was suggested that this pathway plays extensive roles in animal biological processes including embryonic development, organization stability, metabolic balance, and cell maintenance (36). The inhibition of the Wnt pathway was considered to induce obesity and thereby cause metabolic disorder due to which it was reported to have the ability to inhibit adipocyte differentiation (37). In the current experiment, the results showed that in HF treatments the expressions of Wnt3a and β-catenin in the liver were significantly decreased which verified the function of the Wnt3a/β-catenin pathway in obesity. It was also observed that supplementation of GSPE improved the expression of Wnt3a and β-catenin protein in the liver. Owing to the lack of available studies, direct comparisons of response to GSPE on the Wnt3a/β-catenin pathway in obese rats are impossible. Recently, similar results were observed by Zang et al. (38) who reported that tea polyphenols activated the Wnt/β-catenin pathway in order to improve the liver fat deposition in obese adult zebrafish. Moreover, Zhang et al. (39) indicated that the miR-103 could target and modulate the Wnt3a by inhibiting the pathway of Wnt3a/β-catenin and reducing the lipidosis in preadipocytes. On the other hand, it was investigated that a large number of β-catenin expressed in the nucleus may cut the expression of adipogenic genes such as PPARγ thereby reducing the fat deposition (40). Therefore, the changes in expression of Wnt3a and β-catenin protein in the liver may be due to the feedback regulation of miR-103 and PPARγ in this experiment.

The activation of β-catenin also regulates the transcription of the apoptosis factors such as Bcl-2 and Bax (41). Also, along with the NAFLD development, hepatocyte apoptosis occurs. Apoptosis is one of the important steps in hepatic injury induced by NAFLD. It has been suggested that the aggravation of NAFLD *via* a complicated process had a high correlation with apoptosis, hepatic injury, fibrosis, and inflammation (42). The injury of the liver may cause excessive apoptosis and necrosis thereby triggering the inflammation and finally leading to fibrosis of the hepatocyte, eventually making the NAFLD worse. Therefore, the apoptosis and inflammation gene factors were also determined in the current experiment. The results show that the supplementation of GSPE reduced the mRNA expressions of Bax and proinflammatory factors TNF-α and IL-1β as well as improved the anti-apoptotic factor of Bcl-2 expression. Bcl-2 and Bax belong to the family of B-cell lymphoma-2. It has been suggested that they are the homologous, antagonist, and are the downstream target protein controlled by the Wnt/β-catenin signaling pathway (43). Some studies have observed that the activation of the Wnt/β-catenin pathway may inhibit apoptosis and hence protect the cells and organs (44, 45). For instance, Huang et al. (44) reported that in an alcoholic liver disease rat model, Wnt agonists stimulated the –catenin transduction and downregulated the expression of pro-apoptotic gene thereby protecting the liver from alcohol-induced apoptosis and damage. On the other hand, macrophage infiltration of these fat tissues such as M1 macrophages will secrete the pro-inflammatory factors including IL-6, TNF-α, and IL-1β aggravating the inflammatory response (46). The Wnt/β-catenin signaling pathway was also considered to have an anti-inflammatory function (47). Hatting et al. (48) suggested that the Wnt/β-catenin pathway played an important part that apoptotic gene knockout mice observed lower inflammatory. Similarly, Ma et al. (49) indicated that the Wnt/β-catenin pathway inhibited the expression of the proinflammatory factor IL-1β. Taken together, the results of this study substantiated the positive contribution of GSPE *via* the Wnt/β-catenin signaling pathway on liver cell apoptosis and inflammation.

Obesity leads to long-term low-intensity inflammation which results in continuous stress on the endoplasmic reticulum in cells (46). Meanwhile, hyper deposition of fat in cells set off the inhibition of protein which also causes the ERS (50). Recent research found that ERS plays a momentous role in NAFLD occurrence and development due to the accommodation of ERS on hepatocyte apoptosis (51). Chen et al. (52) observed that the ATF6 signaling pathway is one of the keys which affects the progression of NAFLD *via* regulating the ERS-induced inflammation and apoptosis. In the current experiment, the supplementation of GSPE significantly restrained the expressions of ATF6 and CHOP in the liver which was similar to previous studies. Gao et al. (53) reported that GSPE protected the nephrotoxicity by cisplatin through inhibition of the ERS-induced apoptosis. Recently, Long et al. (54) indicated that proanthocyanidins have positive effects in the small intestine of mice that protected the epithelial cells from zearalenone-induced apoptosis by ERS. Moreover, an *in vitro* study observed that the inhibition of the Wnt3a/β-catenin pathway enhanced the ERS and thereby improved the apoptosis of preadipocytes. Therefore, based on the results of this experiment, it was speculated that the suppression of ERS caused by hepatocyte apoptosis may be due to the positive expressions of the Wnt3a/β-catenin pathway.

CONCLUSIONS

Long-term high-fat feeding caused non-alcoholic fatty liver disease and severe liver problems including megalohepatia, steatosis, inflammation, and hepatocyte apoptosis in rats. The supplementation of grape seed proanthocyanidin alleviated these symptoms. The results of the current experiment sustained that grape seed proanthocyanidin addition up-regulated the expression of the Wnt3a/β-catenin signaling pathway, thereby restraining the liver cell endoplasmic reticulum stress and hepatocyte apoptosis. It also promoted liver lipid metabolism including downregulating the expression of genes of lipid synthesis and inhibiting the liver fat deposition in rats. Furthermore, microRNA-103 may play a role in this signal-regulated pathway and this may be a worthy point for further research. In summary, liver protective effects were observed in grape seed proanthocyanidin and the current experiment provides a reference for the application of grape seed proanthocyanidin as a natural feed additive.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The animal study was reviewed and approved by Animal Care and Use Committee of Northeast Agricultural University, People's Republic of China.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

FUNDING

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Characterization of differentially expressed and lipid metabolism-related lncRNA-mRNA interaction networks during the growth of liver tissue through rabbit models

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Background: Characterization the long non-coding RNAs (lncRNAs) and their regulated mRNAs involved in lipid metabolism during liver growth and development is of great value for discovering new genomic biomarkers and therapeutic targets for fatty liver and metabolic syndrome.

Materials and methods: Liver samples from sixteen rabbit models during the four growth stages (birth, weaning, sexual maturity, and somatic maturity) were used for RNA-seq and subsequent bioinformatics analyses. Differentially expressed (DE) lncRNAs and mRNAs were screened, and the *cis*/*trans*-regulation target mRNAs of DE lncRNAs were predicted. Then the function enrichment analyses of target mRNAs were performed through Gene ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways, respectively. The target protein interaction (PPI) and lncRNA-mRNA co-expression networks were constructed using string version 11.0 platform and R Stats. Finally, six lncRNAs and six mRNAs were verified taking RT-qPCR.

Results: Liver Oil Red O detection found that the liver showed time-dependent accumulation of lipid droplets. 41,095 lncRNAs, 30,744 mRNAs, and amount to 3,384 DE lncRNAs and 2980 DE mRNAs were identified from 16 cDNA sequencing libraries during the growth of liver. 689 out of all DE lncRNAs corresponded to 440 DE mRNAs by cis-regulation and all DE mRNAs could be regulated by DE lncRNAs by trans-regulation. GO enrichment analysis showed significant enrichment of 892 GO terms, such as protein binding, cytosol, extracellular exsome, nucleoplasm, and oxidation-reduction process. Besides, 52 KEGG pathways were significantly enriched, including 11 pathways of lipid metabolism were found, like Arachidonic acid metabolism, *PPAR* signaling pathway and Biosynthesis of unsaturated fatty acids. After the low expression DE mRNAs and lncRNAs were excluded, we further obtained the 54 mRNAs

were regulated by 249 lncRNAs. 351 interaction pairs were produced among 38 mRNAs and 215 lncRNAs through the co-expression analysis. The PPI network analysis found that 10 mRNAs such as 3β-Hydroxysteroid-Δ24 Reductase (*DHCR24*), lathosterol 5-desaturase (*SC5D*), and acetyl-CoA synthetase 2 (*ACSS2*) were highly interconnected hub protein-coding genes. Except for *MSTRG.43041.1*, the expression levels of the 11 genes by RT-qPCR were the similar trends to the RNA-seq results.

Conclusion: The study revealed lncRNA-mRNA interation networks that regulate lipid metabolism during liver growth, providing potential research targets for the prophylaxis and treatment of related diseases caused by liver lipid metabolism disorders.

KEYWORDS

lipid metabolism, liver, lncRNA, mRNA, rabbit model

Background

As the veritable metabolic factory, the liver is the largest solid organ of mammals, which performs important metabolic functions in the pathophysiological process of the body and is responsible for the metabolism of lipids, sugars, protein and vitamins in the body (1–3). Lipid metabolism is a dynamic biological process, including the synthesis, accumulation and distribution of lipid to various specific tissues, which is one of the most important metabolic functions of the liver (4). Liver is susceptible to a variety of pathogenic factors, causing a variety of acute and chronic liver diseases. Dysregulation of lipid homeostasis in liver is strongly related to the hepatic steatosis and metabolic syndrome. For example, dysregulation of lipid metabolism in liver is crucial to the development of non-alcoholic liver disease (NAFLD) and is connected with metabolic syndromes such as obesity and insulin resistance (5–7). Fatty liver disease and its metabolic syndrome have become an important source of global morbidity and mortality, posing a serious threat to global public health and human health (8, 9). But this moment, the pathogenesis of fatty liver disease is still not completely clear. Thus, in-depth analysis of the related regulatory mechanisms of liver lipid metabolism is consequent to prevent and treat fatty liver disease and its metabolic syndrome.

Non-coding RNAs (nRNAs) are crucial regulators for controlling geneexpression and transducing cellular signals (10). As the most abundant part of transcriptional genome, long non-coding RNAs (LncRNAs) indirectly regulate gene expression through transcriptional regulation, post-transcriptional modification and regulation of miRNA activity, thus changing cell physiology and function (11, 12). In the liver, lncRNA can regulate liver growth, development, metabolism, protein decomposition and other important functions (13). It coordinates the formation of lipid, metabolism and transportation of fatty acids, cholesterol and phospholipids, and the formation of high-density lipoprotein (HDL) and low-density lipoprotein (LDL). The expression changes of lncRNAs in liver are associated with many diseases, and dysregulation of lncRNA leads to abnormal lipid metabolism, which leads to the occurrence of diseases such as NAFLD (7). In addition, the expression of lncRNA showed high degree of tissue and cell specificity (4, 14). Because of this specificity, lncRNAs may be more suitable targets for the treatment of related diseases than existing protein-coding genes (15). At the same time, as a functional molecule, LncRNA may be a biomarker for better feedback of disease status (16). Therefore, the regulation of lncRNA expression has huge potential in the gene prevention and treatment of fatty liver disease, and plays important indicative parts in the diagnosis and prognosis of the disease (17). The function of lncRNA is defined by being predicted its interaction with mRNA. Compared with mRNA and miRNA, there is still a big gap in our understanding to the function of lncRNA, and we have little understanding about the regulation effects of most lncRNAs and their functions in development, physiology and diseases (18). Besides, the potential mechanism of lncRNA mediated the biological process of liver lipid metabolism is still unclear. Therefore, studying the interaction between lncRNA and coding genes related to lipid metabolism during the growth and development of liver has great values for identifying new genomic biomarkers and therapeutic targets for metabolic diseases.

Abbreviations: NRNA, noncoding RNA; lncRNA, long noncoding RNA; DE, differentially expressed; GO, gene ontology; KEGG, Kyoto Encyclopedia and Genomes; PPI, protein-protein interaction; DHCR24, 3β-Hydroxysteroid-Δ24 Reductase; SC5D, lathosterol 5-desaturase; ACSS2, acetyl-CoA synthetase 2; NAFLD, non-alcoholic liver disease; HDL, high-density lipoprotein; LDL, low-density lipoprotein.

Animal model is an important carrier to study the pathogenesis, therapeutic drugs and prevention strategies for diseases. Currently, the model animals major used in studies related to fatty liver disease are mice and rats. Due to the poor sequence homology and conservatism of lncRNA among different species, the molecular and biological effects of lncRNA obtained in mice and rats are difficult to be applied to related studies in humans (4, 19). Therefore, it is necessary to elucidate the functional regulation of lncRNAs in liver lipid metabolism with the help of a variety of model animals. Rabbits are widely used in biomedical field (20). For example, the rabbit model of hereditary hyperlipidemia with familial hypercholesterolemia has made great contributions to elucidate the pathophysiology about human hypercholesterolemia, lipoprotein metabolism and atherosclerosis (21). In addition, there are few studies describing the expression pattern of lncRNAs in liver under physiological state no matter in animal models or human. Therefore, exploring the lncRNAs and mRNAs expression profiles connected with the regulation of lipid metabolism during the growth and development of rabbit liver will further enrich and improve the research vectors and biomarkers of fatty liver disease, and provide more effective therapeutic targets for human fatty liver disease.

Therefore, with the help of RNA-seq, bioinformatics and molecular biology methods, our research used rabbits as animal models to explore the changes of lncRNA and mRNA transcriptomes in rabbits' liver from birth, weaning, sexual maturity, and finally to somatic maturity, and screened, clarified and verified the lncRNA-mRNA interaction networks that may regulate liver lipid metabolism. We finally obtained 38 Differentially expressed (DE) mRNAs as ACSS2 and 215 DE lncRNAs like *MSTRG.30424.1* that may make vital roles in the regulation of lipid metabolism during rabbit liver growth.

Materials and methods

Animal model preparation and collection of liver tissue

According to the growth and development cycle of rabbit, 0 (birth), 35 (weaning), 85 (youth) and 120 (adult) days old healthy purebred Hyla rabbits were used in our study. In order to eliminate the interference of diet, after the rabbits were born, the lactation diet of female rabbits kept the same. After being weaned, all male rabbits used were given water and food freely under the same diet (16% protein, 10.8MJ/kg) and feeding environment. In our study, rabbits were euthanized using inhalation anesthesia (isoflurane) and exsanguination. Four rabbits' samples of liver tissue were collected for each stage, snap frozen in liquid nitrogen, and stored at −80°C for subsequent detection.

Oil Red O staining of liver tissue

Oil Red O staining for frozen sections of liver tissue were performed according to the steps of the reference (22). Lipid droplets of liver section (red in color) were observed and photographed using the OLYMPUS CX31 (OLYMPUS Corp., Japan).

Preparation and sequencing of rabbit liver tissue samples

Total RNA of liver samples was obtained using Trizol (Invitrogen, Carlsbad, CA, USA), and was quantified using NanoDrop ND-1000. The RNA integrity (RIN number > 7.0) was assessed by Agilent 2100. Then the RNA libraries were constructed and sequenced by LC-BIO Co., Ltd (Hangzhou, China). Briefly, take 5 μg RNA of per sample and remove rRNA using the Ribo-Zero^{TM} rRNA Removal Kit. First-strand and second-strand cDNA was synthesized using Reverse transcriptase, E.coli DNA polymerase I and RNase H, respectively. And then the cDNA libraries were constructed by PCR amplification and purification, and the average insertion of the final libraries was 300 bp (±50 bp). At last, sequencing through Illumina NovaseqTM 6000, and 150bp paired-end reads were generated.

Transcripts assembly and lncRNA identification

Removing the adapter sequence and low-quality reads from the raw data using Cutadapt (1.9) and FastQC(V0.10.1) (23), the high-quality Valid reads were aligned to the rabbit reference genome OryCun2.0 (http://ftp://ensembl.org/pub/release-102/fasta/oryctolagus_cuniculus/) using histat2 (2.0.4) (24). Assemble the mapped reads of each sample using StringTie (1.3.4) (25) with default parameters and merge to reconstruct all transcriptomes from samples using gffcompare (github.com/gpertea/gffcompare/). And then StringTie was used to perform expression level for mRNAs by calculating FPKM (26). Afterwards, transcripts that overlapped with known mRNAs and shorter than 200 bp were discarded. CPC(0.9-r2) (27) and CNCI(2.0) (28) were used to eliminate all transcripts with CPC score <-1 and CNCI score <0, and remained were regared as lncRNAs.

Screening of differentially expressed (DE) mRNAs and lncRNAs

Using StringTie calculated expression levels of mRNAs and lncRNAs through their FPKMs. The DE mRNAs and lncRNAs

were screened with log2 (fold change) ≥1/≤-1 and with *p*-value < 0.05 using R package DeSeq2 (29).

Prediction and functional analysis about target mRNAs of lncRNAs

To clarify the function of lncRNAs, we predicted the *cis*/*trans*-regulation target mRNAs of the DE lncRNAs. Coding genes in 100-kb upstream and downstream were selected by Python script, and these were considered the potentially *cis*-regulation target mRNAs, while using LncTar predicted the potentially *trans*-regulation target mRNAs. Gene ontology (GO) (30) and Kyoto Encyclopedia of genes and Genomes (KEGG) (31) pathway analyses of the target mRNAs of candidate DE lncRNAs were then performed, respectively.

Construction of regulation target protein interaction network

To further study the interaction among the selected mRNAs which were related to lipid metabolism, the PPI network of regulation target proteins was constructed using string version 11.0 platform (32). the species (protein species) and the minimum interaction threshold was set as "Oryctolagus cuniculus" (Rabbit) and "medium confidence" 0.4, respectly. And the remaining parameters remained at the default settings. Then, we used the software of Cytoscape (V3.7.2) (33) to study the topological properties of PPI network and draw the diagram.

Co-expression network analysis of lncRNA-mRNA

In order to investigate the regulatory network in response to lipid metabolism during the liver growth, co-expression network of DE lncRNA-mRNA was created with the help of R package Stats. Use OmicStudio tools (https://www.omicstudio.cn/tool) to select key node DE lncRNA and DE mRNA for visualization, and screen key lncRNAs and mRNAs through this network.

Validation of DE lncRNAs and mRNAs by RT-qPCR

Primers for the lncRNAs, mRNAs and internal control (GAPDH) (Additional File 1) were designed using Primer-BLAST. Total RNA of sample was converted to cDNA by using a PrimeScript^{TM} RT Reagent Kit (TAKARA, Dalian, China). The reaction mix was comprised of 1 μl template cDNA, 0.5 μl of 10 μM forward and reverse primers, 5 μl SYBR Premix Ex Taq^{TM} II (TAKARA), and 3 μl ddH_2O at a final volume of 10 μl. Using a Rotor gene 6000 PCR System (QIAGEN, Hiden, Germany), the reactions were proceeded as follows: 95°C for 30 s, 39 cycles of 95°C for 10 s, 60°C for 1 min, 72°C for 1 min, and melting curve analysis was performed from 58°C to 90°C in 1.5°C increments. Using the $2^{-\Delta\Delta Ct}$ method (34) calculated the relative gene expression levels.

Statistical analysis

In our study, Body weight, Liver weight and RT-qPCR verification results were expressed as mean ± standard deviation (mean ± SD). Data were subjected to analysis of variance using SPSS 26.0 statistical software (SPSS Inc., Chicago, IL, USA). First, the data were tested for a normal distribution and homogeneity of variance. And the differences between the two groups were compared using independent samples *t*-tests. Differences were considered significant at $P < 0.05$.

Results

Growth performance and liver lipid deposition during the rabbit growth

The results showed that the body and liver weight of rabbits were increased significantly ($P < 0.05$) with the growth of age (Additional File 2). Liver Oil Red O detection found that the liver showed time-dependent accumulation of lipid droplets, and by day 120, there were obvious large lipid droplets around the liver cells (Figure 1).

Overview of RNA-seq

We constructed 16 cDNA libraries from the Liver tissues of 0(R0), 35(R35), 85(R85), and 120(R120) days old rabbits. After being sequenced using the Illumina Novaseq^{TM} 6000, and 208.66 Gb raw reads were obtained. Then filtering adapter sequences and low-quality reads, 56,027,094 to 91,373,562 valid reads were obtained from libraries, with the effective ratio and GC content of libraries ranging from 80.82–95.58% and 48.5-52% (Additional File 3). Majority (69.80–77.64%) of valid reads were mapped to the rabbit reference genome, 53.02–60.56% of these had unique genomic positions (Additional File 4). The results showed that the obtained RNA-seq data met the quality control requirements, which could be used for subsequent analysis.

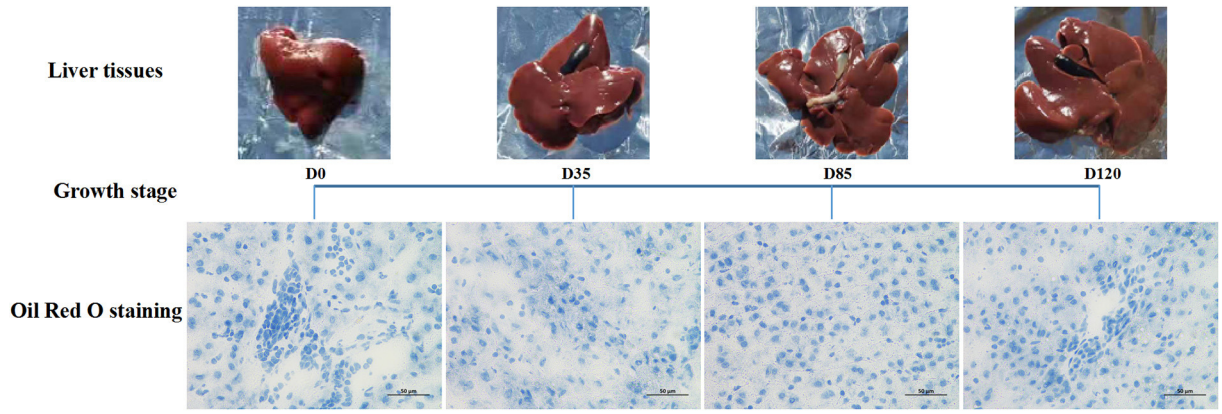

FIGURE 1
Detection results of Oil Red O in rabbit liver at different growth stages.

Identification of lncRNAs and mRNAs in rabbit liver tissue

We obtained 41,095 lncRNAs (Additional File 5A) and 30,744 mRNAs (Additional File 5B) from RNA-seq, and lncRNAs exhibited a higher expression than mRNAs (Additional Figure 1A). All lncRNAs and mRNAs were distributed in 23 chromosomes (Additional Figure 1B). The average length (1579 bp) of the lncRNAs was considerably shorter than the mRNAs (2268 bp) (Additional Figure 1C). Furthermore, the exon number of lncRNAs (average 1) were less than the mRNAs (average 12) (Additional Figure 1D), and the open reading frame (ORF) size in the mRNAs was longer than the lncRNAs (most were within 100 bp) (Additional Figure 1E). In addition, PCA analysis about all of mRNAs and lncRNAs showed that the groups of R0 and R35 had obvious clustering tendency, but samples in groups of R85 and R120 had high similarity.

Screening of DE lncRNAs and DE mRNAs

The DE lncRNAs and mRNAs were calculated and identified through FPKM using StringTie and R DeSeq2. A total of 3384 DE lncRNAs and 2980 DE mRNAs were screened ($p < 0.05$) during the liver growth when the four growth stages were compared in pairs (Additional File 6). The DE lncRNAs and DE mRNAs across the different libraries were clustered using the PCA analysis (Figure 2), and the clustering results were completely consistent with the clustering trends of total RNAs. When comparing R0 vs R35, R35 vs R85 and R85 vs R120, 1125, 476 and 416 DE lncRNAs and 1503, 792 and 220 DE mRNAs were detected, respectively (Figure 3). From the results, with the growth and development of the liver, the number of DE lncRNAs and mRNAs decreased gradually. Venn diagrams constructed with these DE lncRNAs and mRNAs showed that 9 DE lncRNAs and 33 mRNAs were common existed in four growth stages (Figure 4).

Functional enrichment network on the target DE mRNAs of DE lncRNAs

The most lncRNAs of rabbit deposited in databases have not yet been functionally annotated. Therefore, prediction of their functions was based on the functions of their *cis*/*trans*-regulated target mRNAs. First, The *cis*-regulated target mRNAs of DE lncRNAs were predicted and 689 out of the 3384 DE lncRNAs corresponded to 440 DE mRNAs. Prediction of the *trans*-regulation showed that all DE mRNAs were regulated by the DE lncRNAs. This indicated that the expression of mRNAs were extremely affected by the change expression of lncRNAs. To better understand the functions of the 2980 DE mRNAs regulated by DE lncRNAs, we performed GO enrichment and KEGG pathway analyses on the mRNAs. GO enrichment analysis showed significant enrichment of 892 GO terms ($P<0.05$), among which, the term of protein binding (GO:0005515) was enriched in the highest with the 821 DE mRNAs. According to the number of DE mRNAs enriched, the significant GO items in the top 20 were displayed, we found the DE lncRNAs affected the growth and development of liver by regulating the expression of mRNAs which play related functions, such as protein binding, cytosol, extracellular exsome, nucleoplasm, and oxidation-reduction process (Figure 5A). In addition, the GO term associated with lipid metabolism like arachidonic acid epoxygenase activity was also significantly enriched. Furthermore, KEGG enrichment analysis was performed. In order to better understand the

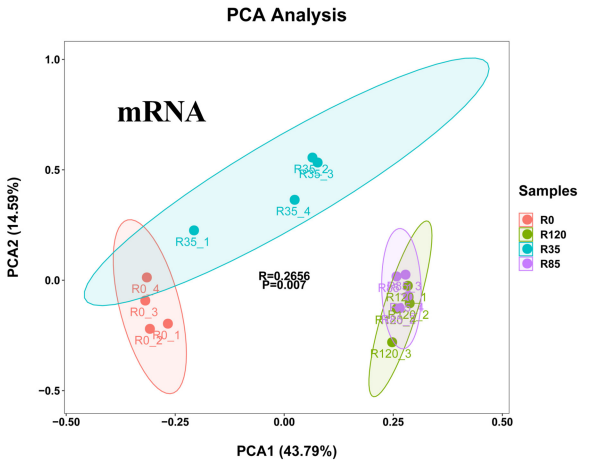

FIGURE 2
The PCA analysis of DE mRNAs and DE lncRNAs from 16 samples.

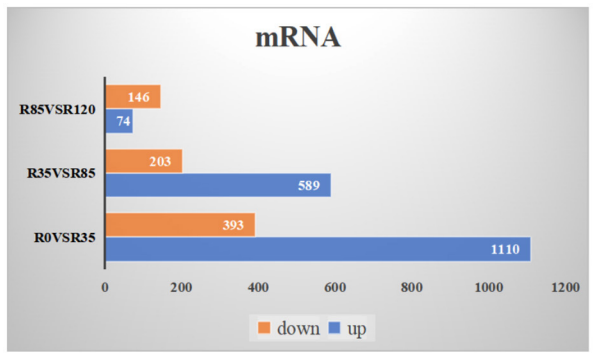

FIGURE 3
The number of up-regulated and down-regulated DE lncRNAs and DE mRNAs in rabbit liver during the adjacent growth stages.

KEGG pathways about regulated DE mRNAs, we summarized all the pathways predicted into six categories, including Cellular Processes, Genetic Information Processing, Metabolism and Organismal Systems (Figure 5B). These six categories were closely related to the function of the liver, and the results suggested that DE lncRNAs play important roles in the regulation of liver growth and development. Besides, Significant analyses of KEGG pathways found that 52 pathways were significantly enriched. Among these pathways, 11 pathways of lipid metabolism were found, including Arachidonic acid metabolism, Fatty acid degradation, *PPAR* signaling pathway and Biosynthesis of unsaturated fatty acids (Figure 5C). The expression level of mRNAs was also an important factor to determine whether they function or not. To this end, we excluded the low expression DE mRNAs (the mean of total expressions < 10 FPKM) which had been predicted in lipid metabolism, and finally got 54 DE mRNAs that may play roles in lipid metabolism during the growth and development of liver (Additional File 7). After excluding the low-expressed lncRNAs (the mean of total expression < 1 FPKM), we found that the 54 DE mRNAs were *cis* or *trans*-regulated by 249 DE lncRNAs in total (Additional File 8). In addition, through the expression trend analysis, we found that twelve of these mRNAs were consistently up-regulated during all the four growth stages of liver, including *ENSOCUG00000001375*, *EPHX1*, *ACSS2*, *CYP39A1*, *PCK2* and *APOA5*. While 6 mRNAs, such as *LDHB*, *FABP7*, *CA2*, *ELOVL2*, *CYP2E1* and *APOA1* were consistently down-regulated.

Interactions of key lncRNAs and mRNAs involved in liver lipid metabolism of rabbit

Based on the co-expression theory and the RNAs being selected in the previous step, we constructed the critical lncRNA-mRNA regulatory network during the growth

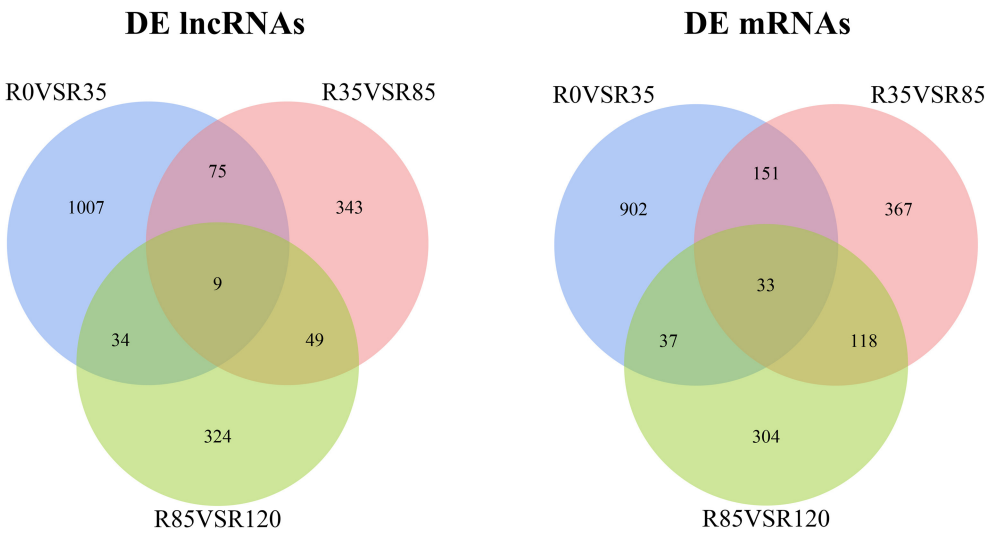

FIGURE 4
Venn diagrams showing the DE lncRNAs and DE mRNAs in rabbit liver during the growth stages.

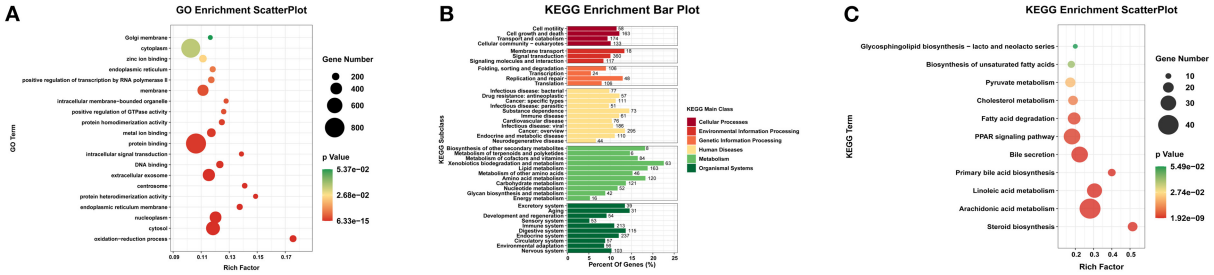

FIGURE 5
GO and KEGG pathway enrichment analysis of 2980 DE mRNAs. **(A)** The top 20 significant terms of GO enrichment analysis (p-value < 0.05). **(B)** Enrichment analysis of KEGG pathway of DE mRNAs. **(C)** The 11 KEGG pathways of lipid metabolism from the total pathways.

and development of liver about lipid metabolism in rabbit (Figure 6). The results showed that a total of 351 interaction pairs were identified among the 38 DE mRNAs and 215 DE lncRNAs, among which 293 positive interactions and 58 negative interactions were identified, indicating that most of the lncRNAs could positively regulate mRNA expression. Among these lncRNAs, *MSTRG.29138.11* had the most interactions with mRNAs, while the mRNA *CA2* was co-regulated by multiple lncRNAs, and most mRNAs only interacted with a single lncRNA, such as *PECR* and *ADH4*. In addition, the vast majority of lncRNAs interacted with mRNAs through trans-regulated, while only *MSTRG.30313.1* by *cis*-regulated, while *MSTRG.2502.1*, *MSTRG.30424.1* and *MSTRG.14429.1* by both *cis* and *trans*-regulated. We also found that some unnamed mRNAs, such as *ENSOCUG00000001375*, *ENSOCUG00000008325*, and *ENSOCUG00000037867* had strong interactions with lncRNAs.

Construction of target protein-protein interaction (PPI) network

To gain further insight into lipid metabolism differing between different stages of liver, we performed the PPI network analysis to identify the potential hub regulatory mRNAs. Based on centrality measures and FC values of the 54 DE mRNAs which obtained in the previous step and the String database analysis, the protein-protein interaction network consisting of 26 nodes and 87 edges was constructed using Cytoscape (Figure 7). We identified highly interconnected hub mRNAs of functional network like *SQLE*, *FDFT1*, *DHCR24*, *LSS*, *SC5D*, *EBP*, *ACSS2*, *CYP51A1*, *CYP7A1* and *CYP2E1*. Among them, *ACSS2* as the seed mRNA was enriched in the Pyruvate metabolism pathway, while *SQLE*, *LSS*, *FDFT1*, *DHCR24*, *EBP* and *SC5D* were enriched in the steroid biosynthesis pathway. In addition, *CYP7A1* was enriched in the *PPAR* signaling

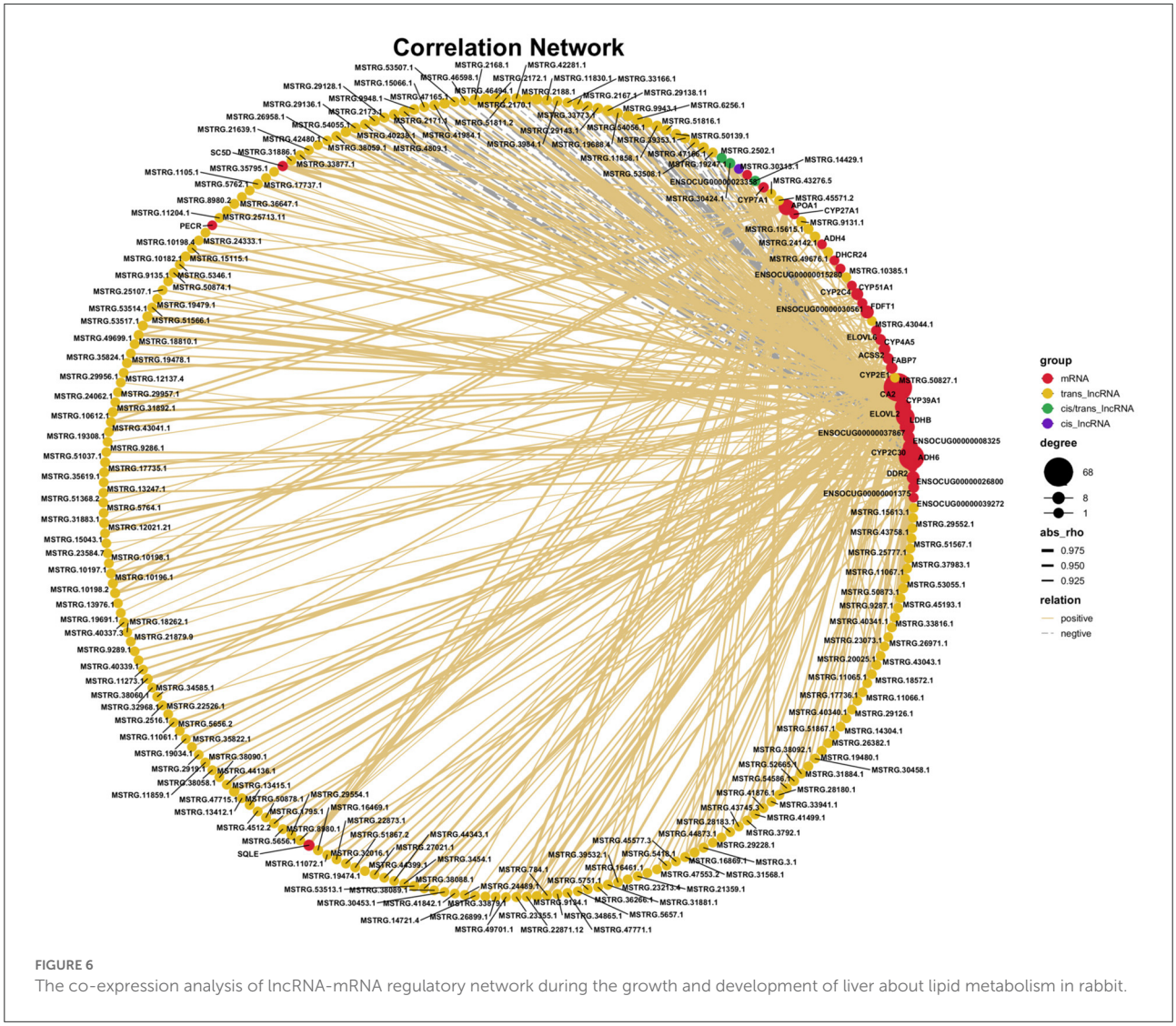

FIGURE 6
The co-expression analysis of lncRNA-mRNA regulatory network during the growth and development of liver about lipid metabolism in rabbit.

pathway. Considering their critical positions in the PPI network, these hub mRNAs are expected to be likely more critical than other mRNAs.

Validation of DE lncRNAs and mRNAs with RT-qPCR

To validate the accuracy of FPKM results from RNA-seq, our study randomly selected six DE lncRNAs and six DE mRNAs to evaluate their expression levels at the four growth stages by RT-qPCR. The RT-qPCR results demonstrated that six lncRNAs (*MSTRG.30313.1*, *MSTRG.29143.1*, *MSTRG.29138.11*, *MSTRG.43041.1*, *MSTRG.15066.1*, *MSTRG.10182.1*) and the mRNAs (*APOA5*, *CYP51A1*, *DHCR24*, *CYP2C4*, *ACSS2*, *APOA1*) were differentially expressed at the four growth stages. In addition, except for *MSTRG.43041.1*, the expression levels of the rest genes had the similar trends with the RNA-seq results (Figure 8). Therefore, the FPKMs obtained from RNA-seq were accurate and reliable with the detections of RT-qPCR.

Discussion

Lipid metabolism include a series of biological transformation processes such as the digestion, absorption, translocation, decomposition, transformation, and excretion of lipids in a biological organism (35). It's one of the body's basic metabolic activities and is important for regulation of the body's blood lipid balance and liver fat deposition (36). LncRNA expressions have tissue and disease specificity, because of this property that lncRNAs have been used in clinical study (37). Besides, lncRNAs have been used as biomarkers in a variety of cancers (38, 39). Disorders of liver lipid metabolism can cause

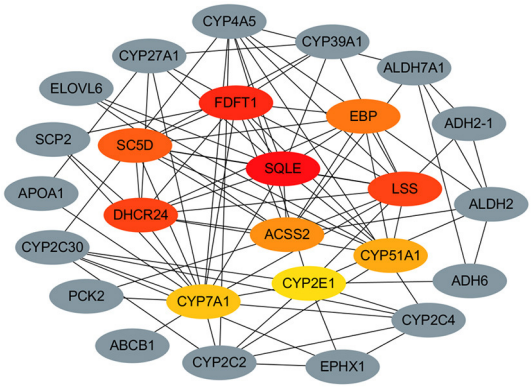

FIGURE 7
The Protein-protein interaction (PPI) network analysis of the hub regulatory mRNAs in lipid metabolism of liver during the rabbit growth stages.

non-alcoholic fatty liver disease, non-alcoholic steatohepatitis and even develop liver cancer and liver cirrhosis. However, mild and early fatty liver has no obvious symptoms, so it is not easy to detect. Using the characteristics of DE lncRNAs between normal individuals and those with abnormal hepatic lipid metabolism, early diagnosis, early treatment and prognosis evaluation of fatty liver can be achieved. The study is to explore the expression profiles and interaction networks of lncRNAs and mRNAs related to the regulation of lipid metabolism during the growth and development of liver, and to provide more effective therapeutic targets for human fatty liver diseases.

At present, there were no reports on the physiological changes of liver and transcriptomics research on regulating lipid metabolism in different growth stages of human or common animal models (mice, rats and rabbits). In this study, Oil red O staining detected rabbit liver tissues at different growth stages, and we found that lipid deposition in liver tissues was closely related to animal age. Therefore, it was of great value to explore the transcriptome variation rules of epigenetic modification refracted behind this natural phenomenon and to find the possible regulatory molecular network and major genes. In this study, RNA-seq was performed on 16 liver tissue samples from four important growth stages of rabbits. Through PCA analysis of transcriptomes from different stages samples, we found that the changes of mRNAs and lncRNAs expression in livers of rabbits liver before 35 days were more obvious than those in later stages (85 days and 120 days). The similarity between the 85 days and 120 days was high, and the number of different lncRNAs and mRNAs gradually decreased by comparison in adjacent growth stages. 35 days after birth was the weaning node of rabbits. Before 35 days, rabbits mainly fed on mother's milk. After 35 days, the diets and living environment of experimental rabbits were consistent in each stage. Gene expression is influenced by many factors such as heredity and environment, this shows that age is an important factor for epigenetic changes of liver without changing external diet and environment.

So far, there have been many mechanisms that lncRNAs regulate mRNAs' expression, including chromatin structure modification, RNA transcription, splicing, editing and translation (40), and participate in the occurrence and development of diseases (41). And *cis*-regulation (the expression of its adjacent genes, participating in the regulation of genes in nucleus) and *trans*-regulation (expression of genes across chromosomes, depending on the free energy required for the formation of secondary structure between sequences) are the main ways of lncRNAs regulating mRNAs. Through the prediction of the *cis*/*trans*-regulation of DE mRNAs by DE lncRNAs, we found that all DE mRNAs were regulated by DE lncRNAs in varying degrees, and the regulatory relationship was complex, while the mutual regulation mainly existed in the form of energy combination. Through the functional enrichment analysis of DE mRNA, we found that these DE mRNA could be classified into six categories: Cellular Processes, Genetic Information Processing, Environmental Information Processing, Human Diseases, Metabolism and organic Systems. The functional attributes of genes and their expression level in organs or cells are important factors to determine their function realization (42). When we excluded some genes with low FPKM, we further obtained that 54 DE mRNAs may play the key roles in lipid metabolism during the growth and development of liver, including *EPHX1*, *ACSS2* and *CYP39A1*, which were continuously up-regulated in four growth stages, and *LDHB*, *CA2*, *ELOVL2*, which were continuously down-regulated, and these DE mRNAs were regulated by 249 lncRNAs. To further screen the DE lncRNAs that regulated these 54 mRNAs, the number of DE lncRNAs was reduced from 249 to 215 again by co-expression analysis, and we found that most of lncRNAs had positive regulation with mRNAs. Among 215 lncRNAs, the mRNAs regulated by *MSTRG.29138.1* was the largest, including *LDHB*, *ELOVL2*, *ADH6*, *FDFT1* and so on.

It is of great value to further identify the potential central regulatory mRNAs and clarify the core target of regulating hepatic lipid metabolism for the prevention and treatment of diseases related to hepatic lipid metabolism disorder. With the help of PPI network analysis, we found hub mRNAs with highly interconnected functional networks from 54 DE mRNAs, including *SC5D*, *EBP*, *ACSS2* and *CYP7A1*, among which *ACSS2* was the seed mRNA. Related studies have found that *ACSS2* is located in the cytoplasm and nucleus of mammals, and which is the main subtype that catalyzes the production of acetyl coenzyme A from free acetic acid to synthesize fatty acids (43), and lacking *ACSS2* significantly reduced body weight and reversed hepatic steatosis in a diet-induced obesity mice model, suggesting that *ACSS2* may have a therapeutic role in the treatment of fatty liver. Silencing of hepatic ACSS2 potently suppresses the conversion of fructose into hepatic

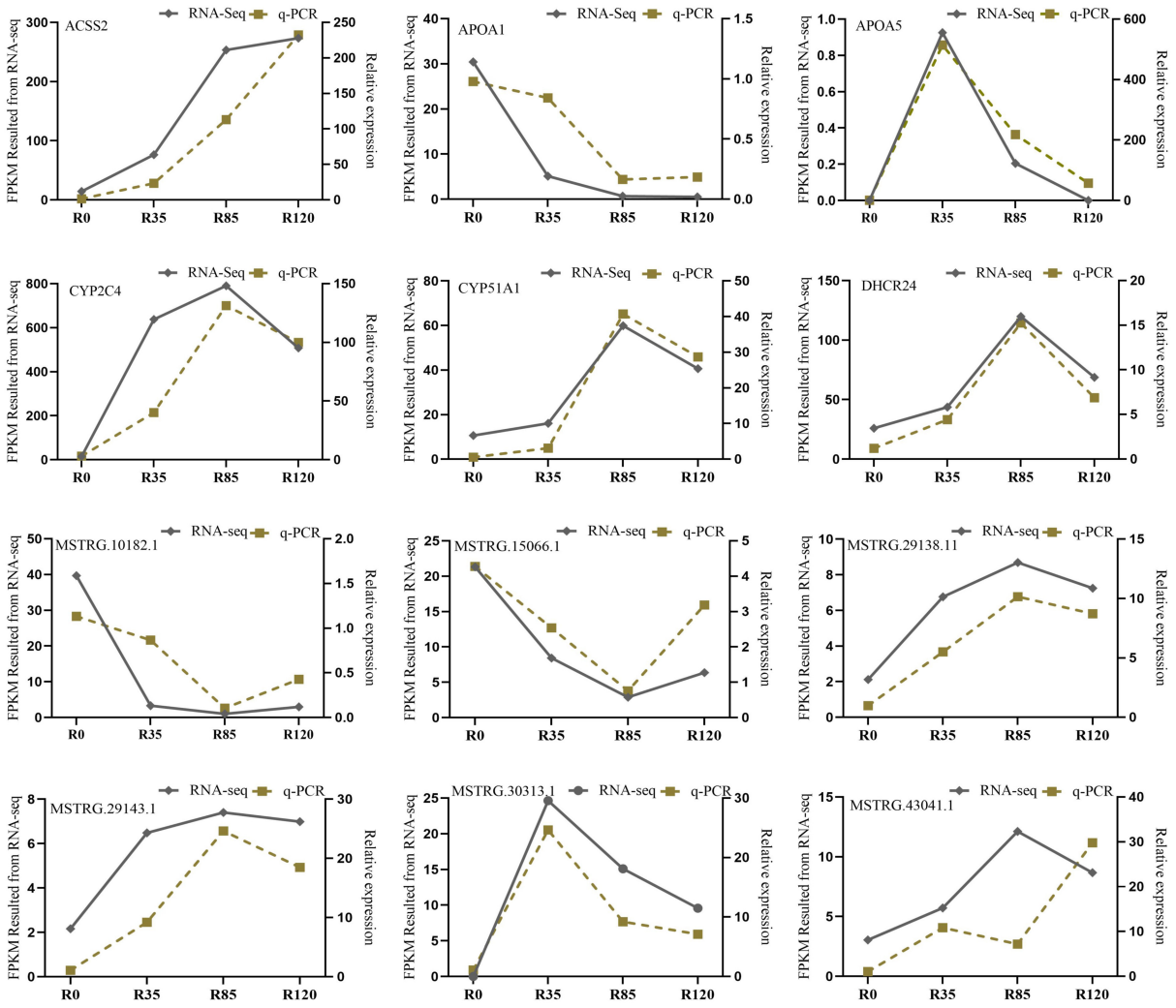

FIGURE 8
RT-qPCR verification for six lncRNAs and six mRNAs in rabbit four growth stages.

acetyl-CoA and fatty acids (44). In addition, ACSS2 may be an important linker in obesity-related myeloma (45). Cytochrome P450 protein is a monooxygenase that catalyzes many reactions of drug metabolism and the synthesis of cholesterol, steroids and other lipids. *CYP51A1* gene encodes a member of cytochrome P450 superfamily of enzymes which is related to lipid metabolism (46). *CYP51A1* is highly expressed in rabbit liver tissue, and its expression gradually increases with age. In the early growth and development of rabbits, a large amount of synthetic cholesterol is required to participate in the growth and development of the body and cell life activities, such as the formation of cholic acid, the formation of cell membranes and the synthesis of hormones. *CYP51A1* is involved in many reactions in the synthesis of cholesterol, steroids, and is the key enzyme in the synthesis of cholesterol and other substances. Our research shows that lncRNA *MSTRG.30424.1*, *MSTRG.28180.1*, *MSTRG.784.1*, *MSTRG.8980.1* and *MSTRG.22526.1* can positively regulate *ACSS2*, LncRNA *MSTRG.43041.1*, *MSTRG.32968.1*, *MSTRG.34585.1* etc. could positively regulate *CYP51A1*. In addition, It was reported that the siRNAs targeting *DHCR24* protect cells from the liposome-induced cell death, probably by reducing production of reactive oxygen species and lowering the cellular cholesterol in the generation (47). Therefore, the function of most hub mRNAs screened by the research has been reported in lipid metabolism, confirming the accuracy of our screening from the side.

In general, this study explored the expression patterns of lncRNA and mRNA transcripts in rabbit livers at different growth stages, and with the help of PPI and other bioinformatics analysis and screening methods, finally confirmed that 215 DE lncRNAs and their regulated 38 mRNAs mediated the role of lipid metabolism during the process of liver growth, which

provided important reference targets for further research at the cellular and animal levels, and also provided a theoretical reference for the regulation of clinical intervention targets of liver lipid metabolism disorders.

Conclusions

In this study, the main key 38 mRNAs affecting liver lipid metabolism were screened in rabbit models at different growth stages, including *ACSS2*, *CYP51A1* and *DHCR24*, and the potential 215 lncRNAs regulating these key mRNAs were also identified, like *MSTRG.30424.1*, *MSTRG.8980.1*, *MSTRG.43041.1*, *MSTRG.32968.1*. It provides transcriptome data reference for the study and application of rabbit model in liver disease, and also provides potential research targets for the prevention and treatment of related diseases caused by liver lipid metabolism disorder.

Data availability statement

Most of the tables in this study are provided as the additional files, and the whole RNA-seq reads of 16 rabbit samples can be found at http://www.ncbi.nlm.nih.gov/sra with the accession codes (BioProject ID: PRJNA865562).

Ethics statement

The animal study was reviewed and approved by Guizhou Medical University experimental animal operation regulations and welfare management committee.

Author contributions

SZho and PL made the same contribution to the paper. PL and GW conceived and designed the research. GW and ML conducted the experiment and wrote the paper. JM and SZha analyzed the data. YW, BW, and HP modified manuscript. All authors read and approved the final manuscript.

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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.2022.998796/full#supplementary-material

ADDITIONAL FIGURE 1
Identification of lncRNA and mRNA expression profiles of liver during the rabbit growth stages. **(A)** Expression level analysis and the total number of lncRNAs and mRNAs. **(B)** The quantitative distribution of lncRNAs and mRNAs in each Chromosome. **(C)** The Length distribution of lncRNAs and mRNAs. **(D)** Exon number distribution of lncRNAs and mRNAs. **(E)** Distribution of open reading frame (ORF) length in lncRNAs and mRNAs.

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Effects of *Chinese yam* polysaccharides on the immune function and serum biochemical indexes of broilers

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The purpose of this experiment was to investigate the effects of *Chinese yam* polysaccharides (CYP) in diets on the immune function of broilers. A total of 360 (1-day-old, sex balance) healthy growing broilers with similar body weight (39.54 ± 0.51 g) were randomly divided into control (0.00 g/kg), CYP I (0.25 g/kg), CYP II (0.50 g/kg), and CYP III (1.00 g/kg) groups. Each group contains 3 replicates with 30 broilers in each replicate, and the feeding trial lasted 48 d. The results showed that compared with the control group, the CYP II group had higher thymus index, serum IgA, complement C3, C4, IGF-I, T_3, T_4, INS, GH, IL-2, IL-4, IL-6, and TNF-α levels ($P < 0.05$) at 28, 48 d, respectively. In addition, the spleen index, serum IgM and IgG concentrations in CYP II group were higher than those in the control group at 28 d ($P < 0.05$). Results indicated that 0.50 g/kg CYP supplementation improved the immune function of broilers, and the CYP has a potential biological function as a green additive in broilers.

KEYWORDS

***Chinese yam* polysaccharides, broilers, immune organ index, cytokine, serum**

Introduction

In intensive breeding and production, improving the nutrient utilization rate and immunity are the key to ensure efficiency and health in the livestock and poultry industry (1, 2). However, antibiotic abuse in actual production has caused a tremendous negative influence on the livestock and poultry industry, such as producing antibiotic residues, inducing bacterial resistance and reducing animal immune function. In 2020, the Chinese government banned the addition of antibiotics to animal feed. Therefore, the research and development of safe, efficient and green antibiotic substitutes for livestock and poultry are necessary. Plant-active polysaccharides are macromolecules with a variety of biological activities that can improve the growth performance and immune function in poultry. At present, these substances have become the research hotspot of antibiotic substitutes for livestock and poultry (3, 4).

Chinese yam (CY) is the dried rhizome of *Dioscoreae* plant that grows all over China and is a traditional Chinese herbal medicine with high edible value (5). The polysaccharide isolated from CY has low toxicity, high biological activity and biological characteristics of promoting growth, regulating immunity and the gastrointestinal tract

and reducing blood sugar (6–8). CYP can induce a specific immune response in animals, increase the number of immune cells, enhance the immune activity and phagocytosis of immune cells and stimulate the production of immunoglobulin and cytokines, thus regulating the immune function *in vivo* and *in vitro* and improving the cellular and humoral immune function of the body (9). CYP also plays essential roles in animals, such as improving mice insulin sensitivity; playing an anti-hyperlipidaemic role (10); promoting the proliferation of spleen lymphocytes; significantly increasing the levels of cytokines, and serum immunoglobulins in mice, and decreasing the content of total cholesterol (TC) and triglyceride (TG) in rat serum (11, 12). Dietary CYP supplementation also significantly increased the contents of tumor necrosis factor α (TNF-α) and IL-2 in the serum of weaned rats, suggesting that CYP can improve the immune response of rats (7).

CYP can improve immune function and serum biochemical indicators in mice, rats and pigs; however, its effect on immune organ index, serum immunoglobulins, complements and cytokines in broilers is unknown. In this study, the hypothesis is that different amounts of CYP could change the immune organ index, serum immunoglobulins, complements and cytokines of broilers, thereby improving their immune function and serum biochemical indexes. Therefore, an experiment was conducted to examine the effect of different amounts of dietary CYP addition on the immune function and serum biochemical indexes of broilers and to provide a theoretical basis for rational dietary CYP addition in broiler production.

TABLE 1 Ingredient and nutrient levels of the basal diet in each feeding phase for broilers.

Items	Composition (%)	
	Day 28	Day 48
Ingredients (%)		
Corn	60.00	63.50
Soybean meal	32.00	29.00
Wheat bran	1.00	-
Soybean oil	1.00	2.00
Fish meal	2.00	1.60
$CaHPO_4$	1.30	1.30
Limestone	1.40	1.30
NaCl	0.30	0.30
Premix[a]	1.00	1.00
Total	100.00	100.00
Nutrient levels (%)		
Metabolic energy, (MJ/kg)[b]	12.13	12.55
Crude protein	21.00	20.00
Calcium	1.00	0.90
Total P	0.65	0.60
Available P	0.45	0.35
Lysine	0.50	0.38
Methionine	1.10	1.00

[a]Premix supplied per kg: VA 3000 IU,VD_3 500 IU, VE 10 IU, VK_3 0.5 mg,VB_6 3.5 mg, VB_1 3.8 mg, D-pantothenic acid 10 mg, folie acid 0.5 mg, biotin 0.15 mg, Fe 80 mg, Cu 8 mg, Zn 75 mg, Mn 60 mg, Se 0.15 mg.
[b]Metabolic energy was calculated by value and others were measured values.

Materials and methods

Experimental diets and animal management

A total of 360 (1 day old, gender-balanced) healthy growing broilers with similar body weight (39.54 ± 0.51 g) were randomly divided into four groups (Control, CYP I, CYP II and CYP III) individually consisting of three replicates with 30 broilers each. The control group was fed a basal diet without CYP, and the CYP I, CYP II and CYP III groups were fed a basal diet added with 0.25, 0.50 and 1.00 g/kg CYP, respectively. Feeding trials were divided into an initial phase (1–28 days) and an end phase (29–48 days). The basal diet was formulated to meet or exceed the NRC (1994) nutrient requirements for broilers. The composition and nutrient levels of the basal diet are shown in Table 1. During the whole trial, all broilers had *ad libitum* access to equally complete feed and water using nipple drinkers. The data on feed intake were a part of another article that is being prepared for publication elsewhere. In simple terms, the average daily feed intake (g/d) of the following: 1–28 d (23.50 ± 0.75 control group, 27.36 ± 0.23 CYP I group, 28.88 ± 0.03 CYP II group and 26.06 ± 0.01 CYP III group), 29-48 d (89.16 ± 1.38 control group, 97.72 ± 0.02 CYP I group, 98.78 ± 0.09 CYP II group and 97.19 ± 0.03 CYP III group),1-48 d (56.33 ± 0.50 control group, 62.54 ± 0.14 CYP I group, 63.83 ± 0.02 CYP II group and 61.62 ± 0.04 CYP III group). The broilers were reared in appropriate confinement cage with controlled air temperature. The light was continuously turned on for 24 hours, and the temperature was held at 32°C for the first 3 days and then gradually cooled until 26 °C from days 4–21. The CYP in the study was purchased from Shanxi Hana Biotechnology Co., Ltd. (Shanxi, China) and had a carbohydrate level of sed from(CYP content a caramong them, monosaccharide types including glucose 99.48%, galactose 0.52%).

Blood sample collection

At 28 and 48 days, 24 broilers (six broilers of similar body weight in each group, two broilers per replicate, gender balance) of similar weight were randomly selected to collect blood samples. After weighing, the blood was collected from the wings' veins of broilers, placed in a centrifuge tube and allowed to coagulate at 4 °C. After centrifugation (3,000 g, 10 min, at

4°C), the serum was collected into sterile tubes and stored at −80 °C until analysis.

Determination of immune organ index

At 28 and 48 days, the experimental broilers were slaughtered. The thymus, bursa of Fabricius (BF) and spleen were dissected from each experimental chicken; the surface blood of the immune organ was wiped dry with clean absorbent paper and the connective tissues surrounding the immune organ were dissected and weighed. The formula for immune organ index was adopted from Chi (13) as follows:

$$Immune\ organ\ index = \frac{Immune\ organ\ weight\ (g)}{Live\ weight\ of\ broiler\ (kg)}$$

Assay of serum immunoglobulins and complements C3 and C4

At 28 and 48 days, the serum concentrations of IgM, IgA and IgG and complements C3 and C4 in experimental broilers were measured using respectively chicken IgM, IgA, IgG ELISA Kits, chicken C3 and C4 ELISA Kits purchased from Nanjing Jiancheng Institute of Biological Engineering (Nanjing, China).

Determination of serum biochemical indicators

At days 28 and 48, the serum concentrations of insulin-like growth factor I (IGF-I), insulin (INS), growth hormone (GH), triiodothyronine (T3) and thyroxine (T4) in chicken were measured using respectively chicken IGF-I, INS, GH, T3, T4 ELISA Kits (Beijing Northern Institute of Biotechnology, Beijing, China).

Assay of serum cytokines

At days 28 and 48, the serum concentrations of TNF-α and serum cytokine interleukin (IL)-2, IL-4 and IL-6 were determined using respectively chicken ELISA kits (Nanjing Jiancheng Institute of Biological Engineering Nanjing, China) in accordance with the manufacturer's instructions.

Statistical analysis

Statistical analysis of variance (ANOVA) was performed using the one-way ANOVA procedure of SPSS 26.0 for Windows (IBM Corp., Chicago, IL, USA). Significant differences among all treatments were measured at $P < 0.05$ by Duncan's multiple range tests. All data were presented as mean ± SEM (standard error of the means).

Results

Immune organ index

As shown in Table 2, the immune organ index of the thymus in the CYP II group was significantly higher than that in the control group at days 28 and 48. Meanwhile, the immune organ index of the spleen in the CYP II group was significantly higher than in the control group at day 28. The immune organ index of BF in the CYP II group was significantly higher than that in the CYP I and CYP III groups at day 48. Meanwhile, no significant difference among the four groups was found for the immune organ index of BF at day 28 and of spleen at day 48.

Serum immunoglobulins and complements C3 and C4

The effects of dietary CYP on the concentrations of serum immunoglobulins and complements C3 and C4 in broilers are shown in Table 3. The serum concentrations of IgA and complements C3 and C4 in the CYP II group were significantly higher than those in the control group at days 28 and 48 ($P < 0.05$). In addition, the serum IgG concentrations in the CYP II group were higher than those in the control, CYP I and CYP III groups at day 28 ($P < 0.05$). Meanwhile, no differences in serum IgM and IgG concentrations were found among all the groups at day 48 ($P > 0.05$).

Serum biochemical indicators

The effects of dietary CYP on serum biochemical indicators in broilers are shown in Table 4. The serum concentrations of IGF-I, INS, GH, T_3 and T_4 in the CYP II group were significantly higher than those in the control group at days 28 and 48 ($P<0.05$). In addition, the serum concentrations of GH, T_3 and T_4 in the CYP II group at day 28 and the serum concentrations of IGF-I and T_3 at day 48 were significantly higher than those in the control, CYP I and CYP III groups ($P < 0.05$).

Serum cytokines

As shown in Table 5, the serum concentrations of TNF-α, IL-2, IL-4 and IL-6 in the CYP II group were significantly higher than those in the control group at days 28 and 48 ($P < 0.05$). In

TABLE 2 Effects of CYP on the immune organ index in broilers (g/kg).

Item	CYP level (g/kg)[1]				SEM	*P*-value
	Control	CYP I	CYP II	CYP III		
Day 28						
Thymus	3.42^{b}	5.02^{a}	5.59^{a}	4.70^{a}	0.427	0.006
BF	0.83	0.97	1.00	0.96	0.960	0.395
Spleen	1.79^{c}	1.85^{bc}	2.21^{a}	2.12^{ab}	0.136	0.039
Day 48						
Thymus	2.82^{b}	3.53^{ab}	4.29^{a}	3.06^{b}	0.351	0.014
BF	0.66^{a}	0.55^{b}	0.60^{ab}	0.43^{c}	0.435	0.004
Spleen	1.86	2.31	2.38	2.24	0.394	0.582

In the same line, values with different lowercase superscripts indicate significant differences, and values with the same lowercase superscripts indicate no significant differences ($P < 0.05$). n = 6. BF, bursa of fabricius.
[1]CYP I, CYP II, CYP III represented the data of broilers fed with 0.25 g, 0.50 g, and 1.00 g CYP per kilogram of diet, respectively.

TABLE 3 Effects of CYP on the concentrations of serum immunoglobulins and complements C3 and C4 in broilers.

Item	CYP level (g/kg)[1]				SEM	*P*-value
	Control	CYP I	CYP II	CYP III		
Day 28						
IgM (ng/mL)	5866.64^{b}	6056.54^{b}	6369.87^{a}	6365.12^{a}	82.707	0.001
IgA (ng/mL)	8588.37^{c}	9049.87^{ab}	9331.47^{a}	8862.14^{bc}	152.481	0.007
IgG (ng/mL)	85.98^{c}	92.09^{b}	96.21^{a}	89.36^{b}	1.374	0.001
C3 (μg/mL)	713.88^{c}	867.57^{a}	874.65^{a}	824.37^{b}	15.540	0.001
C4 (μg/mL)	458.04^{c}	478.37^{b}	560.58^{a}	556.06^{a}	8.206	0.001
Day 48						
IgM (ng/mL)	5097.55	5092.81	5197.25	5140.28	72.155	0.481
IgA (ng/mL)	7915.66^{b}	8158.15^{ab}	8447.57^{a}	8087.75^{b}	146.233	0.038
IgG (ng/mL)	77.95	78.02	79.05	78.09	1.367	0.832
C3 (μg/mL)	747.88^{c}	845.62^{ab}	859.78^{a}	810.21^{b}	15.612	0.001
C4 (μg/mL)	519.02^{b}	511.79^{b}	570.52^{a}	561.48^{a}	7.564	0.001

In the same line, values with different lowercase superscripts indicate significant differences, and values with the same lowercase superscripts indicate no significant differences ($P < 0.05$). n = 6.
[1]CYP I, CYP II, CYP III represented the data of broilers fed with 0.25 g, 0.50 g, and 1.00 g CYP per kilogram of diet, respectively.

addition, the serum concentrations of IL-2 and IL-6 in the CYP II group were significantly higher than those in the CYP I and CYP III groups at days 28 and 48 ($P < 0.05$).

Discussion

The thymus, BF and spleen are the most critical immune organs of chickens. The development of immune organs is closely related to the immune organ index, which is usually used to reflect the immune function and health status of animals (14). In this experiment, 0.50 g/kg CYP supplementation significantly increased the immune organ index of broilers. As a result, the immune level of broilers was regulated. Numerous evidences demonstrated that plant-active polysaccharides can increase the index and enhance the status of animal immune organs. Song et al. (15) showed that 5 mg/kg supplementation of medicinal mushroom *Antrodia camphorata* polysaccharides significantly increased the immune organ index of spleen and BF in aseptic chickens compared with those in the control group after 14 days. Liang et al. (16) reported that 200 mg/ml supplementation of Taishan *Robinia pseudoacacia* polysaccharides can significantly increase the index of the spleen, BF and thymus and promote the growth and development of immune organs in chickens. Nan et al. (17) found that 50, 100 and 200 mg/kg supplementation of *Lycium barbarum* polysaccharides (LBP) can increase the immune organ index of the thymus and spleen in mice and improve the animal's immune function. Zhao et al. (18) also

TABLE 4 Effects of CYP on the serum biochemical indicators in broilers.

Item	CYP level (g/kg)[1]				SEM	*P*-value
	Control	CYP I	CYP II	CYP III		
Day 28						
IGF-I (μg/L)	36.11^{b}	38.62^{a}	38.99^{a}	35.91^{b}	0.655	0.002
INS (mU/L)	17.00^{b}	19.02^{a}	19.42^{a}	17.03^{b}	0.310	0.001
GH (μg/L)	18.24^{d}	19.59^{c}	21.51^{a}	20.21^{b}	0.243	0.001
T_3 (pmol/L)	314.21^{c}	340.65^{b}	368.02^{a}	338.20^{b}	6.176	0.001
T_4 (pmol/L)	925.12^{b}	964.36^{b}	1151.82^{a}	925.12^{b}	24.939	0.001
Day 48						
IGF-I (μg/L)	34.80^{c}	36.51^{b}	41.09^{a}	37.71^{b}	0.487	0.001
INS (mU/L)	16.77^{c}	17.56^{b}	20.18^{a}	19.76^{a}	0.225	0.001
GH (μg/L)	20.77^{b}	22.48^{a}	23.35^{a}	22.96^{a}	0.348	0.001
T_3 (pmol/L)	296.08^{d}	308.68^{c}	346.50^{a}	331.13^{b}	5.198	0.001
T_4 (pmol/L)	925.12^{c}	1125.67^{a}	1181.25^{a}	1030.84^{b}	17.676	0.001

In the same line, values with different lowercase superscripts indicate significant differences, and values with the same lowercase superscripts indicate no significant differences ($P < 0.05$). $n = 6$.
[1]CYP I, CYP II, CYP III represented the data of broilers fed with 0.25 g, 0.50 g, and 1.00 g CYP per kilogram of diet, respectively.

TABLE 5 Effects of CYP on the serum levels of cytokine TNF-a and IL-2, IL-4 and IL-6 levels in broilers.

Item	CYP level (g/kg)[1]				SEM	*P*-value
	Control	CYP I	CYP II	CYP III		
Day 28						
TNF-α (ng/L)	60.64^{b}	61.15^{b}	65.77^{a}	63.89^{a}	1.157	0.007
IL-2 (ng/L)	143.34^{d}	152.06^{c}	170.44^{a}	161.40^{b}	2.472	0.001
IL-4 (ng/L)	168.19^{b}	184.45^{b}	204.74^{a}	202.81^{a}	12.733	0.046
IL-6 (ng/L)	40.78^{d}	44.09^{c}	51.62^{a}	49.31^{b}	0.573	0.001
Day 48						
TNF-α (ng/L)	64.79^{b}	68.27^{a}	70.04^{a}	68.96^{a}	1.105	0.007
IL-2 (ng/L)	179.47^{c}	184.38^{c}	214.17^{a}	204.19^{b}	2.347	0.001
IL-4 (ng/L)	182.04^{c}	195.40^{b}	202.32^{a}	181.88^{c}	1.821	0.001
IL-6 (ng/L)	42.65^{d}	45.47^{c}	51.58^{a}	49.00^{b}	0.625	0.001

In the same line, values with different lowercase superscripts indicate significant differences, and values with the same lowercase superscripts indicate no significant differences ($P < 0.05$). $n = 6$.
[1]CYP I, CYP II, CYP III represented the data of broilers fed with 0.25 g, 0.50 g, and 1.00 g CYP per kilogram of diet, respectively.

found that 0.6 and 1.2 g/kg mulberry leaf polysaccharides (MLPs) additives could significantly increase the thymus and spleen indexes and help enhance the immune performance of piglets. Wang et al. (19) confirmed that 200 mg/kg dietary supplementation of *Camellia oleifera* cake polysaccharides increased the spleen weight and index of yellow feather broilers after 42 days compared with those in the control group, indicating that oil tea polysaccharides have positive effects on the immunity of broilers. Li et al. (20) observed that 50 mg/mL supplementation of Taishan *Pinus massoniana* pollen polysaccharide could significantly increase the immune organ index of the thymus, BF and spleen, thereby improving the immunity efficacy of immunosuppressed chickens. Combined with the above findings on plant-active polysaccharides, the present results showed that CYP can increase the relative weight and index of immune organs by promoting their growth and development and further improving their immune performance.

As important immunologically active substances, immunoglobulins (IgM, IgA, and IgG) and complements (C3, C4) are the main components of humoral immunity, which can enhance the body's immune response. The experiment results showed that adding 0.50 g/kg CYP to the diet can increase the serum levels of IgM, IgA, IgG, C3 and C4 in broilers. Previous study reported that polysaccharides are

natural active components in plants and one of the best options for future immune enhancers in animals. Long et al. (21) observed that 2000 mg/kg supplementation of LBP can significantly increase the serum IgM and IgA concentrations of chickens. This discovery is beneficial in enhancing the immune function of broilers. Zhao et al. (18) also found that adding 0.6 g/kg MLPs to the diet significantly increases serum IgG levels in weaned piglets, thus effectively improving their immune capacity. Liu et al. (22) confirmed that 0.2 g/L supplementation of *Hericium erinaceus* polysaccharides can significantly increase the serum levels of IgM, IgA, IgG and complements C3 and C4 in Muscovy duck, thus improving its immune function. Wu (4) showed that 1 g/kg supplementation of *Astragalus membranaceus* polysaccharides (AMP) increased the serum levels of IgM, IgA and IgG in broilers, indicating that AMP can be used as an immunostimulator to improve the immune function of broilers. Chen et al. (23) reported that adding 4000 mg/kg of LBP to the diet significantly increased the serum IgM and IgG levels of weaned piglets compared with those of the control group, indicating that LBP can enhance the immune status of weaned piglets. All of these results suggest that the increase of serum immunoglobulins and complements may be related to the kind of polysaccharides, type of animals, addition amount of polysaccharide, feeding period and feeding method.

This experimental study found that adding 0.50 g/kg CYP to the diet can increase the serum concentrations of IGF-I, INS, GH, T_3 and T_4 in broilers. These results indicated that CYP might improve the growth and metabolism of broilers by regulating their serum hormone levels. A previous study found that a daily injection of 100 mg/kg *Schisandra challenges* acidic polysaccharides for 8 weeks effectively increased the fasting INS concentrations in rats with type 2 diabetes (24). Wu et al. (25) confirmed that 0.2% supplementation of *Radix rehmanniae preparata* polysaccharides could increase the serum GH level in alpaca, thus improving its growth performance. Zeng et al. (26) also found that the 50 mL injection of APS for 21 consecutive days had a positive effect on increasing the serum concentrations of T_3 and T_4 in heat-stressed cows. These results showed that different plant-active polysaccharides could improve the serum hormone levels of animals to varying degrees and were consistent with the present experimental results. However, how the yam polysaccharide regulates the serum hormone level of broilers and its potential mechanism need to be further studied.

When the body is invaded by pathogens, it will initiate an immune response and protect itself by releasing a series of cytokines (11). TNF-α and IL-6 cytokines play eliminate inflammation, and TNF-α can influence other cells to produce additional inflammatory cytokines that will participate in the body's immune response (27, 28). IL-2 and IL-4 are essential regulators of cellular immune function and are secreted by the Th1 and Th2 cell subgroups, respectively (29, 30). Increasing evidences indicated that plant-active polysaccharides could regulate the levels of cytokines, such as TNF-α, IL-2, IL-4 and IL-6. Long et al. (21) found that supplementing 2000 mg/kg LBP in the diet can significantly increase the levels of TNF-α and IL-4 compared with those in the control group, thus confirming that LBP can enhance the immune activity of chickens. Park et al. (31) observed that the supplementation of *Platycodon grandiflorum* polysaccharides could increase the ability of mouse dendritic cells to secrete TNF-α, IL-2 and IL-6. Zhang et al. (32) reported that 50 and 100 mg/kg APS supplementation can promote chicken's lymphocyte proliferation and enhance the immune response of TNF-α and IL-2. Li et al. (20) observed that 50 mg/mL supplementation of Taishan *Pinus massoniana* pollen polysaccharides can significantly increase the IL-2 level of immunosuppressed chickens, proving that these polysaccharides could promote the immune regulation ability of cytokines. Mirzaie et al. (33) showed that the supplementation of *Chlorella Vulgaris* polysaccharides could induce peripheral blood mononuclear cells and promote IL-2 expression in broilers. In the present study, 0.50 g/kg CYP supplementation increased the serum concentrations of TNF-α, IL-2, IL-4 and IL-6. Our results were similar to the above reports and indicated that CYP can improve the immune activity and promote the immune regulation ability of cytokines.

In our research, the concentration of immune parameters, serum IgM, IgA, IgG, C3, C4, IL-2, IL-4, IL-6 and TNF-α, were decreased when the concentration of CYP was increased to 1.00 g/kg, Similar results have been reported in previous studies (4, 21). The reason may be that the high concentration of plant-active polysaccharides affects the emulsification, thus reducing the immune parameters of these experiment. However, the mechanism by which high concentrations of plant-active polysaccharides lead to decreased immune parameters requires further study.

Conclusion

The dietary supplementation of 0.50 g/kg CYP improved the immune organ index and the serum immunoglobulin, complement and cytokine levels of broilers. However, with the dietary supplementation of 1.00 g/kg CYP, the promoting effect of CYP tended to decrease. These data suggested that the dietary supplementation of 0.50 g/kg CYP can effectively enhance the immune function of broilers.

Data availability statement

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

Ethics statement

The animal study was reviewed and approved by Animal Protection and Utilization Committee of Henan Institute of Science and Technology.

Author contributions

JD: investigation, writing-original manuscript, and writing–review and editing. JZ, YC, and SW: data curation and formal analysis. MS: conceptualization, methodology, and supervision. ZM: project administration and funding acquisition. 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

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An *Artemisia ordosica* extract: Effects on growth performance, immune, and inflammatory response in lipopolysaccharide-challenged broilers

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Artemisia ordosica has been applied as a traditional Chinese/Mongolian medicine for treating csertain inflammatory ailments. This study was conducted to investigate the effect of *Artemisia ordosica* alcohol extract (AOAE) supplemented in diets on growth performance, immune, and inflammatory response in lipopolysaccharide (LPS)-challenged broilers. A total of 240 one-day-old Arbor Acre male broilers were randomly allotted into 5 groups with 6 replicates ($n = 8$), which were basal diet group (CON), LPS-challenge and basal diet group (LPS), LPS-challenge and the basal diet added with low (500 mg/kg), middle (750 mg/kg), and high (1,000 mg/kg) dose of AOAE groups (AOAE-L, AOAE-M, and AOAE-H), respectively. On d 16, 18, 20, 22, 24, 26, and 28, all broilers were injected intra-abdominally either with LPS or an equivalent amount of saline. Results showed that dietary AOAE alleviated the LPS-induced decrease in average daily gain and average daily feed intake in the broilers ($P < 0.05$). Dietary AOAE supplementation reversed the increased spleen index and the decreased bursa index in LPS-challenged broilers ($P < 0.05$). Moreover, feeding AOAE could mitigate the elevation of IL-1β in serum, liver, and spleen, IL-2 in serum and liver, IL-6 in serum and spleen, and the decrease of IgG in spleen, IgM in serum, liver, and spleen, and IL-4 in serum of the LPS-challenged broilers ($P < 0.05$). This study also showed that AOAE supplementation alleviated the increase of mRNA expression of *TLR4*, *MyD88*, *TRAF6*, *NF-κB p65*, *NF-κB p50*, *IL-1β*, and *IL-6*, and the decrease of gene expression of *IκBα* and *PPARγ* in liver and/or spleen of broilers challenged by LPS ($P < 0.05$). We speculated that AOAE administration could effectively alleviate LPS-induced inflammation via decreasing over-production of proinflammatory cytokines, ultimately relieving the growth inhibition of broilers caused by LPS. In conclusion, 1,000 mg/kg AOAE has a strong capacity

to enhance immunity and inhibit inflammation, and can be used as a potential novel feed additive with applications in treating inflammation-related diseases and bacterial infection in broilers.

KEYWORDS

Artemisia ordosica **alcohol extract, broiler, lipopolysaccharide challenge, growth performance, immunomodulation**

Highlights

- 1,000 mg/kg AOAE supplementation alleviated LPS-induced inflammation in broilers.
- 1,000 mg/kg AOAE supplementation relieved LPS-induced growth inhibition of broilers.
- The results of this study provided a theoretical basis for further developing *Artemisia ordosica* and its extract including AOAE as a feed additive in poultry diets to improve growth performance and immune function.

Introduction

Over the past decades, the modern broiler farming business has grown rapidly because of the advantages of poultry meat, including high production efficiency, low cost, no religious restriction, and multiple consumption options (1). Referring to related data and reports, the global demand for poultry meat is likely to grow at a peak rate of 121% between 2005 and 2050 (2). Admittedly, broilers can reach market weight in a shorter period of time due to genetic selection, while providing more meat with a higher feed efficiency than ever before via intensive farming model. However, in reality, as intensive breeding has continued to grow, the stress response caused by high farming density has followed, especially birds are more susceptible to a variety of stressors (such as nutritional, physiological/pathological and environmental) than other domestic species. Oxidative stress and inflammatory response are two of the physiological consequences of such stressors in broilers, which have an intense link and influence each other (3). This situation may not only affect broiler welfare but also have adverse effects on antioxidant status and immunity and increase the risks of facing biological damage, disease threats and even mortality, which ultimately result in poor performance and economic losses. According to statistics, the average annual economic burden induced by the prevalence of disease-causing pathogens and their metabolites pose in the global poultry industry is $3 to $6 billion (4). Consequently, with the rapid growth of the broiler farming industry and the fact that broilers continuously suffer from some type of stress, it is extremely urgent to supplement an immune modulator to enhance immune function, thus mitigating the adverse effects of stressors.

Antibiotics have been used as feed additives since 1950s to prevent and control diseases and indirectly accelerate the growth of animals, as well as have made a huge contribution to the poultry industry (5). Nonetheless, it is worth noting that the abuse of antibiotics not only affects the safety of animal products but also induces the emergence of a number of antibiotic resistant human pathogens and causes the deterioration of the ecological environment (6). What's more, following the rise of customers' attention to health and safety issues, the modern livestock production system has also begun to more consider the concept of clean, green, and ethical animal production practices (7). Considering these facts, the prohibition of supplementing antibiotics in diets of livestock have been implemented in many countries of the European Union since 2006, and China has instituted a comprehensive restriction on the use of antibiotics in poultry since 2020 as well (8, 9). Thus, it is imperative to study and develop green, economic, and effective alternatives to antibiotic used in poultry production. Recently, there is increasing interest in natural products, particular to traditional medicinal plants, as a source of alternative to antibiotics in feeds. It has been proven that many of the plant secondary metabolites can modulate the immune responses and disrupt the proinflammatory cascade through antioxidant mechanisms and/or variations in cell signaling, and ultimately, enhance the health of animals and their resistance to pathogens (10). Therefore, the form of herbal extract alternatives is widely applied to protect animals to relieve stress and improve health and performance in the Western as well as in many Asian countries, including China and India (10).

The immune and inflammatory reactions of broilers stimulated by stressors are the main pathogenesis of various immune-inflammatory diseases. Therefore, alleviating

Abbreviations: LPS, lipopolysaccharide; ADG, average daily gain; ADFI, average daily feed intake; F, G, feed-to-gain ratio; IL, interleukin; Ig, immunoglobulin; TLR4, toll like receptor 4; MyD88, myeloid differentiation factor 88; TRAF6, tumor necrosis factor receptor associated factor 6; NF-κB p65, nuclear factor kappa B p65; NF-κB p50, nuclear factor kappa B p50; IκBα, inhibitor of NF-κB alpha; IL-1β, interleukin 1 beta; IL-6, interleukin 6; PPARγ, peroxisome proliferator-activated receptor γ.

inflammation might be an efficient approach to treat inflammation-related diseases. Medicinal plants and their extracts have long been applied in traditional Chinese medicine and have shown potent pharmacological effects on metabolic and inflammatory diseases. *Artemisia ordosica* alcohol extract (AOAE) is isolated from *Artemisia ordosica* Krasch (*A. ordosica*, *Compositae* family, *Artemisia* genus), which is a traditional Chinese/Mongolian medicine. *A. ordosica* is a main and representative plant in dry areas of East Asia, especially in the north and northwest of China, such as Inner Mongolia, Xinjiang, and the rest (11). The whole plant of *A. ordosica*, even root, has been utilized as a folk medicine to treat rheumatoid arthritis, cold headache, sore throat, carbuncle, swollen boil, and nasal bleeding, etc. (12). In addition, owing to high nutritional value and also rich in flavonoid (13), terpenoids (14), polysaccharides (15), sterols (16), coumarins (17), acetylenes (18), and other bioactive compositions, mainly flavonoid and terpenoid compounds (19–23), *A. ordosica* and their extracts have multiple pharmacological activities, including antimicrobial (24), antioxidation (25), anti-inflammation (26), immunomodulation (27), and therefore can be efficiently applied in poultry and animal production to promote health. Our previous studies have verified that *A. ordosica* aqueous extracts, mainly comprised of flavonoids, terpenoids, organic acids, and polysaccharides, could effectively improve the growth and antioxidant capacity and modulate the immune function of broilers and weanling piglets (26, 27). However, as we have explored, in comparison with ethanol extraction technique, water extraction is lower in extraction rate and the contents of effective composition in extract, while the impurity components are higher. Consequently, as reported by Ghalia (28), an ethanol extract usually shows greater potential for improvement as compared to aqueous extract, which may be attributed to that the ethanolic extracts contain higher concentrations of alkaloids, phenols, steroids, tannins, flavonoids, or glycosides (29).

The endotoxin lipopolysaccharide (LPS), a primary component of the cell wall of gram-negative bacteria, can be recognized by immune cells as a pathogen-associated molecular pattern and consequently induces an inflammatory response, which reduces the feed intake and body weight gain, resulting in the animal's growth being inhibited (30). Intraperitoneal or intravenous injection of LPS can be used to effectively model oxidative stress and inflammatory damage caused by bacterial infection. Our previous study also successfully built inflammatory immune response model of broilers by injecting LPS, meanwhile, verified the *A. ordosica* aqueous extract in the basal diet could effectively alleviate LPS-induced immune overresponse in broilers by lessening the inflammatory cytokines and stress hormone (26). However, whether *A. ordosica* alcohol extract (AOAE) exerts beneficial effects on growth performance and immune function in broilers still remains unclear.

Therefore, the present study used ethanol, which is much less toxic than methanol, as a safer solvent to extract the bioactive components of *A. ordosica*, and aimed to investigate whether AOAE in diets could alleviate inflammatory damage and growth inhibition of broilers induced by LPS, so as to provide a theoretical basis for the application of *A. ordosica* and its alcohol extract in poultry production.

Materials and methods

This study was conducted after the approval by the Animal Care and Use Committee of Inner Mongolia Agricultural University and performed following the national standard Guideline for Ethical Review of Animal Welfare (GB/T 35892-2018).

Preparation of AOAE

Fresh *A. ordosica* (aerial part) was collected from Erdos (40.41° N and 110.03° E, Inner Mongolia, China) in July. Raw materials were washed with distilled water and shade-dried at room temperature. The dried materials were smashed and sieved (60 mesh), then the powder was degreased and removed pigments by petroleum ether in the Soxhlet apparatus for 12 h and dried again at room temperature to reduce petroleum ether residues. AOAE was prepared using the method described by Guo et al. (31). Briefly, after a series of above-mentioned processing, 30 g dry powder was steeped in 900 mL 60% ethanol aqueous solution (solid: ethanol = 1: 30), ultrasonic-assisted extraction for 1 h at 200 W power, heated reflux at 50°C to obtain the extracting solution. The resulting solution was filtered through a 0.45 μm filter and the filtrate was concentrated using a rotary vacuum evaporator (RE-5298, Shanghai Yarong Biochemical Instrument Factory, Shanghai, China), and then lyophilized by a vacuum evaporate to prepare the powder which was stored at −20°C until use. The total flavonoid content was determined according to Wang et al. (32), using rutin as a standard. The phenol sulfuric acid method was applied to evaluate total polysaccharide content based on Di et al. (33), and D-glucose was used as a reference standard. Results were expressed as rutin equivalents (mg RE/g) for total flavonoid, D-glucose equivalents (mg GE/g) for total polysaccharide, and the content of total flavonoid and total polysaccharide in AOAE was 556.1 mg RE/g and 145.9 mg GE/g, respectively.

Birds, experimental design, and diets

A total of 240 similar-sized (38.14 ± 0.36 g) healthy one-day-old Arbor Acres male broilers were purchased from a local commercial hatchery in Hohhot, Inner Mongolia, China, and

were randomly divided into five treatment groups with six replicates for each group and eight broilers in each replicate. Using a completely randomized trial design, the five treatments were as follows: (1) control group (CON), broilers received a basal diet and treated with 0.9% sterile saline; (2) LPS group (LPS), broilers received a basal diet and underwent LPS-challenge; (3) low-dose of AOAE group (AOAE-L), broilers received a basal diet supplemented with 500 mg/kg AOAE and underwent LPS-challenge; (4) middle-dose of AOAE group (AOAE-M), broilers received a basal diet supplemented with 750 mg/kg AOAE and underwent LPS-challenge; (5) high-dose of AOAE group (AOAE-H), broilers received a basal diet supplemented with 1,000 mg/kg AOAE and underwent LPS-challenge. According to our previous study, when AOAE level in basal diet was 750 mg/kg, broilers under normal rearing conditions had better growth performance, immunity and antioxidant capacity, therefore, the diet containing 750 mg/kg AOAE was chosen in the current experiment as a medium dosage treatment group. The experiment lasted for 42 days, divided into the starter phase (d 1 to 15), stress period (d 16 to 28) and convalescence (d 29 to 42). During stress period (on d 16, 18, 20, 22, 24, 26 and 28), the broilers were injected intraperitoneally either with LPS solution (*Escherichia coli*, serotype O55: B5, L2880; Sigma-Aldrich, St. Louis, MO, USA) at the dose of 750 μg/kg of body weight (BW) (LPS was dissolved in sterile saline at a concentration of 100 μg/mL) or with an equal dose of 0.9% sterile saline.

The feeding trial was conducted on the experimental farm of Inner Mongolia Agricultural University, Hohhot, China. According to the method reported by De Oliveira and Lara (34), the incremental lighting system was adopted in the whole experimental period. The temperature of the experimental room was set at 33°C for the first 3 days and then gradually reduced by 3°C every week, and reached a final temperature of 21°C. The relative humidity and ventilation were maintained at about 50–60% and 0.2–0.5 m/s, respectively. All broilers were routinely immunized and had *ad libitum* consumption of diet and water throughout the trial. The vaccination procedure was conducted as follows: the broilers were vaccinated with Newcastle disease and infectious bronchitis combined vaccine on d 7 and 20, Newcastle disease, infectious bronchitis and avian influenza triple vaccine on d 10, infectious bursal disease vaccine on d 14 and 24. All diets were fed in mash form and were based on corn-soybean meal and were formulated to meet or slightly exceed National Research Council (35) recommendations and nutrients recommendations of Feeding Standard of Chicken, China (NY/T 33-2004) (36) (Table 1).

TABLE 1 Composition and nutrient levels of the basal diet (as-fed basis), %.

Items	1 to 21 days of age	22 to 42 days of age
Ingredients		
Corn	52.50	58.80
Soybean meal	40.00	33.80
Soybean oil	3.00	3.00
Dicalcium phosphate	1.90	1.80
Limestone	1.08	1.22
Salt	0.37	0.37
Lysine	0.05	0.03
Methionine	0.19	0.07
Premix[1)]	0.80	0.80
Choline chloride	0.11	0.11
Total	100.0	100.0
Nutrient levels[2)]		
Metabolic energy (MJ/kg)	12.42	12.62
Crude protein	21.77	19.65
Calcium	1.00	1.02
Available phosphorus	0.44	0.42
Lysine	1.34	1.15
Methionine	0.55	0.40
Cystine	0.40	0.36

[1)] Premix provided the following per kilogram of diet: vitamin A 9000 IU, vitamin D_3 3000 IU, vitamin E 26 mg, vitamin K_3 1.20 mg, vitamin B_1 3.00 mg, vitamin B_2 8.00 mg, vitamin B_6 4.40 mg, vitamin B_{12} 0.012 mg, nicotinic acid 45 mg, folic acid 0.75 mg, biotin 0.20 mg, calcium pantothenate 15 mg, Fe 100 mg, Cu 10 mg, Zn 108 mg, Mn 120 mg, I 1.5 mg, Se 0.35 mg.
[2)] Crude protein was measured value, while others were all calculated values.

Sample collection and preparation

Broilers were weighed on days 1, 15, 28, 42, and the feed consumption on each replicate basis was recorded on days 15, 28, and 42 to calculate the average daily gain (ADG), average daily feed intake (ADFI) and feed-to-gain ratio (F: G) for each period.

On d 28 and 42, one broiler was randomly selected from each replicate to collect blood sample from the wing vein using a vacuum non-anticoagulant tube after anesthesia with sodium pentobarbital, and then euthanized by cervical dislocation and dissected to collect the spleen, thymus, bursa of Fabricius and to weigh them after stripping fat. Serum sample was detached and collected via centrifugation at 3,000 × g for 15 min at 4°C and then immediately stored at −20°C until further analysis. The values for immune organ index were calculated by the following equation:

$$\textit{Organ index} = \textit{organ weight}\ [g]/\textit{BW}\ [kg]$$

Spleen and liver samples were collected according to the above methods and frozen immediately in liquid nitrogen, then stored at −80°C for preparation of homogenate and total RNA isolation.

Determination of immune indexes in serum and tissue samples

Liver and spleen tissue samples were minced and homogenized with ice-cold saline (wt/vol, 1: 9), then centrifuged at 4,000 × g for 15 min at 4°C. Interleukin-1 beta (IL-1β), interleukin-2 (IL-2), interleukin-4 (IL-4), interleukin-6 (IL-6), immunoglobulin A (IgA), immunoglobulin G (IgG), and immunoglobulin M (IgM) concentrations in the serum and tissue homogenate supernatant were analyzed using ELISA kits (Quanzhou Ruixin Biological Technology Co., Ltd. Fujian, China) following the manufacturer's instructions. Coomassie brilliant blue assay was used to determine the protein of the homogenate according to the instructions of the commercial kits (Nanjing Jiancheng Institute of Bioengineering, Nanjing, China).

Total RNA extraction and reverse transcription

Total RNA from liver and spleen samples was obtained using Trizol reagent (TaKaRa Biotechnology Co. Ltd, Dalian, China). The whole extraction process was carried out in a closed sterile environment without enzymes to avoid contamination. The purity and quantity of the total RNA were assessed with a spectrophotometer (Pultton P200CM, San Jose, CA, USA). Subsequently, the total RNA was treated with DNase I (TaKaRa) to remove DNA.

Total RNA was reverse transcribed to cDNA on LifeECO (TC-96/G/H(b)C, BIOER, Hangzhou, China) using TB® Green qPCR method with a Prime Script™ RT reagent kit with gDNA Eraser (TaKaRa Biotechnology Co. Ltd., Dalian, China). The reactions were incubated for 15 min at 37°C, followed by 5 s at 85°C.

Quantitative Real-time PCR

Real-time PCR was performed using the QuantStudio®5 real-time PCR Design & Analysis system (LightCycler® 480 II, Roche Diagnostics, USA) with a TB® Premix Ex Taq™ Kit (Takara Biotechnology Co. Ltd., Dalian, China). The reactions were: 95°C for 30 s (hold stage), followed by 40 cycles of 95°C for 5 s, 60°C for 30 s, and 72°C for 20 s (PCR stage), then 95°C for 15 s, 60°C for 1 min, 95°C for 15 s (melt-curve stage). A subsequent dissociation stage produced a melting curve to verify the specificity of the amplified products as described in the previous report (37). The mRNA expression of each gene was normalized to that of β-actin. The fold change relative to the control group was analyzed according to the $2^{-\Delta\Delta CT}$ method. The specific sequences of primers are listed in Table 2.

Statistical analysis

Data obtained are expressed as the mean with standard deviation (SD). The analyses of all data were performed by one-way ANOVA using GLM procedure of SAS 9.2 (SAS Institute Inc., Cary, NC, USA) with replicate as the experimental unit. Tukey's multiple range test was used to compare the mean values ($P < 0.05$) to show the significant differences.

Results

Growth performance

As shown in Table 3, dietary AOAE showed no significant effect on growth performance in broilers before LPS challenge (days 1 to 15) ($P > 0.05$). Compared with the control group, the broilers with LPS intraperitoneal injection alone presented a significant decrease in ADG and ADFI during the stress period and convalescence ($P < 0.05$). In contrast, the group that was supplemented with 1,000 mg/kg AOAE exhibited significant increase in ADG and ADFI ($P < 0.05$) compared with the LPS group, and had no significant difference compared to the control group ($P > 0.05$). Moreover, in the convalescence (days 29 to 42), broiler ADG in the AOAE-M group (LPS-challenged broilers fed a basal diet supplemented with 750 mg/kg AOAE) was higher than that in the LPS group ($P < 0.05$). Over the whole experimental period, there was no significant difference in the feed-to-gain ratio among broilers fed with AOAE or LPS-challenged ($P > 0.05$).

Immune organ index

The effects of AOAE on the relative weight of immune organs in broilers challenged with LPS are shown in Table 4. As described in Table 4, on day 28, the spleen index markedly increased while the bursa of Fabricius index markedly decreased in the LPS group relative to broilers in the CON group, whereas this negative effect was obviously alleviated by feeding 750 and 1,000 mg/kg AOAE ($P < 0.05$). Likewise, compared with the control group, LPS caused increase of spleen index which was significantly inhibited by the treatments with AOAE at both 750 and 1,000 mg/kg on day 42 ($P < 0.05$). Broilers in AOAE-M and AOAE-H group (LPS-challenged broilers fed a basal diet supplemented with 1,000 mg/kg AOAE) had comparable relative weight of spleen (d 28 and 42) to broilers in the CON group ($P > 0.05$). There was no obvious difference in thymus index between LPS- and/or AOAE-treated broilers over the whole experimental period ($P > 0.05$).

TABLE 2 Primer sequences and parameter.

Genes	GenBank accession No	Primer sequences, 5′-3′	Length, bp	References
TLR4	NM_001030693	F-TTCAGAACGGACTCTTGAGTGG	131	Xing et al. (15)
		R-CAACCGAATAGTGGTGACGTTG		
MyD88	NM_001030962	F-CCTGGCTGTGCCTTCGGA	198	Xing et al. (15)
		R-TCACCAAGTGCTGGATGCTA		
TRAF6	XM_421089	F-GAGTGTCCAAGGCGTCAAGTCTG	243	Lu et al. (38)
		R-GTGTCGTGCCAGTTCATTCCTC		
NF-κB p65	D13721	F-CAGCCCATCTATGACAACCG	151	Xing et al. (15)
		R-CAGCCCAGAAACGAACCTC		
NF-κB p50	NM_205134	F-GAAGGAATCGTACCGGGAACA	80	Cheng et al. (39)
		R-CTCAGAGGGCCTTGTGACAGTAA		
IκBα	NM_001001472.2	F-GGCAGATGTGAACAAGGTGA	118	Yang et al. (40)
		R-TATCTGCAGGTCAGCTGTGG		
IL-1β	NM_204524	F-CAGCCTCAGCGAAGAGACCTT	84	Xing et al. (15)
		R-ACTGTGGTGTGCTCAGAATCC		
IL-6	HM179640	F-AAATCCCTCCTCGCCAATCT	106	Xing et al. (15)
		R-CCCTCACGGTCTTCTCCATAAA		
PPARγ	NM_001001460.1	F-TCGCATCCATAAGAAAAGCA	176	Zhang et al. (41)
		R-CTTCTCCTTCTCCGCTTCGT		
β-actin	NM_205518	F-GCCAACAGAGAGAAGATGACAC	118	Xing et al. (15)
		R-GTAACACCATCACCAGAGTCCA		

TLR4, toll like receptor 4; MyD88, myeloid differentiation factor 88; TRAF6, tumor necrosis factor receptor associated factor 6; NF-κB p65, nuclear factor kappa B p65; NF-κB p50, nuclear factor kappa B p50; IκBα, inhibitor of NF-κB alpha; IL-1β, interleukin 1 beta; IL-6, interleukin 6; PPARγ, peroxisome proliferator-activated receptor γ; β-actin, beta-actin; F, forward primer; R, reverse primer.

TABLE 3 Effect of AOAE on growth performance of broilers challenged with LPS.

Items	Treatments					*P*-value
	CON	LPS	AOAE-L	AOAE-M	AOAE-H	
Starter phase (days 1 to 15)						
BW at day 1, g	38.07 ± 0.36	38.21 ± 0.49	38.17 ± 0.31	38.10 ± 0.46	38.18 ± 0.23	0.9672
ADG, g/d	26.45 ± 2.54	26.01 ± 2.60	27.12 ± 2.15	27.83 ± 1.48	28.50 ± 1.05	0.2514
ADFI, g/d	36.08 ± 2.98	36.37 ± 2.32	35.24 ± 3.93	36.99 ± 2.44	37.91 ± 1.22	0.5317
F: G	1.37 ± 0.03	1.40 ± 0.06	1.30 ± 0.14	1.33 ± 0.03	1.33 ± 0.06	0.2193
Stress period (days 16 to 28)						
BW at day 15, g	434.81 ± 38.03	428.33 ± 38.74	444.91 ± 32.11	455.48 ± 22.03	465.62 ± 15.90	0.2464
ADG, g/d	58.35 ± 3.96^{a}	49.22 ± 3.69^{c}	52.18 ± 5.20^{bc}	52.16 ± 4.82^{bc}	56.00 ± 4.12^{ab}	0.0120
ADFI, g/d	92.89 ± 7.34^{a}	80.30 ± 3.27^{c}	81.91 ± 9.36^{bc}	84.45 ± 7.03^{abc}	90.13 ± 9.45^{ab}	0.0378
F: G	1.60 ± 0.15	1.64 ± 0.14	1.58 ± 0.25	1.63 ± 0.14	1.61 ± 0.13	0.9803
Convalescence (days 29 to 42)						
ADG, g/d	85.73 ± 8.58^{ab}	73.17 ± 6.51^{c}	79.19 ± 5.15^{bc}	84.34 ± 9.43^{ab}	89.91 ± 5.56^{a}	0.0054
ADFI, g/d	160.37 ± 11.49^{a}	142.97 ± 11.82^{b}	155.55 ± 9.11^{ab}	155.05 ± 14.14^{ab}	164.90 ± 6.93^{a}	0.0252
F: G	1.88 ± 0.15	1.96 ± 0.14	1.97 ± 0.16	1.84 ± 0.11	1.84 ± 0.15	0.3613

1) BW, body weight; ADG, average daily gain; ADFI, average daily feed intake; F: G, feed-to-gain ratio.
2) AOAE, Artemisia ordosica alcohol extract; CON, non-challenged broilers fed a basal diet; LPS, lipopolysaccharide-challenged broilers fed a basal diet; AOAE-L, lipopolysaccharide-challenged broilers fed a basal diet supplemented with 500 mg/kg AOAE; AOAE-M, lipopolysaccharide-challenged broilers fed a basal diet supplemented with 750 mg/kg AOAE; AOAE-H, lipopolysaccharide-challenged broilers fed a basal diet supplemented with 1,000 mg/kg AOAE.
3) Each value is shown as mean ± SD ($n = 6$); Different superscript letters (a, b and c) within the same row indicate significant difference between experimental groups ($P < 0.05$).

TABLE 4 Effect of AOAE on relative weight of immune organs in broilers challenged with LPS.

Items	Treatments					*P*-value
	CON	LPS	AOAE-L	AOAE-M	AOAE-H	
28 d						
Spleen index	0.93 ± 0.13^{c}	1.60 ± 0.21^{a}	1.39 ± 0.22^{b}	1.09 ± 0.16^{c}	0.96 ± 0.13^{c}	<0.0001
Thymus index	2.19 ± 0.33	2.52 ± 0.39	2.33 ± 0.30	2.29 ± 0.30	2.27 ± 0.26	0.4965
Bursa index	2.67 ± 0.37^{a}	1.73 ± 0.30^{c}	1.86 ± 0.13^{c}	2.25 ± 0.26^{b}	2.60 ± 0.45^{ab}	<0.0001
42 d						
Spleen index	1.07 ± 0.12^{b}	1.28 ± 0.13^{a}	1.18 ± 0.12^{ab}	1.11 ± 0.11^{b}	1.05 ± 0.11^{b}	0.0135
Thymus index	2.42 ± 0.44	2.39 ± 0.41	2.44 ± 0.30	2.39 ± 0.39	2.38 ± 0.31	0.9983
Bursa index	1.81 ± 0.21	1.80 ± 0.16	1.83 ± 0.17	1.84 ± 0.27	1.83 ± 0.25	0.9973

1) Index = organ weight (g)/body weight (kg).
2) AOAE, Artemisia ordosica alcohol extract; CON, non-challenged broilers fed a basal diet; LPS, lipopolysaccharide-challenged broilers fed a basal diet; AOAE-L, lipopolysaccharide-challenged broilers fed a basal diet supplemented with 500 mg/kg AOAE; AOAE-M, lipopolysaccharide-challenged broilers fed a basal diet supplemented with 750 mg/kg AOAE; AOAE-H, lipopolysaccharide-challenged broilers fed a basal diet supplemented with 1,000 mg/kg AOAE.
3) Each value is shown as mean ± SD (n = 6); Different superscript letters (a, b, and c) within the same row indicate significant difference between experimental groups ($P < 0.05$).

TABLE 5 Effect of AOAE on serum cytokines and immunoglobulins in broilers challenged with LPS.

Items	Treatments					*P*-value
	CON	LPS	AOAE-L	AOAE-M	AOAE-H	
28 d						
IL-1β, pg/mL	53.10 ± 3.85^{c}	69.57 ± 1.86^{a}	64.19 ± 3.51^{ab}	58.75 ± 6.72^{bc}	55.39 ± 5.82^{c}	<0.0001
IL-2, pg/mL	37.22 ± 2.07^{c}	45.36 ± 5.98^{a}	42.80 ± 2.83^{ab}	39.30 ± 1.41^{bc}	38.97 ± 5.25^{bc}	0.0100
IL-4, pg/mL	22.95 ± 3.20^{a}	17.88 ± 2.93^{c}	19.04 ± 1.17^{bc}	20.68 ± 2.37^{abc}	21.06 ± 1.33^{ab}	0.0104
IL-6, pg/mL	5.05 ± 0.16^{c}	9.97 ± 0.36^{a}	8.85 ± 0.30^{b}	5.41 ± 0.57^{c}	5.37 ± 0.60^{c}	<0.0001
IgA, μg/mL	35.57 ± 2.75	36.64 ± 1.80	36.91 ± 2.89	35.34 ± 4.01	35.85 ± 2.62	0.8552
IgG, μg/mL	258.45 ± 17.01	290.14 ± 28.03	284.71 ± 25.42	260.55 ± 45.62	262.79 ± 33.85	0.2807
IgM, μg/mL	130.73 ± 12.70^{a}	107.10 ± 4.86^{c}	115.30 ± 7.50^{bc}	125.13 ± 9.75^{ab}	126.65 ± 4.47^{a}	0.0003
42 d						
IL-1β, pg/mL	54.77 ± 8.45	59.94 ± 7.91	60.06 ± 9.51	60.05 ± 2.47	58.01 ± 6.13	0.6740
IL-2, pg/mL	36.40 ± 4.49	37.42 ± 3.95	35.61 ± 3.47	37.29 ± 2.62	36.38 ± 5.33	0.9377
IL-4, pg/mL	20.57 ± 3.00	19.51 ± 1.82	20.14 ± 2.13	19.07 ± 2.70	19.00 ± 1.98	0.7362
IL-6, pg/mL	5.01 ± 0.31	5.22 ± 0.65	5.13 ± 0.44	5.36 ± 0.25	5.52 ± 0.26	0.2495
IgA, μg/mL	32.29 ± 3.64	34.56 ± 5.11	31.29 ± 7.46	32.71 ± 5.25	31.23 ± 4.67	0.8190
IgG, μg/mL	314.14 ± 15.04	283.96 ± 10.68	283.19 ± 49.98	299.14 ± 39.19	292.13 ± 48.08	0.5792
IgM, μg/mL	150.55 ± 22.80	142.67 ± 8.93	142.24 ± 9.99	146.08 ± 12.90	155.11 ± 18.61	0.5719

1) IL-1β, interleukin 1 beta; IL-2, interleukin 2; IL-4, interleukin 4; IL-6, interleukin 6; IgA, immunoglobulin A; IgG, immunoglobulin G; IgM, immunoglobulin M.
2) AOAE, Artemisia ordosica alcohol extract; CON, non-challenged broilers fed a basal diet; LPS, lipopolysaccharide-challenged broilers fed a basal diet; AOAE-L, lipopolysaccharide-challenged broilers fed a basal diet supplemented with 500 mg/kg AOAE; AOAE-M, lipopolysaccharide-challenged broilers fed a basal diet supplemented with 750 mg/kg AOAE; AOAE-H, lipopolysaccharide-challenged broilers fed a basal diet supplemented with 1000 mg/kg AOAE.
3) Each value is shown as mean ± SD (n = 6); Different superscript letters (a, b, and c) within the same row indicate significant difference between experimental groups ($P < 0.05$).

Effects of AOAE on serum cytokines and immunoglobulins in broilers challenged with LPS

As indicated in Table 5, on day 28, compared with the CON group, LPS challenge decreased the contents of IgM and IL-4 and increased the contents of IL-1β, IL-2 and IL-6 in serum of broilers; however, dietary AOAE supplementation obviously alleviated the decrease of IgM and IL-4 and the increase of IL-1β, IL-2 and IL-6 in the serum of broilers challenged with LPS, and the AOAE-H group had the best alleviatory effect ($P < 0.05$).

The effect of AOAE addition and LPS challenge on serum cytokines and immunoglobulins in broilers during convalescence is also presented in Table 5. Neither the AOAE addition nor the LPS challenge had any effect on serum immunoglobulins and cytokines of broilers on day 42 ($P > 0.05$).

TABLE 6 Effect of AOAE on hepatic cytokines and immunoglobulins in broilers challenged with LPS.

Items	Treatments					*P*-value
	CON	LPS	AOAE-L	AOAE-M	AOAE-H	
28 d						
IL-1β, pg/mg prot.	9.4 ± 1.28^{c}	31.98 ± 1.69^{a}	23.42 ± 1.44^{b}	9.96 ± 0.48^{c}	9.45 ± 1.12^{c}	<0.0001
IL-2, pg/mg prot.	4.19 ± 0.27^{c}	9.86 ± 0.48^{a}	9.28 ± 0.43^{ab}	9.03 ± 1.06^{ab}	8.61 ± 1.54^{b}	<0.0001
IL-4, pg/mg prot.	2.33 ± 0.16	2.44 ± 0.25	2.51 ± 0.12	2.58 ± 0.24	2.37 ± 0.27	0.2689
IL-6, pg/mg prot.	0.51 ± 0.02	0.54 ± 0.04	0.52 ± 0.04	0.53 ± 0.07	0.49 ± 0.07	0.4774
IgA, μg/mg prot.	6.54 ± 0.23	5.94 ± 0.54	6.49 ± 0.56	6.80 ± 0.62	6.66 ± 0.92	0.1845
IgG, μg/mg prot.	47.99 ± 3.56	48.56 ± 2.79	48.34 ± 4.94	48.95 ± 3.96	48.26 ± 7.88	0.9979
IgM, μg/mg prot.	13.08 ± 1.48^{bc}	11.35 ± 0.95^{c}	13.25 ± 1.63^{abc}	13.52 ± 0.94^{ab}	15.11 ± 2.33^{a}	0.0075
42 d						
IL-1β, pg/mg prot.	13.04 ± 2.02	15.06 ± 2.58	14.69 ± 2.24	14.37 ± 2.70	12.63 ± 2.38	0.3447
IL-2, pg/mg prot.	6.01 ± 0.80^{ab}	6.76 ± 0.55^{a}	6.76 ± 1.24^{a}	6.81 ± 0.96^{a}	5.27 ± 0.83^{b}	0.0244
IL-4, pg/mg prot.	3.26 ± 0.67	3.49 ± 0.42	3.82 ± 0.93	3.55 ± 0.61	3.03 ± 0.56	0.3227
IL-6, pg/mg prot.	0.66 ± 0.11	0.70 ± 0.04	0.73 ± 0.16	0.77 ± 0.11	0.62 ± 0.16	0.2713
IgA, μg/mg prot.	9.68 ± 0.57	8.15 ± 0.93	8.39 ± 2.21	10.06 ± 1.28	10.19 ± 2.70	0.1569
IgG, μg/mg prot.	73.01 ± 1.39	70.18 ± 5.79	77.42 ± 19.14	71.77 ± 10.17	72.82 ± 18.60	0.9031
IgM, μg/mg prot.	20.75 ± 2.26	18.25 ± 2.98	20.10 ± 3.87	20.37 ± 2.82	20.10 ± 6.36	0.8329

1) IL-1β, interleukin 1 beta; IL-2, interleukin 2; IL-4, interleukin 4; IL-6, interleukin 6; IgA, immunoglobulin A; IgG, immunoglobulin G; IgM, immunoglobulin M.
2) AOAE, Artemisia ordosica alcohol extract; CON, non-challenged broilers fed a basal diet; LPS, lipopolysaccharide-challenged broilers fed a basal diet; AOAE-L, lipopolysaccharide-challenged broilers fed a basal diet supplemented with 500 mg/kg AOAE; AOAE-M, lipopolysaccharide-challenged broilers fed a basal diet supplemented with 750 mg/kg AOAE; AOAE-H, lipopolysaccharide-challenged broilers fed a basal diet supplemented with 1,000 mg/kg AOAE.
3) Each value is shown as mean ± SD (n = 6); Different superscript letters (a, b, and c) within the same row indicate significant difference between experimental groups ($P < 0.05$).

Effects of AOAE and LPS treatment on the hepatic cytokines and immunoglobulins in broilers

As illustrated in Table 6, on day 28, stimulation with LPS alone caused a noticeable increase of IL-1β and IL-2 production in liver compared with the control group, whereas AOAE addition dramatically suppressed the overproduction of hepatic IL-1β (500, 750 and 1,000 mg/kg AOAE) and IL-2 (1,000 mg/kg AOAE) in LPS-treated broilers ($P < 0.05$). In addition, the hepatic IgM content in the AOAE-M and AOAE-H groups was significantly higher than that in the LPS group ($P < 0.05$). On day 42, the hepatic IL-2 content in AOAE-H group was lower than that in LPS group ($P < 0.05$), but had no difference from the CON group ($P > 0.05$).

AOAE regulated the expression of TLR4/NF-κB pathway related genes in liver of broilers challenged with LPS

To determine whether the anti-inflammatory action of AOAE was mediated through the TLR4/NF-κB signaling pathway, the gene expression of *TLR4*, *MyD88*, *TRAF6*, *NF-κB p65*, *NF-κB p50*, *IκBα*, *IL-1β*, *IL-6,* and *PPARγ* was assessed by quantitative real-time PCR analysis. As summarized in Figure 1, on day 28, compared to the control group, LPS administration increased the gene expression of *TLR4*, *MyD88*, *TRAF6*, *NF-κB p65,* and *IL-1β*, but decreased the gene expression of *IκBα* and *PPARγ* in the liver tissue of broilers ($P < 0.05$); however, the negative changes in mRNA expression of the aforementioned genes were markedly reversed by adding AOAE in diets, except for *TLR4*, which decreased significantly only with 750 and 1,000 mg/kg AOAE supplementation ($P < 0.05$). On day 42, hepatic *NF-κB p50* mRNA expression in AOAE-M and AOAE-H groups was lower than that in the LPS group ($P < 0.05$), but had no difference from the CON group ($P > 0.05$).

Effects of AOAE and LPS treatment on the splenic cytokines and immunoglobulins in broilers

As illustrated in Table 7, on day 28, the broilers that were injected with LPS only exhibited a marked increase of the IL-1β and IL-6 in spleen compared with the control group, whereas AOAE addition remarkably reduced the upregulated levels of IL-1β (500, 750, and 1,000 mg/kg AOAE) and IL-6 (1,000 mg/kg AOAE) ($P < 0.05$). Similarly, the contents of splenic IgG and IgM were significantly decreased under LPS challenge, while AOAE supplementation markedly reversed

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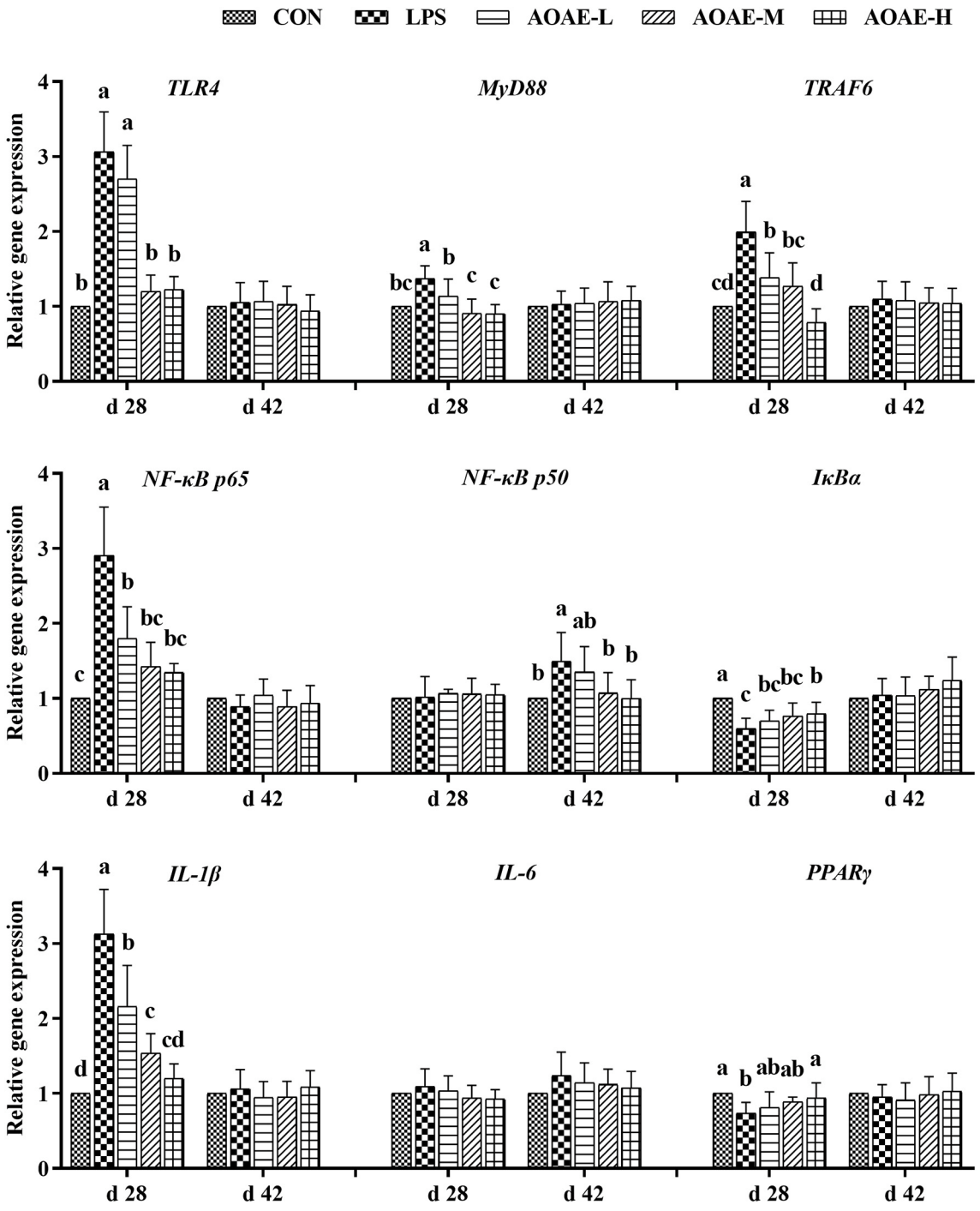

FIGURE 1
Effect of AOAE on the expression of TLR4/NF-κB pathway related genes in liver of broilers challenged with LPS (1) TLR4, toll like receptor 4; MyD88, myeloid differentiation factor 88; TRAF6, tumor necrosis factor receptor associated factor 6; NF-κB p65, nuclear factor kappa B p65; NF-κB p50, nuclear factor kappa B p50; IκBα, inhibitor of NF-κB alpha; IL-1β, interleukin 1 beta; IL-6, interleukin 6; PPARγ, peroxisome proliferator-activated receptor γ. (2) AOAE, *Artemisia ordosica* alcohol extract; CON, non-challenged broilers fed a basal diet; LPS, lipopolysaccharide-challenged broilers fed a basal diet; AOAE-L, lipopolysaccharide-challenged broilers fed a basal diet supplemented with 500 mg/kg AOAE; AOAE-M, lipopolysaccharide-challenged broilers fed a basal diet supplemented with 750 mg/kg AOAE; AOAE-H, lipopolysaccharide-challenged broilers fed a basal diet supplemented with 1,000 mg/kg AOAE. (3) The gene expression for *β-actin* was used as a housekeeping gene. The relative expression levels from the control group were used as reference values. Each value is shown as mean ± SD (n = 6). Bars that do not share the same letters (a, b, c, and d) are significantly different ($P < 0.05$) from each other.

TABLE 7 Effect of AOAE on splenic cytokines and immunoglobulins in broilers challenged with LPS.

Items	Treatments					*P*-value
	CON	LPS	AOAE-L	AOAE-M	AOAE-H	
28 d						
IL-1β, pg/mg prot.	13.09 ± 2.19^{c}	33.32 ± 3.77^{a}	26.19 ± 2.33^{b}	23.23 ± 3.49^{b}	14.73 ± 2.32^{c}	<0.0001
IL-2, pg/mg prot.	6.40 ± 0.97	8.06 ± 0.92	7.75 ± 1.31	7.59 ± 1.44	7.33 ± 1.57	0.2354
IL-4, pg/mg prot.	4.01 ± 0.59	3.93 ± 0.57	4.56 ± 0.76	4.48 ± 0.75	4.51 ± 0.52	0.2928
IL-6, pg/mg prot.	0.59 ± 0.11^{c}	2.71 ± 0.49^{a}	2.75 ± 0.37^{a}	2.70 ± 0.18^{a}	0.97 ± 0.17^{b}	<0.0001
IgA, μg/mg prot.	9.13 ± 1.09	8.22 ± 0.74	8.70 ± 1.39	9.11 ± 1.52	8.78 ± 0.47	0.6152
IgG, μg/mg prot.	84.32 ± 10.57^{a}	65.68 ± 9.61^{b}	60.18 ± 11.25^{b}	63.03 ± 11.32^{b}	87.41 ± 16.79^{a}	0.0009
IgM, μg/mg prot.	27.78 ± 1.86^{a}	16.93 ± 1.96^{c}	22.00 ± 1.79^{b}	26.33 ± 5.30^{a}	26.71 ± 3.71^{a}	<0.0001
42 d						
IL-1β, pg/mg prot.	11.36 ± 1.30^{b}	15.27 ± 3.13^{a}	12.17 ± 2.16^{b}	11.47 ± 1.69^{b}	12.55 ± 1.65^{b}	0.0214
IL-2, pg/mg prot.	8.60 ± 0.85	7.86 ± 1.65	8.00 ± 1.19	7.76 ± 1.17	7.85 ± 1.81	0.8300
IL-4, pg/mg prot.	4.12 ± 0.47	3.92 ± 0.72	3.66 ± 0.58	4.27 ± 0.59	4.54 ± 0.91	0.2318
IL-6, pg/mg prot.	0.70 ± 0.09	0.71 ± 0.11	0.73 ± 0.06	0.69 ± 0.10	0.71 ± 0.10	0.9742
IgA, μg/mg prot.	6.96 ± 1.13	7.35 ± 0.72	6.85 ± 1.10	6.73 ± 0.97	7.56 ± 1.50	0.6689
IgG, μg/mg prot.	72.84 ± 4.72^{ab}	64.50 ± 7.08^{b}	66.13 ± 9.40^{b}	73.13 ± 7.98^{ab}	83.72 ± 16.62^{a}	0.0224
IgM, μg/mg prot.	27.26 ± 4.97^{a}	19.14 ± 2.86^{c}	21.23 ± 3.60^{bc}	22.60 ± 3.27^{bc}	24.35 ± 3.14^{ab}	0.0087

1) IL-1β, interleukin 1 beta; IL-2, interleukin 2; IL-4, interleukin 4; IL-6, interleukin 6; IgA, immunoglobulin A; IgG, immunoglobulin G; IgM, immunoglobulin M.
2) AOAE, Artemisia ordosica alcohol extract; CON, non-challenged broilers fed a basal diet; LPS, lipopolysaccharide-challenged broilers fed a basal diet; AOAE-L, lipopolysaccharide-challenged broilers fed a basal diet supplemented with 500 mg/kg AOAE; AOAE-M, lipopolysaccharide-challenged broilers fed a basal diet supplemented with 750 mg/kg AOAE; AOAE-H, lipopolysaccharide-challenged broilers fed a basal diet supplemented with 1000 mg/kg AOAE.
3) Each value is shown as mean ± SD (n = 6); Different superscript letters (a, b, and c) within the same row indicate significant difference between experimental groups ($P < 0.05$).

the downregulated levels of IgG (1,000 mg/kg AOAE) and IgM (500, 750, and 1,000 mg/kg AOAE) ($P < 0.05$). On day 42, the splenic IgG and IgM contents in AOAE-H group were higher than that in the LPS group ($P < 0.05$), but had no difference from the CON group ($P > 0.05$). In addition, the IL-1β level in LPS group was significantly higher than that in the other groups during convalescence ($P < 0.05$).

AOAE regulated the expression of TLR4/NF-κB pathway related genes in spleen of broilers challenged with LPS

The splenic gene expression data from the broilers are shown in Figure 2. On day 28, LPS markedly elevated the gene expression of *TLR4*, *MyD88*, *TRAF6*, *NF-κB p65*, *IL-1β*, and *IL-6*, whereas decreased the gene expression of *IκBα* in the spleen tissue of broilers ($P < 0.05$); however, dietary AOAE supplementation obviously alleviated negative changes induced by LPS, and the AOAE-H group had the best alleviatory effect ($P < 0.05$). On day 42, the splenic *NF-κB p65* and *IL-1β* mRNA expression in LPS group were significantly higher than those in the other groups ($P < 0.05$).

Discussions

In this experiment, we used a classical LPS-stimulated model to induce the inflammation in broilers by intraperitoneal administration of LPS and evaluated the effect of AOAE on LPS induced inflammatory immune response broiler model. As observed in the present investigation, *E. coli* LPS injection caused the inflammatory reaction and obviously inhibited the growth performance of broilers, which suggested that the LPS simulation model had been successfully established, consistent with our previous study (26, 42).

In the current study, we found that LPS challenge (LPS group) decreased the ADG and ADFI compared with that in the unchallenged broilers (CON group), which was consistent with the findings of Li et al. (26) and Yang et al. (42), where LPS-induced growth retardation was reported in broilers exposed to 500 μg/kg BW LPS. This phenomenon might be partly due to dietary nutrients being diverted from maintaining growth to maintaining the stability of the immune system, and partly to the excessive production of inflammatory cytokines (IL-1β and IL-6) under inflammation feed information back to the central nervous system to suppress the animal's appetite. Simultaneously, we also observed that, diet supplemented with 1,000 mg/kg of AOAE could improve the ADG and ADFI in broilers stimulated by LPS both in stress period and convalescence, thereby alleviating the inhibition of the growth

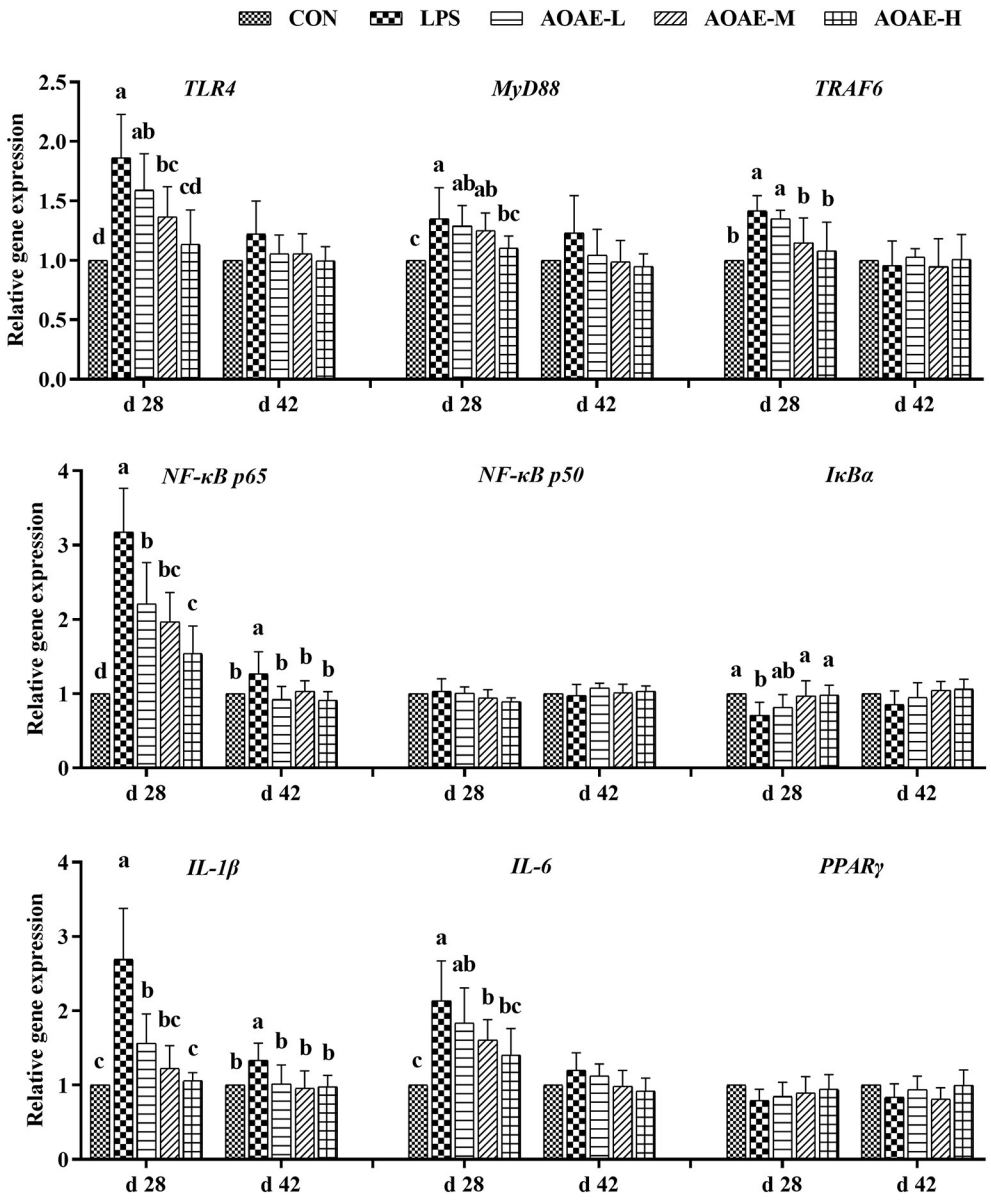

FIGURE 2
Effect of AOAE on the expression of TLR4/NF-κB pathway related genes in spleen of broilers challenged with LPS. (1) TLR4, toll like receptor 4; MyD88, myeloid differentiation factor 88; TRAF6, tumor necrosis factor receptor associated factor 6; NF-κB p65, nuclear factor kappa B p65; NF-κB p50, nuclear factor kappa B p50; IκBα, inhibitor of NF-κB alpha; IL-1β, interleukin 1 beta; IL-6, interleukin 6; PPARγ, peroxisome proliferator-activated receptor γ. (2) AOAE, Artemisia ordosica alcohol extract; CON, non-challenged broilers fed a basal diet; LPS, lipopolysaccharide-challenged broilers fed a basal diet; AOAE-L, lipopolysaccharide-challenged broilers fed a basal diet supplemented with 500 mg/kg AOAE; AOAE-M, lipopolysaccharide-challenged broilers fed a basal diet supplemented with 750 mg/kg AOAE; AOAE-H, lipopolysaccharide-challenged broilers fed a basal diet supplemented with 1000 mg/kg AOAE. (3) The gene expression for β-actin was used as a housekeeping gene. The relative expression levels from the control group were used as reference values. Each value is shown as mean ± SD ($n = 6$). Bars that do not share the same letters (a, b, c and d) are significantly different ($P < 0.05$) from each other.

performance of broilers caused by LPS challenge, which was consistent with the previous studies (26, 42). This may be related to the fact that AOAE contained flavonoids and polysaccharides that were able to alleviate LPS-induced inflammation by suppressing the release of proinflammatory cytokines, ultimately improving the growth performance. Furthermore, Xing et al. (27) reported that flavonoids and polysaccharides of *A. ordosica* aqueous extract might contribute to the positive effects on growth performance by improving the apparent nutrient digestibility of weanling piglets in a dose-dependent manner. Another study indicated that LPS and *A. ordosica* extract (rich in flavonoids and phenolic acids) exhibited an interaction for

the content of insulin-like growth factor-1 (IGF-1) in serum; in other words, *A. ordosica* extract (rich in flavonoids and phenolic acids) could also relieve the growth inhibition of broilers challenged with LPS by promoting the secretion of growth-promoting hormones (26). More specifically, it has been proven that flavone can promote the combination of growth hormone and hepatic growth hormone receptor and then induce growth promotion (43). These results may also partially explain our findings.

The thymus, bursa of Fabricius, and spleen are important immune organs in birds, which all play a key function in defending the body against external or internal irritants or pathogens through different immune response mechanisms. B-lymphocytes mature in the bursa of Fabricius, while the thymus is responsible for the differentiation and proliferation of T-lymphocytes (44, 45). As the largest peripheral immune organ and an initiating site for antibody production after antigenic stimulation, the spleen can regulate cellular and humoral immunity in poultry via activation of B-lymphocytes, T-lymphocytes, and macrophages (46). Hence, their relative development is indispensable in evaluating the poultry immune system and health condition. Consistent with the results of Ahiwe et al. (47), in the stress period of the current study, compared with the CON group, an increase in spleen index and a decline in bursa index were found in the broilers of LPS group after LPS challenge. The increase in the relative weight of spleen observed in LPS group may be associated with the compensatory splenic hyperplasia induced by LPS, which resulted in the activation of inflammatory cells and the large-scale production of inflammatory cytokines (48). Furthermore, as the most responsive immune organ of broiler for microbial infections, in the stress period of the present study, compared with the CON group, the bursa was observed a decrease in the relative weight in response to LPS challenge, which may be due to the occurrence of bursa immune overresponse caused by the presence of LPS antigen that disrupted histo-morphological integrity, as well as pro-apoptotic factors that induce apoptosis and chronic atrophy of the bursa (47). The increase of spleen index and decrease of bursa index in LPS-challenged broilers were noticeably alleviated after dietary supplementation with 1,000 mg/kg AOAE, which demonstrated that AOAE might be able to have beneficial effects on homeostatic mechanisms associated with the immune response of immune organs under inflammatory conditions. These results were in agreement with a previous study of flavonoid-rich ethanolic extract, which reported a significant improvement in heart, thymus and bursa size of coccidia-challenged broilers (49). The reason for this improvement induced by AOAE supplementation in diets of broilers challenged with LPS might be associated with the ability of flavonoids to optimize immunity by promoting the phagocytic activity of macrophages, therefore, alleviating the damage of visceral organs caused by pathogenic bacteria and viruses (50).

The immune protective properties and immunomodulatory potential of flavonoids from medicinal plants in living systems have been reported. Zhong et al. (13) also found that flavonoids (3,5,3′,4′-tetrahydroxy-6,7-dimethoxyflavone, 5,3′,4′-trihydroxy-7-methoxyflavanone, 5,7,4′-trihydroxy-6-methoxyflavone) isolated from *A. ordosica* exhibited strong inhibitory activities for the formation of nitric oxide (NO) in rat macrophages *in vitro*. Unfortunately, to our knowledge, little or no previous study has attempted to examine the *in vivo* alleviating effects of AOAE on LPS induced inflammatory reaction. The present study found that, compared with the CON group, intraperitoneal injection with 750 μg/kg BW LPS caused overproduction of IL-1β, IL-2, and IL-6 in the serum of broilers in LPS group during stress period, which could prove the successful induction of inflammatory reaction in broilers. Meanwhile, we observed a decreased IL-4 and IgM level in serum after LPS challenge in LPS group broilers, indicating that LPS challenge disturbed the balance of the proinflammatory and anti-inflammatory systems. However, dietary supplementation of both 750 and 1,000 mg/kg AOAE were able to decrease the level of these cytokines and increase the content of IL-4 and IgM in broilers challenged by LPS, which could protect against LPS-induced inflammatory responses. These benefits were possibly due to the immunomodulatory characteristic of AOAE, mainly provided by flavonoids. This hypothesis was also supported by the previous findings of Niu et al. (51), who reported that naringenin from *A. ordosica* could increase the level of the anti-inflammatory factor IL-4, while decrease the inflammatory factor tumor necrosis factor α (TNF-α). Similarly, another study reported that *Artemisia anomala* ethanolic extract, the most potent ingredients of which were flavonoid compounds, had anti-inflammatory effects by inhibiting nod-like receptor family pyrin domain containing 3 (NLRP3) inflammasome activation (52). Combined with these results and based on our study, it is reasonable to infer that AOAE, whose main active ingredient is flavonoid, can inhibit LPS-induced proinflammatory responses to alleviate inflammation.

To gain further insight into the mitigating effects of AOAE against LPS-induced excessive inflammation in broilers, we selected the liver and spleen as the target organs. Undoubtedly, in addition to the spleen, the liver is also a major immune organ and plays a pivotal role in protein synthesis and detoxification processes, and has a high metabolic rate of nutrients from dietary feed (53). Many bioactive compounds, such as flavonoids and polysaccharides, can reach the liver and exert a variety of biological functions via the portal vein after absorption along the gastrointestinal tract (54, 55). As the target organ of LPS, the liver, while defending against bacteria and getting rid of their toxic products, such as LPS, is susceptible to damage from endotoxins and live bacteria (56). Immunoglobulins, as a vital part of the humoral immune system, are all produced by B lymphocytes and have many important biological functions, including recognizing and

resisting pathogen invasion, enhancing immune ability, and neutralizing toxins, while also playing an important role in the regulation of inflammation (57). LPS stimulation has been confirmed to influence the expression level of immunoglobulins and inhibit the humoral immune response (58). Our results showed that intraperitoneal LPS injection led to a decrease in IgM and IgG in LPS group compared to the CON group, which was in line with previous studies. IgM is the first antibody generated during the primary antibody response post-infection and hence may be depleted or reduced to decrease or counter the antigen level when it encounters the antigens (59). IgG, also referred to as IgY in poultry, is the most abundant immunoglobulin and generated mainly in T-cell-dependent secondary antibody responses, which promotes immune cells to neutralize bacterial toxins (60, 61). In the current study, the LPS-induced decrease in IgM and IgG levels were manifestly moderated by the presence of AOAE with the level of 1,000 mg/kg, indicating the immune promotion effects of AOAE supplementation. Consistently, it was reported that supplementation with quercetin was able to ameliorate the decline in the levels of serum IgG, IgM, and IgE in rats exposed to doxorubicin (62). According to the results of this experiment, it is speculated that AOAE with flavonoids as the main active component can effectively alleviate the negative effects of inflammation caused by LPS stimulation via regulating the production of immunoglobulins, which help to resist further damage. Nevertheless, we observed that the immunoglobulin levels in the serum returned to normal, but the low levels in the spleen continued throughout the convalescence, suggesting that spleen recover more slowly after parenchymatous injury, whereas the recovery process could be accelerated by diet addition of 1,000 mg/kg AOAE.

Proinflammatory cytokines have a significant contribution in the initiation of inflammatory pathogenesis and the development of inflammatory pathology (63). In the present study, our results showed that, compared with the CON group, the LPS group had higher IL-1β and IL-2 in liver and IL-1β and IL-6 in spleen. However, we also found that hepatic IL-1β, IL-2, and splenic IL-1β, IL-6 of broilers challenged by LPS were lowered by 1,000 mg/kg AOAE supplementation during stress period, which indicated that AOAE exerted a certain degree of palliation for organ inflammation induced by LPS, and this effect was continuous, even in the convalescence after LPS stimulation ended. In addition, compared with the LPS group, the gene expression levels of hepatic and splenic IL-1β and splenic IL-6 in AOAE-M group and AOAE-H group were significantly decreased and restored to the levels of CON group in stress period and/or convalescence, which partially supports this finding. A similar result was reported for bovine mammary epithelial cells by Song et al. (64), who found that the ethanol extract of *Artemisia annua* (rich in flavonoids and phenolic acids) could exert anti-inflammatory effects by downregulating the mRNA levels of inflammatory cytokines, including TNF-α, IL-1β, and IL-6, in a dose-dependent manner. In our research, we found that AOAE may act as a protective agent and prevent LPS induced hepatic and splenic inflammatory injury to a certain extent, as evidenced by alleviating LPS-induced immune overresponse through suppressing the transcriptional expression and the secretion of inflammatory cytokines, which ultimately improved the systemic anti-inflammatory response.

Toll-like receptors (TLRs), acting as sentinels of pathogens, are a class of transmembrane proteins that play a crucial part in activating immunity and controlling inflammation, especially TLR4 (65). As the pattern recognition receptor of LPS, TLR4 mediates inflammatory responses via combining with MyD88 to activate various intracellular signaling pathways, including the NF-κB transduction cascades, upon LPS challenge (66). Normally, functional NF-κB dimers combine with its inhibitor protein IκB in the cytosol and act as an inactive form (67). When under inflammatory stimuli, NF-κB will dissociate from IκB, which is triggered by another kinase, IκB kinase (IKK), and subsequently transfer into the nucleus, where it upregulates the expression of proinflammatory cytokines, including IL-1β, IL-6, TNF-α, and IL-2, eventually exaggerates the initial inflammatory responses (67). Numerous studies have suggested that NF-κB, a crucial transcription factor for inflammation progression, is involved in the immunoregulation activity of flavonoids and polysaccharides isolated from a variety of plants (50, 61). As an extension, we speculated that AOAE has a similar anti-inflammatory effect against the inflammatory disorders caused by LPS-challenge and primarily explored the possible action mechanism by detecting the mRNA expressions of eight candidate key nodes in the TLR4/NF-κB signaling pathways in liver and spleen, including *TLR4*, *MyD88*, *TRAF6*, *NF-κB p50*, *NF-κB p65*, *IκBα*, *IL-1β,* and *IL-6*. In the present study, the results showed that compared with the CON group, when broilers exposed to LPS (LPS group), the gene expression levels of *TLR4*, *MyD88*, *TRAF6*, *NF-κB p65*, *IL-1β*, and *IL-6* were increased, while the gene expression level of *IκBα* was decreased in liver and spleen. However, 1,000 mg/kg AOAE inhibited the gene over-expression of *NF-κB p50* and *NF-κB p65* through decreasing *TLR4*, *MyD88*, and *TRAF6* gene expression, but increasing *IκBα* gene expression. Accordingly, as discussed above, the content of proinflammatory cytokines, such as IL-1β and IL-6, and the related gene expression were key to alleviate inflammation, and could be significantly reduced and restored to normal levels by AOAE. In addition, peroxisome proliferator activated-receptors (PPARs) have been shown to interact with NF-κB pathways and are believed to act as a class of anti-inflammatory proteins that have the ability to regulate inflammatory gene expression (68). Among them, PPARγ is known as a significant anti-inflammatory factor that can inhibit NF-κB by preventing its binding to target sequences (69). The data from the current study indicated that dietary AOAE supplementation for broilers alleviated the inhibitory effect of LPS on *PPARγ*, which was similar to the finding of Lv

et al. (70), who reported that genistein treatment upregulated the mRNA expressions of *PPARα* in LPS-challenged chicks. It is therefore reasonable to assume that negative inflammation response regulators can also be activated by AOAE when the host is infected by pathogenic bacteria in a bid to moderate or restore immune homeostasis. The presence of flavonoids and polysaccharides in AOAE might be responsible for these benefits, as was previously described in broilers, in which the supplementation of *Artemisia argyi* flavonoids or *Artemisia ordosica* polysaccharides lowered serum inflammatory cytokines and improved immune function (21, 50). What we stress here is that the advantages of flavonoids should contribute indispensably to AOAE. Flavonoids isolated from *Artemisia ordosica* have been confirmed *in vitro* in both antioxidant and anti-inflammatory aspects (13, 20). Niu et al. (51) also found that the naringenin from *A. ordosica* could ameliorate the allergic rhinitis symptoms by increasing the IL-4 and decreasing the TNF-α, which showed an anti-allergic rhinitis effect. Additionally, as the main component in AOAE was total flavonoid (556.1 mg rutin/g AOAE), it is reasonable to speculate that AOAE may have the same anti-inflammatory effect as rutin which has been proven to ameliorate dextran sulfate sodium-induced colitis in mice by attenuating proinflammatory gene expression (71). The compounds that accounts for the anti-inflammatory effect of AOAE need to be clarified in the near future. Moreover, whether the anti-inflammatory effect of entire AOAE on LPS-exposed broilers is greater than that of the single active ingredient needs a continued study to confirm.

Actually, inflammation is generally not harmful to the pathogen and, in contrast, benefits the pathogen by providing an additional nutritional supply through the increased blood supply (72). The present results suggested the possibility that AOAE could suppress the LPS-mediated excessive production of inflammatory cytokines and recover the physiological balance. This might be related to the mediating of MyD88-dependent TLR4/NF-κB signaling pathway. As reported, the *Artemisia annua* ethanol extract (rich in flavonoids and phenolic acids) ameliorated inflammation by regulating NF-κB pathways (64). However, the effects of AOAE on the NF-κB signaling pathway still need to be validated by further studies. It is worth noting that the presence of different active constituents in herbal extract can simultaneously act on different targets in the inflammatory pathway (73). Increasing evidences demonstrate that the excess production of ROS is usually associated with high levels of proinflammatory cytokines, suggesting that crosstalk between the NF-κB and other pathways including nuclear factor erythroid 2-related factor 2 (Nrf2) may be an important regulatory mechanism in many cellular responses to various stresses, such as oxidative stress and immunological stress.

Collectively, *A. ordosica* has a great deal of advantages, such as abundant resources, low price, rich nutritional value and high pharmacological activity, and possesses great application and development value. Unfortunately, as far as we know, studies on the effects of *A. ordosica* and its extract as a feed additive on growth performance and health of livestock are limited, especially the study of *A. ordosica* alcohol extract in poultry. Consequently, further studies of different *in vitro* and *in vivo* disease models should be carried out to completely evaluate the role and the exact mechanism of AOAE in antioxidant and immunomodulatory.

Conclusion

In conclusion, the present study reported for the first time that AOAE protected against LPS-induced inflammation in an animal model. The results obtained from this study showed that LPS injection inhibited growth performance of broilers and led to an inflammatory response manifested as high levels of IL-1β and IL-6 and low levels of IgG and IgM, which negatively affected several parameters including splenic and bursal relative weight. However, dietary AOAE supplementation could reverse these effects to improve growth performance of LPS-challenged broilers. Based on the results of our study, we preliminarily speculated that AOAE with flavonoids as the main active component could alleviate LPS-induced inflammatory response, which might be related to the mediating of TLR4/NF-κB pathways. Therefore, under the conditions of this experiment, the recommended level of AOAE in broiler diet was 1,000 mg/kg. AOAE deserves further investigation to develop a natural feed additive in poultry diets and improve growth performance and immune function in broilers.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/supplementary material.

Ethics statement

The animal study was reviewed and approved by the Animal Care and Use Committee of Inner Mongolia Agricultural University and performed following the national standard Guideline for Ethical Review of Animal Welfare (GB/T 35892-2018).

Author contributions

Conceptualization and supervision: BS and SY. Investigation: LS. Sample collection: LS, YG, and YC. Data curation: LS and YC. Writing—original draft preparation: LS and LZ. Writing—review & editing: LS, YXi, and SG. Supervision: YXu and XJ. 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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Positive effects of Mulberry leaf extract on egg quality, lipid metabolism, serum biochemistry, and antioxidant indices of laying hens

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Plant extracts are becoming a hot topic of research by animal husbandry practitioners following the implementation of a global policy to restrict antibiotic use in animal production. Mulberry leaf extract has received considerable attention as a new plant extract. Mulberry leaf polysaccharides and flavonoids are its main constituents, and these substances possess immunoregulatory, hypoglycemic, antioxidant, and anticoagulant properties. It is however less common to use them in poultry production. Therefore, we investigated the effects of adding MLE to the diet of laying hens on egg quality, lipid metabolism, serum biochemistry, and antioxidant indices in this study. A total of 288 Lohmann Silber layers, aged 38 weeks, were randomly assigned to four groups (six replicates of 12 hens each). Hens were fed a basal diet supplemented with 0 (control diet), 0.4, 0.8, or 1.2% MLE for 56 d. Results showed that the addition of 0.4–1.2% MLE to the diet improved aspartate transaminase (AST) activity in the serum of laying hens, reduced low-density lipoprotein (LDL-C) content in the serum, and significantly decreased yolk triglyceride (TG) and total cholesterol (TC) contents ($P < 0.05$). No adverse effects were observed on production performance ($P > 0.10$). MLE (0.4 and 1.2%) significantly reduced the TG and TC levels in the liver ($P < 0.05$). MLE (0.8 and 1.2%) significantly increased glutathione peroxidase (GSH-Px) activity in the serum, decreased alanine transaminase (ALT) activity, TG and TC content in the serum, and improved egg yolk color ($P < 0.05$). MLE (1.2%) significantly increased high-density lipoprotein (HDL-C) content and superoxide dismutase (SOD) activity in the serum and enhanced eggshell strength ($P < 0.05$). The liver-related lipid metabolism gene assay revealed that the relative mRNA expression of PPARα and SIRT1 in the liver was significantly upregulated and that of FASN and PPARγ was significantly decreased after the addition of MLE. In contrast, the relative mRNA expression of SREBP-1c in the liver dramatically decreased after the addition of 0.8 and 1.2% MLE ($P < 0.05$). The addition of MLE to the diet improved egg quality and the economic value of hens by increasing antioxidant capacity and lipid metabolism. The most appropriate

amount of MLE to be added to the diet of laying hens was 0.8%. Our study provides a theoretical reference for the application of MLE in egg production and to promote the healthy and sustainable development of the livestock and poultry industry under the background of antibiotic prohibition.

KEYWORDS

mulberry leaf extract (MLE), laying hen, egg quality, antioxidant indexes, lipid metabolism

Introduction

Modern egg farming has benefited from highly intensive farming methods, which increasing efficiency, convenience, and effectiveness for farmers. However, this has also put egg-laying hens at risk of inherited diseases related to lipid metabolism. The use of antibiotics to treat these diseases is not the best solution, and the concept of healthy consumption drives consumers to prefer purchasing green and antibiotic-free poultry products. Eggs are one of the most readily available high-quality proteins, but the lipid and high cholesterol content (∼30% of the nutrient content) of egg yolks have become an issue of concern for consumers (1). Excessive cholesterol intake has adverse effects on the body and increases the risk of developing diabetes (2), especially for people with underlying diseases, such as heart disease (3). Therefore, finding alternatives to antibiotics to balance the product market demand has become a part of the modern farming industry.

Plant extracts have become a hot topic for industry research as a natural feed additive due to decrees issued by countries including China to restrict the growth of antibiotics in livestock production (4, 5). Most plants contain anti-nutritional elements, such as tannins and phytic acids, and chemical extraction can be used to eliminate these effects on livestock and poultry and improve the palatability of feed. Previous studies have shown that the addition of natural mineral elements, such as iodine and iron, to the diet of laying hens can improve egg quality (6, 7). The use of natural plant extracts for animal production has many benefits. The addition of 1.5 g/kg of ginger powder to the diet of Japanese quails improved their performance and egg quality (8). The addition of cinnamon oil to the diet of poultry can balance the gastrointestinal microenvironment, optimize lipid metabolism, and thus increase production performance and immune function (9). The addition of tartary buckwheat extract to the diet of ewes and lambs can alleviate oxidative stress and enhance production performance (10).

Mulberry (*Morus alba* L.) is a deciduous tree belonging to the family Moraceae. It is distributed worldwide, mostly in Asian countries, including China, Japan, and Korea, where is used in traditional industries, such as sericulture (11, 12). The leaves of mulberry plants contain biologically active substances, such as polysaccharides, flavonoids, and alkaloids, which contribute to lowering triglycerides, antioxidants, immunity, and so on (13). Mulberry leaf extract (MLE) has several applications in animal production. MLE can reduce blood glucose levels in mice, which is likely due to active ingredients that stimulate adipocyte proliferation and differentiation. Adipogenic transcription factors and downstream gene expression are likely regulated in the same manner (14). The addition of 200–1,600 mg/kg of mulberry leaf flavonoids to the diet of fattening pigs significantly improves their growth performance and meat quality and positively affects lipid metabolism (15). However, the application of MLE in egg production has not been extensively studied. The addition of 0.5% mulberry leaf powder to the diet of Hendrix hens can improve egg yolk weight, shell weight, Haugh unit, yolk color, and antioxidant status (16). It was found that 4 mg of mulberry leaf polysaccharide supplement fed to chicks vaccinated against Newcastle disease virus triggered an immune response and resulted in high levels of antibodies for several weeks post vaccination (17). The addition of 60 mg/kg mulberry leaf flavonoids to the diet of older breeders improved eggshell thickness and shell strength by affecting calcium transport in the shell glands (18). As of now, mulberry leaf extract is primarily used *in vitro* or on rats, with relatively few animal production studies and even fewer studies on laying hens. Therefore, this study aimed to investigate the effects of MLE on egg quality, antioxidants, and lipid metabolism. It also aimed to determine the optimal ratio of MLE in hen diets to provide a theoretical reference for the application of MLE in egg production and to promote the healthy and sustainable development of the livestock and poultry industry under the background of antibiotic prohibition.

Abbreviations: PPARα, peroxisome proliferators-activated receptor-α; PPARγ, peroxisome proliferators-activated receptor-γ; SIRT1, Silent information regulator 1;FASN, fatty acid synthetase; SREBP-1c(SREBF1), sterol regulatory element-binding protein-1c; ALB, albumin; GLB, globulin; TG, triglyceride; TC, total cholesterol; VLDL-C, very-low-density lipoprotein; LDL-C, Low-Density Lipoprotein; HDL-C, high-density lipoprotein; ALT, alanine transaminase; AST, aspartate transaminase; CAT, catalase activity; T-AOC, total antioxidant capacity; SOD, superoxide dismutase; GSH-Px, glutathione peroxidase; MDA, malondialdehyde.

Materials and methods

Birds, diets, and management

A single-factor design was used for the experiment. A total of 288 38-week-old Lohmann Silber layers with good health and similar growth were randomly divided into four groups, each dietary treatment had 6 replicates with 12 hens each. The pre-trial and trial periods were 14 and 56 d, respectively. All chickens were fed a basic diet during the pre-trial period. In the trial period, the control group was fed a basic diet, and the experimental group was fed a basic diet supplemented with various concentrations of MLE, namely 0.4, 0.8, and 1.2%. MLE was purchased as a dark green powder with silica as the carrier from Xiangda Hezhong Biotechnology Co., Ltd. (Hebei, China). The extraction method used was hot-water extraction, the main components were mulberry leaf polysaccharides (20%), mulberry leaf flavonoids (3%), and alkaloids (2%). The basal diet was formulated according to the NRC (1994) to meet the nutrient requirements of laying hens (19) (Table 1). The experimental site was the animal husbandry teaching base of Hebei Agricultural University, and laying hens were caged with three and a half open steps. Hens were allowed to eat and drink freely. Natural ventilation and natural and artificial light were used. The light/dark schedule was 16/8 h, and the light intensity used was 15 lx. Eggs were collected at 15:00 every day, and the mental state and death of the chickens were recorded. The chicken coop was cleaned regularly.

TABLE 1 Ingredients and chemical composition of basal diet.

Items	Content/%
Ingredients	
Corn	66.40
Soybean meal	25.00
Wheat bran	2.20
Vegetable oil	0.80
NaCl	0.30
$CaHPO_4$	1.50
Fish meal	2.80
Premix[a]	1.00
Total	100.00
Nutrient levels[b]	
ME/(MJ/kg)	12.38
CP	16.57
Ca	3.60
AP	0.45
Lys	0.86
Met	0.38

[a]The premix provided the following per kg of the diet: VA 12 000 IU, VB_1 6 mg, VB_2 7 mg, VB_6 7 mg, VB_{12} 0.34 mg, VD 4 500 IU, VE 20 IU, VK 3.2 mg, biotin 5 mg, folic acid 1.1 mg, nicotinic acid 50 mg, Cu (as copper sulfate) 9 mg, Fe (as ferrous sulfate) 50 mg, Mn (as manganese sulfate) 100 mg, Zn (as zinc sulfate) 85 mg, I (as potassium iodide) 90 mg, Se (as sodium selenite) 0.30 mg.
[b]Nutrient levels were all calculated values.

Sample collection

Two chickens were randomly selected from each replicate on the 56th day of the experiment, and 48 chickens were fasted for 24 h with free access to water. Afterwards, blood was collected from the wing vein, kept at 20–25°C, centrifuged at 3,000 rpm for 15 min, and the supernatant was stored at −20°C. These chickens were then slaughtered according to the animal welfare slaughtering procedure, the livers were removed and weighed, and ∼2 g of the left side of the livers were snap frozen in liquid nitrogen. The frozen liver samples were stored at −80°C.

Egg quality characteristics

Twenty eggs were randomly selected from each group on day 28 and 58 of the experiment to determine the egg quality. Eggshell strength was measured using an egg force reader (EFR-01, ORKA Technology Co., Ltd., Herzliya, Israel); egg yolk color was measured using a yolk color chart (Robotmation, Co., Ltd., Tokyo, Japan); Vernier calipers were used to measure eggshell thickness at the blunt end, sharp end, and middle part after the eggshell membrane was peeled off, and the average thickness value was determined. Egg long and short diameters were measured using an egg form coefficient measuring instrument (NFN385, FHK Corp., Tokyo, Japan), the ratio of long diameter to short diameter was measured using an egg shape index, and protein height and Haugh unit was measured using an egg multitester (model EA-01, ORKA Technology Co., Ltd., Herzliya, Israel) (20). The yolk was separated and weighed, the proportion of yolk was calculated, and the yolk moisture content was calculated by mixing three yolks and then freeze-drying (21).

Plasma indices

An enzyme labeling instrument (Bio Tek Instruments, Inc., Vermont, VT, USA) was used to determine the levels of serum albumin (ALB; cat. NO. A045-3-2), malondialdehyde (MDA; Cat. NO. A003-1-2), total protein (TP; Cat. NO. A045-3-2), triglyceride (TG; cat. NO. A110-1-1), total cholesterol (TC; Cat. NO. A111-1-1), high-density lipoprotein cholesterol (HDL-C; Cat. NO. A112-1-1), low-density lipoprotein cholesterol (LDL-C; Cat. NO. A113-1-1), very-low-density lipoprotein (VLDL; cat. NO. JL15942), aspartate transaminase (AST; cat. NO.C010-2-1), alanine transaminase (ALT; cat. NO.C09-2-1), superoxide dismutase (SOD; Cat. NO. A001-3-2), glutathione peroxidase (GSH-Px; cat. NO. A005-1-2), catalase activity

TABLE 2 List of gene primer sequences.

Genes	NCBI ID no.	Primer sequence (5′-3′)	Product length (bp)
PPARα	NM_001001464.1	F-AGTAAGCTCTCAGAAACTTTGTTG	108
		R-ACATTGGTGATAGCAAGTGGC	
PPARγ	NM_001001460.1	F-CCAGCGACATCGACCAGTTA	109
		R-CTTGCCTTGGCTTTGGTCAG	
FASN	NM_205155.4	F-GCGGGCAAAGACTCACAATG	112
		R-GGTGCGGTGATCTCCTTCAA	
SIRT1	NM_001004767.2	F-CTTCTCCAAGATGGCGGACG	120
		R-CCGTCTTCCGAGTTCAGGC	
SREBP-1C (SREBF1)	NM_204126.3	F-GAGCACCTCCTGGAGAAAGC	88
		R-CATCCGAAAAGCACCCCTCT	
β-actin	NM_205518.2	F-CGGACTGTTACCAACACCCA	115
		R-TCCTGAGTCAAGCGCCAAAA	

PPARα, peroxisome proliferators-activated receptor-α; PPARγ, peroxisome proliferators-activated receptor-γ; SIRT1, Silent information regulator 1; FASN, fatty acid synthetase; SREBP-1c(SREBF1), sterol regulatory element-binding protein-1c.

TABLE 3 Effects of mulberry leaf extract on serum biochemical indices of laying hens.

Items	Control group	Mulberry leaf extract added levels (%)			*P*-value		
		0.4	0.8	1.2	ANOVA	Linear	Quadratic
ALB/(g/L)	26.14 ± 2.25	26.33 ± 1.47	26.89 ± 2.06	28.11 ± 2.19	0.224	0.046	0.108
GLB/(μg/mL)	49.05 ± 5.45	49.50 ± 4.19	50.36 ± 2.79	51.83 ± 2.41	0.512	0.134	0.310
TG/(mmol/L)	23.27 ± 2.08^{a}	22.99 ± 2.12^{a}	21.58 ± 1.95^{ab}	20.54 ± 2.01^{ab}	0.041	0.005	0.017
TC/(mmol/L)	4.55 ± 0.65^{a}	4.36 ± 0.51^{ab}	4.01 ± 0.39^{bc}	3.75 ± 0.32^{c}	0.013	0.001	0.004
VLDL-C/(mmol/L)	8.42 ± 2.07	7.44 ± 2.61	7.36 ± 1.26	6.80 ± 1.37	0.442	0.541	0.092
LDL-C/(mmol/L)	0.64 ± 0.05^{a}	0.51 ± 0.06^{c}	0.59 ± 0.06^{b}	0.58 ± 0.05^{b}	<0.001	0.277	0.007
HDL-C/(mmol/L)	0.85 ± 0.12^{b}	0.86 ± 0.03^{b}	0.89 ± 0.08^{b}	0.97 ± 0.11^{a}	<0.001	<0.001	<0.001
ALT/(U/L)	310.58 ± 25.32^{a}	296.24 ± 28.16^{ab}	279.25 ± 25.83^{bc}	263.25 ± 24.02^{c}	0.006	<0.001	0.002
AST/(U/L)	64.85 ± 7.21^{a}	58.41 ± 6.13^{b}	56.97 ± 5.13^{b}	53.15 ± 4.13^{b}	0.004	<0.001	0.001

Different letter in each row indicates a significant difference ($p < 0.05$).
ALB, albumin; GLB, globulin; TG, triglyceride; TC, total cholesterol; VLDL-C, very-low-density lipoprotein; LDL-C, Low-Density Lipoprotein; HDL-C, high-density lipoprotein; ALT, alanine transaminase; AST, aspartate transaminase.

(CAT; Cat. NO. A007-1-1), and total antioxidant capacity (T-AOC; cat. NO. A015-1-2). VLDL kits were purchased from Shanghai Jianglai Biotechnology Co. Ltd. (Shanghai, China). The remaining kits were purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, Jiangsu, P.R. China). These kits were used according to the manufacturer's instructions (22, 23).

Liver and egg yolk lipid analysis

The liver samples were thawed, homogenized at a constant temperature of 0°C using a high-speed homogenizer, and the TG and TC levels were determined. The freeze-dried egg yolks were homogenized at a constant temperature of 0°C, and the TG and TC levels were determined (24).

Gene expression

Quantitative real-time PCR was performed to analyze the relative mRNA expression of genes related to liver lipid metabolism. Primers used in this study are listed in Table 2. The β-actin gene was used as an internal reference. Real-time PCR was performed using a fluorescence quantitative PCR system (SLAN-96P, Shanghai Hongshi Medical Technology Co., Ltd., Shanghai, China). Relative mRNA expression of related genes was analyzed using the $2^{-\Delta\Delta Ct}$ method (16). Quantitative

real-time PCR was performed by the Huaying Institute of Biotechnology in Beijing, China (25).

Statistical analysis

The data were analyzed using one-way ANOVA (LSD) with Duncan's method for multiple comparisons between groups (26). Orthogonal polynomial contrasts were used to estimate the linear and quadratic effects of the various amounts of MLE added. All data were analyzed using SPSS (version 25.0; IBM Inc., New York, US), and images were created using GraphPad Prism version 8.0.2 for Windows (GraphPad Software, La Jolla California USA, www.graphpad.com). The results are presented as the mean ± standard deviation (*SD*), and statistical significance was set at $P < 0.05$ (27).

Results

Serum biochemical indices

The addition of MLE to the diet significantly reduced serum AST activity and LDL-C levels ($P < 0.05$) (Table 3). The addition of 0.8 and 1.2% MLE to the diet significantly increased serum ALT activity ($P = 0.006$) and decreased the TG ($P < 0.05$) and TC content ($P < 0.05$), whereas the addition of 1.2% MLE significantly increased serum HDL-C content ($P < 0.05$). The serum levels of TG, TC, AST, and ALT decreased significantly, and HDL-C increased significantly (linear or quadratic, $P < 0.05$) with increasing levels of Mulberry leaf extract in the diet. No statistically significant differences were found in other serum indicators ($P > 0.10$).

Egg quality

The egg quality-related characteristics are listed in Table 4. Up to day 28, the addition of 1.2% MLE to the diet significantly improved the eggshell strength ($P < 0.05$), and the addition of 0.8 and 1.2% MLE significantly improved the egg yolk color ($P < 0.05$). The yolk color and protein height increased significantly, and yolk weight decreased significantly (linear $P < 0.05$) as the level of MLE added to the diet increased. However, other characteristics were not statistically different ($P > 0.10$). Up to day 56, the addition of 1.2% MLE to the diet significantly improved eggshell strength ($P < 0.05$) and egg yolk color ($P < 0.05$). The yolk percentage decreased significantly (linear $P < 0.05$), and the yolk color increased significantly (quadratic, $P < 0.05$) with increasing levels of MLE added to the diets.

Serum antioxidant capacity

The indicators related to the serum antioxidant capacity are listed in Table 5. The addition of MLE to the diet, compared to the control group, tended to increase CAT activity ($P = 0.057$). The addition of 1.2% MLE to the diet significantly increased SOD activity ($P < 0.05$), and the addition of 0.8 and 1.2% MLE to the diet significantly increased GSH-Px activity ($P < 0.05$). As the level of MLE added to the diet increased, serum CAT, SOD, and GSH-Px activities increased significantly (linear or quadratic, $P < 0.05$), T-AOC capacity increased significantly (linear $P < 0.05$), and MDA content increased significantly (quadratic, $P < 0.05$).

Liver and yolk lipid profile

Compared to the control group, the addition of MLE to the diet significantly reduced the TG ($P < 0.05$) and TC content in egg yolk ($P < 0.05$) (Table 6). The addition of 0.4 and 1.2% MLE to the diet significantly reduced the TG content in the liver ($P < 0.05$), and the addition of 0.4 and 0.8% MLE significantly reduced the TC content in the liver ($P < 0.05$). The TG content in the liver and the TG and TC contents in egg yolk showed linear and quadratic changes, respectively, with an increase of dietary MLE ($P < 0.05$).

Lipid metabolism

The relative mRNA expression of PPARα and SIRT1 was significantly upregulated in MLE treatment groups than that in the control group ($P < 0.05$) (Figure 1). The relative mRNA expressions of FASN, PPARγ, and SREBP-1c were significantly decreased ($P < 0.05$) in the liver after the addition of MLE at 0.8 and 1.2%.

Discussion

Mulberry trees are suitable for cultivation in most regions and have many uses, including for consumption, and ornate and medicinal uses. Biologically active substances such as polysaccharides, flavonoids, and alkaloids are extracted from mulberry leaves and can be used in several applications. Mulberry leaf polysaccharides have been shown to display a variety of pharmacological effects including antioxidant, hypoglycemic, and immune-boosting properties (17, 28). This study was conducted to evaluate the effects of MLE on laying hens with regards to serum biochemical parameters, egg quality, antioxidant properties, and lipid metabolism.

Serum biochemical indicators can reveal the metabolism and health status of body (29). ALT and AST activities are often used to determine the health status of the heart and

TABLE 4 Effects of mulberry leaf extract on egg quality of laying hens.

Items	Control group	Mulberry leaf extract added levels (%)			*P*		
		0.4	0.8	1.2	ANOVA	Linear	Quadratic
D28							
Egg weight/g	59.81 ± 4.11	59.35 ± 3.97	59.19 ± 4.30	59.85 ± 3.84	0.942	0.715	0.838
Eggshell strength/N	39.71 ± 8.43^{b}	41.08 ± 5.81^{b}	44.65 ± 7.93^{ab}	47.20 ± 5.98^{a}	0.010	0.082	0.186
Yolk color	8.33 ± 0.91^{b}	8.72 ± 0.67^{ab}	8.94 ± 0.80^{a}	9.00 ± 0.69^{a}	0.049	0.023	0.053
Eggshell thickness/mm	0.34 ± 0.26	0.35 ± 0.26	0.36 ± 0.18	0.35 ± 0.15	0.109	0.232	0.141
Egg shape index	1.32 ± 0.04	1.33 ± 0.06	1.31 ± 0.03	1.32 ± 0.03	0.375	0.459	0.468
Haugh unit	83.48 ± 5.49	83.06 ± 5.47	83.31 ± 5.89	83.35 ± 6.14	0.997	0.633	0.626
Egg yolk weight	16.94 ± 1.72	16.65 ± 1.60	16.12 ± 1.00	16.03 ± 1.17	0.185	0.046	0.128
Eggshell weight	8.06 ± 0.84	7.90 ± 0.82	7.91 ± 0.51	8.33 ± 0.52	0.230	0.545	0.402
Protein height	6.00 ± 1.37	6.25 ± 1.33	6.67 ± 1.08	7.00 ± 1.17	0.205	0.049	0.122
Egg yolk ratio/(%)	27.78 ± 0.84	26.28 ± 0.90	27.18 ± 0.95	29.08 ± 3.81	0.153	0.110	0.127
Egg yolk moisture content/(%)	48.28 ± 5.12	48.51 ± 3.29	46.26 ± 4.70	44.13 ± 4.51	0.313	0.738	0.217
D56							
Egg weight/g	59.46 ± 4.44	59.15 ± 4.15	59.73 ± 4.94	59.49 ± 4.12	0.985	0.930	0.924
Eggshell strength/N	39.73 ± 4.47^{b}	42.64 ± 5.30^{ab}	43.25 ± 5.68^{ab}	44.58 ± 5.35^{a}	0.048	0.233	0.040
Yolk color	9.56 ± 0.78^{b}	10.17 ± 0.98^{a}	10.33 ± 1.03^{a}	10.50 ± 0.62^{a}	0.006	0.125	0.011
Eggshell thickness/mm	0.34 ± 0.03	0.36 ± 0.03	0.36 ± 0.02	0.34 ± 0.03	0.219	0.076	0.206
Egg shape index	1.32 ± 0.23	1.33 ± 0.23	1.29 ± 0.21	1.31 ± 0.15	0.931	0.968	0.825
Haugh unit	77.10 ± 6.54	77.85 ± 7.23	78.17 ± 7.97	77.99 ± 6.11	0.131	0.393	0.424
Egg yolk weight	18.63 ± 2.41	18.32 ± 1.71	18.53 ± 1.45	18.73 ± 1.48	0.891	0.360	0.632
Eggshell weight	7.78 ± 0.70	7.86 ± 0.63	7.99 ± 0.45	7.85 ± 0.65	0.791	0.360	0.411
Protein height	5.90 ± 1.62	5.76 ± 2.09	6.05 ± 1.50	6.20 ± 1.38	0.869	0.642	0.774
Egg yolk ratio/(%)	27.85 ± 1.03	26.51 ± 0.88	27.16 ± 0.91	27.33 ± 0.71	0.110	0.014	0.052
Egg yolk moisture content/(%)	48.32 ± 3.72	48.41 ± 2.81	46.50 ± 5.88	43.89 ± 9.13	0.523	0.793	0.058

Different letter in each row indicates a significant difference ($p < 0.05$).

TABLE 5 Effects of mulberry leaf extract on serum antioxidant capacity of laying hens.

Items	Control group	Mulberry leaf extract added levels (%)			*P*		
		0.4	0.8	1.2	ANOVA	Linear	Quadratic
CAT/(U/mL)	18.25 ± 0.98	19.07 ± 1.51	19.87 ± 1.35	20.10 ± 1.71	0.057	0.007	0.023
T-AOC/mM	3.54 ± 0.42	3.68 ± 0.41	4.02 ± 0.39	3.75 ± 0.42	0.121	0.018	0.057
SOD/(U/mL)	151.42 ± 8.96^{b}	155.33 ± 10.77^{b}	161.10 ± 7.29^{ab}	165.64 ± 10.53^{a}	0.029	0.002	0.010
GSH-Px/(U/mL)	108.36 ± 9.81^{c}	115.74 ± 11.19^{bc}	126.25 ± 13.32^{ab}	130.25 ± 12.37^{a}	0.003	<0.001	0.001
MDA/(nmol/mL)	8.82 ± 1.70	8.10 ± 1.13	7.37 ± 1.10	7.11 ± 1.83	0.151	0.492	0.006

Different letter in each row indicates a significant difference ($p < 0.05$).
CAT, catalase activity; T-AOC, total antioxidant capacity; SOD, superoxide dismutase; GSH-Px, glutathione peroxidase MDA, malondialdehyde.

liver. The transaminase activity of the liver is higher than that of the blood and the liver cell membrane ruptures during liver injury. The release of transaminase into the blood increases transaminase activity in the blood (30). Researchers have found that the addition of 1.0% Chinese herbal mixture to the diet of laying hens can reduce serum ALT contents (31). Salvia polysaccharides were added to drinking water at a concentration of 0.5–2.0 g/L and showed a significant reduction in ALT and AST activities in chicken serum (32). Our findings were consistent with the results of these studies. The AST and ALT activities decreased in the current study with MLE addition. These results combined with other data from this experiment show that mulberry leaf polysaccharides may exhibit antioxidative properties, which helps to reduce the liver damage caused by hens laying egg over a long period of time. Serum contents of TG, TC, LDL-C, and HDL-C were used to determine whether lipid metabolism in the animals was normal. High-energy diets fed to hens during

TABLE 6 Effects of mulberry leaf extract on lipid metabolism parameters of laying hens.

Location	Items	Control group	Mulberry leaf extract added levels (%)			*P*		
			0.4	0.8	1.2	ANOVA	Linear	Quadratic
Liver	TG/(mmol/L)	2.68 ± 0.69^{a}	1.76 ± 0.20^{b}	2.06 ± 0.71^{ab}	1.97 ± 0.65^{b}	0.046	0.015	0.049
	TC/(mmol/L)	1.05 ± 0.19^{a}	0.80 ± 0.08^{b}	0.96 ± 0.17^{b}	0.85 ± 0.20^{ab}	0.052	0.062	0.177
Yolk	TG/(mmol/L)	4.07 ± 0.80^{a}	3.02 ± 0.21^{b}	2.56 ± 0.40^{b}	2.92 ± 0.74^{b}	0.002	0.014	0.001
	TC/(mmol/L)	5.31 ± 0.71^{a}	1.54 ± 0.29^{c}	3.71 ± 1.29^{b}	3.95 ± 0.66^{b}	<0.001	<0.001	<0.001

Different letter in each row indicates a significant difference ($p < 0.05$).
TG, triglyceride; TC, total cholesterol.

the peak laying period can easily lead to lipid metabolism-related diseases in the late laying period, thereby reducing economic efficiency. A previous study showed that the addition of 0.5% MLE to the diet reduced serum TG, TC, and LDL-C levels in rats (33). The results from another study supported this finding (34). The present study demonstrated that the addition of MLE to the diet was associated with significant reductions in serum TG, TC, and LDL-C contents and significant increases in HDL-C contents. This indicates that the increase of lipolytic capacity may be due to the hypoglycemic effect of mulberry leaf polysaccharides. Studies suggested that mulberry leaf polysaccharides may reduce blood glucose by affecting the activity of related enzymes, improving glucose and lipid metabolism, and regulating the related lipid metabolism signaling pathways (35, 36). The cause of these results were further investigated.

Egg-laying hens exhibit fast metabolisms during the peak egg-laying period, which results in the rapid accumulation of a large number of free radicals in the body, leading to lipid peroxidation. This inhibits the activity of various antioxidant enzymes, causing oxidative stress and cellular tissue damage, resulting in accelerated aging of the body and adverse effects on production performance. Therefore, during peak egg production, we should take the initiative to alter the diet to avoid the premature aging of laying hens. The antioxidant enzymes SOD, GSH-Px, and CAT are the important parts of the *in vivo* antioxidant system. The T-AOC contents indicate the status of the non-enzymatic reactive oxygen defense system of body, whereas MDA contents reflect the rate and intensity of lipid peroxidation in the body (37). The addition of antioxidative substances to the feed will help improve the ability to scavenge free radicals of body and maintain the redox balance (38). According to the results of the experiment, mulberry leaf powder significantly increased the GSH-Px activity in the serum of Xiangcun black pigs (39). In the current study, supplementing the diet with MLE significantly increased the serum levels of SOD and GSH-Px, indicating that MLE has antioxidative properties. Many *in vitro* tests have demonstrated the scavenging effect of mulberry leaf polysaccharides on free radicals, such as 1,1-diphenyl-2-picrylhydrazyls (DPPH), hydroxyl (OH^{-}), and superoxide (O_2^{-}) (36, 40). It has been hypothesized that mulberry leaf polysaccharides also play an antioxidative role in laying hens.

In the production of modern laying hens, producers prefer using natural plant additives to obtain higher egg quality to comply with local regulations and policies on the use of additives. However, producers can improve egg quality and functional differences using other competing products to obtain great economic benefits. Studies have shown that dietary administration of 100 mg/kg of Yucca schidigera extract could significantly improve egg quality (41). Additionally, researchers have found that the addition of mulberry leaf powder to the diet significantly improved egg yolk color, but adding more than 10% mulberry leaf powder negatively affected egg quality (42). One study found that yolk weight, eggshell weight, eggshell strength, eggshell thickness, yolk color, and Haugh units increased in all MLE supplemented groups after adding 1% MLE to the diet of laying hens (16), which is consistent with the results of our present experiment, where adding MLE the diet caused egg quality-related indicators to be affected linearly and quadratically. The addition of mulberry leaf flavonoids to the diet enhanced eggshell strength by increasing the antioxidant capacity of the uterine shell gland and calcium deposition (significantly upregulating the expression of related genes, namely ESRpha, ESRbeta, KCNA1, OPN, CABP-28K, and CDH6) (18). In this current study, adding MLE to the diet improved shell strength and yolk color on days 28 and 56. Increasing eggshell strength within a certain range is beneficial for reducing the damage rate of eggs during transportation and reducing loss. The measures to improve the eggshell strength have been reported. This result may be related to the active ingredients, mulberry leaf polysaccharides, and mulberry leaf flavonoids in the MLE. The further research and confirmation were required. Yolk color is one of the most critical indicators of egg quality. There is a strong relationship between egg yolk color and egg quality. A dark yolk color implies better egg quality; therefore, eggs with a darker yolk color are preferred by consumers (43, 44). The carotenoid content of the laying hens diet is the main factor affecting the yolk color. Several studies have shown that carotenoids found naturally

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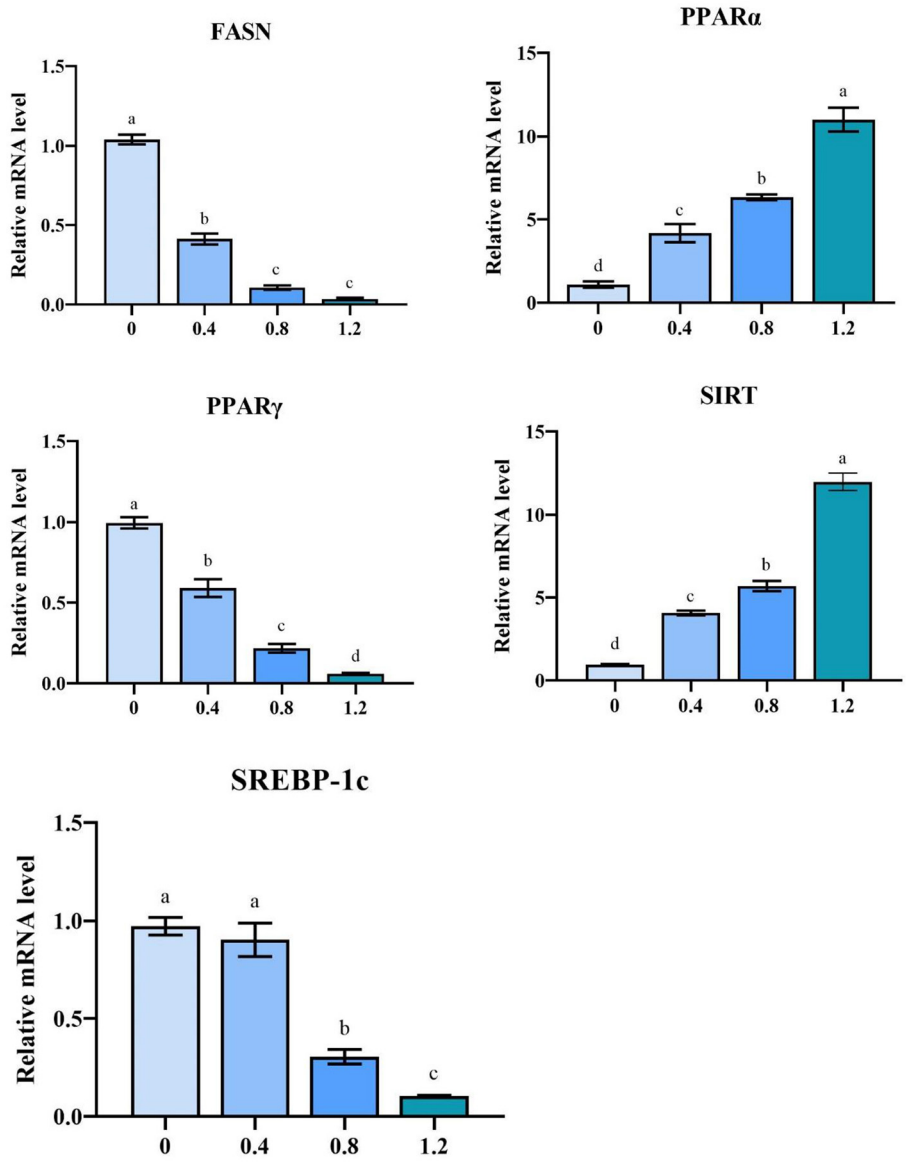

FIGURE 1
The effect of MLE on the mRNA expression of the laying hen hepatic (FASN, SIRT, PPARγ, SREBP-1c and PPARα) genes (mean ± MSE). Columns with different superscript letters are significantly different ($P < 0.05$).

in plant-based diets of laying hens are transferred to the yolk of the eggs laid. Different diet components can also influence the yolk's color, such as the lipid structure and the type and amount of carotenoids (45–47). The darker yolk color in this experiment is presumed to be due to the impact of MLE on the lipid metabolism of laying hens, promoting both the absorption of fat-soluble carotenoids and their deposition in the yolk.

The liver is an essential organ for lipid metabolism in poultry and an integral part of the *ab initio* synthesis of fatty acids, with nutrients entering the liver through the portal vein after absorption in the small intestine (48). The diet of laying hens contains only a small amount of cholesterol, and the cholesterol of body is mainly synthesized through the liver; two-thirds of the cholesterol is metabolized through eggs, and the rest is metabolized through fecal and bile acid metabolic pathways. In this experiment, TG and TC contents were significantly reduced in both the liver and egg yolk of the test groups, further indicating that MLE positively affects lipid metabolism in poultry. Most plant polysaccharides have hypolipidemic effects. The addition of 1–2 g/kg mannan-oligosaccharides to the diet significantly reduced serum TG and LDL contents in laying

hens (49). The addition of 0–20 g/kg sumac and ginger to the diet significantly reduced TC contents in egg yolk and serum (50). To further validate these results, we measured the protein expression of relevant lipid metabolism genes in the liver in response to available experimental data.

Peroxisome proliferators-activated receptors (PPARs) are ligand-activated receptors in the nuclear hormone receptor superfamily and are present as three isoforms (51). PPARα is the main transcription factor that regulates mitochondrial fatty acid β-oxidation genes and is negatively correlated with IMF content (52). PPARγ promotes liver energy storage and adipocyte differentiation and is potentially regulated by SREBP-1C to regulate lipid synthesis (53, 54). SREBP-1C preferentially regulates the biosynthesis of fatty acids, phospholipids, and triglycerides and can activate the fatty acid synthase gene (FASN) (55). FASN is a rate-limiting enzyme for fat regeneration capacity and is involved in fat deposition and phospholipid synthesis in animals; its elevated expression level leads to a significant increase in triglycerides *in vivo* (56). Silent information regulator 1 (SIRT1) is an NAD^+-dependent deacetylase involved in regulating lipid metabolic processes, acts as a negative regulator of TG synthesis, and is capable of stimulating fatty acid oxidation (57). Our experimental results showed that MLE might affected liver lipid metabolism in laying hens by influencing the SIRT/PPAR signaling pathway. Additionally, it reduces the expression of its target gene-FASN by inhibiting the expression of the transcription factor SREBP-1C, thereby reducing lipid synthesis. It has been shown that 0.8 g/kg·d MLP inhibited adipocyte differentiation and triglyceride synthesis by affecting the PPAR-γ-C/EBP-α signaling pathway in rats (58), and the addition of 5% MLP to the diet of fattening pigs resulted in a decrease in FAS and a significant increase in hormone-sensitive adiponectin and leptin receptors (59). Other plant extracts can also affect lipid metabolism in livestock by modulating the SIRT/PPAR pathway. Green tea extract can reduce abdominal fat accumulation in broiler chickens by downregulating PPARγ expression in abdominal adipose tissue (60). The addition of genistein to laying hen diets inhibits fatty acid synthesis and enhances β-oxidation in the liver by modulating the PPAR-LXRα-SREBP1c-ACC/FAS/FAT pathway (61).

Conclusion

In conclusion, adding 0.8% MLE to the diet of laying hens could improve egg quality and antioxidant capacity, regulate lipid metabolism, reduce the probability of lipid metabolism-related diseases in the egg-laying period, and extending the egg-laying cycle, Obtain higher economic benefits when promoting the application in the future. The study will provide a theoretical reference for the application of MLE in egg production and promoting the healthy and sustainable development of the livestock and poultry industry under the background of antibiotic prohibition.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/supplementary material.

Ethics statement

The animal study was reviewed and approved by the Animal Use and Ethical Committee of Hebei Agricultural University (University Identification Number: HB/2019/03). Written informed consent was obtained from the owners for the participation of their animals in this study.

Author contributions

BZ, DC, HC, and ZW: design and complete the experiment. BZ, DW, HC, and YC: statistics and contributions. HC and XS: provide experimental guidance. 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

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Dietary supplementation with a mixture of herbal extracts during late gestation and lactation improves performance of sows and nursing piglets through regulation of maternal metabolism and transmission of antibodies

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The dietary inclusion of phytogenic feed additives to improve the performance and health of sows is considered to be safe, effective and environmentally friendly, thus gaining growing popularity among new strategies. This study was designed with three trials aimed to determine the effective supplemental levels of *Scutellaria baicalensis* and *Lonicera japonica* mixed extracts (SLE) in sow diets based on production performance and explore its related mechanisms of action based on serum metabolites, antioxidant capacity, and immune profile of sows and nursing piglets. Trials 1 and 2 were conducted to determine the effective dose and ratio of SLE by supplementation of various proportions and doses of SLE to sows diets from the late pregnancy to weaning, with litter performance at farrowing and weaning and disease conditions being evaluated. Trial 3 was conducted to further explore the mechanisms of action of SLE as evaluated by serum immunity and antioxidants indices in late gestation and lactation sows. The results of trials 1 and 2 showed that dietary supplementation of 1.0 g/kg SLE (50% *S. baicalensis* extract, 30% *L. japonica* extract, and 20% wheat bran fiber as carrier) enhanced the number of piglets born alive, litter birth weight, litter weight gain, and average daily feed intake of sows during lactation, while decreased diarrhea of suckling piglets. In Trial 3, compared with the control group, dietary SLE supplementation increased ($P < 0.05$) sow serum glucose (GLU), triglyceride (TG), total cholesterol (TC), prolactin (PRL) and interleukin-10 (IL-10) concentrations, and total superoxide dismutase (T-SOD)

activities at the farrowing, and increased ($P < 0.05$) sow serum prolactin, leptin, and insulin concentrations at d 14 of lactation. Fat concentrations in sow colostrum and in milk on day 14 of lactation, both IgA and IgG concentrations in colostrum, and both IL-10 and IgA concentrations in piglet serum at d 14 of lactation were all increased ($P < 0.05$) following dietary SLE supplementation. Altogether, dietary supplementation with the appropriate levels of SLE promoted health and growth of suckling piglets, which was associated with the improvement of maternal metabolism and transmission of antibodies.

KEYWORDS

***scutellaria baicalensis*, *lonicera japonica*, sows, performance, immunity**

Introduction

Milk yield is one of the most important factors limiting neonatal piglet growth (1). Poor milk yield is the most frequent cause of breastfeeding failure (2). For sow-reared piglets, maximum weight gain is limited to as early as seven days after farrowing (3, 4). Besides, genetic selection for highly prolific sows has increased nutrient requirements during gestation and lactation to allow an increased milk yield to support large piglets (4). However, the vigorous metabolism of high-yielding sows during lactation generated extensive reactive oxygen species (ROS) to cause oxidative stress in the body, which resulted in reduced feed intake and milk yield of sows. The growth rate of suckling piglets largely depends on sow milk yield, but the amount of milk produced by sows did not improve significantly, resulting in the slow growth of piglets and the increase of weak and dead piglets (5). The limited nutrient intake would leave sows under severe catabolic status and reduce reproductive performance concurrently (6). One way to increase sow milk yield would be to stimulate mammary development. But sows enter a critical period of mammary gland development and rapid fetal growth at 75 days later in gestation. Maternal metabolic intensity increases and ROS accumulate in the body, which results in increased maternal oxidative stress and immunosuppression, and increased stillbirth and postpartum inflammation. In addition, maternal oxidative stress and low immune status will reduce the content of immune factors in colostrum and change the milk composition, increase the risk of diarrhea in piglets, and cause poor growth and development and even death of piglets. Therefore, optimizing feed intake during lactation, slowing down progressive oxidative stress, and improving immune function are the keys to improving the reproductive performance of sows. It is becoming more and more urgent to find an additive that can improve the milking ability and immune function of sows.

Several herbs and spices are assumed to have beneficial effects on milk secretion. Many herbal products are currently used in the European Union and elsewhere by the feed industry as feed additives (7). Traditional Chinese medicine (TCM), after a long period for being screened, is considered natural, low/non-toxic, showing short resistance and less residue. And its extracts have more functions including antibacterial and antioxidant activities, improving immunity, and regulating hormone secretion (8). *Scutellaria baicalensis* (*S. baicalensis*) Georgi and *Lonicera japonica* (*L. japonica*) Thunb. are two widely used traditional Chinese herbal medicines, and are officially listed in the Chinese Pharmacopeia. *S. baicalensis* roots have been used as anti-inflammatory and anticancer agent, for the treatment of bacterial and viral infections of the respiratory and the gastrointestinal tract, and because of its cholagogic, diuretic, and detoxifying properties. Baicalin is the most abundant component of *S. baicalensis* extracts, which could alleviate the adverse effect of heat stress and showed anti-allergic, anti-tumor, anti-inflammatory, and antioxidant activities (9, 10). *Lonicera japonica* contains a variety of organic acids, essential oils, flavones, saponins, and iridoids. Among them, chlorogenic acid and essential oils are the primary pharmacological compounds in *L. japonica*. Modern pharmacological studies showed that *L. japonica* and its extracts possessed wide pharmacological actions, such as antibacterial, anti-inflammatory, antiviral, antiendotoxin, antioxidant, antipyretic, and excitation of nerve centers (11–13). In clinical practice, the *L. japonica* extracts have been used for the treatment of fever, heatstroke, bloody flux, sores, carbuncles, furunculosis, headache, and some infectious diseases (11).

In weaning and growing-finishing pigs, many positive reports are available on the herbal extract supplementation diets (14–17). However, there is little information on responses of gestation and lactation sows to dietary herbal extract supplementation. Therefore, the objectives of the present study were to determine the effect of dietary herbal supplements on performance of sows and their progeny, and explore the underlying mechanisms as evaluated by serum metabolites,

antioxidant capacity, and immune function of sows and suckling piglets.

Materials and methods

Ethics approval

The protocol of this study was approved by the Animal Care and Use Committee of Animal Nutrition Institute, Sichuan Agricultural University (Ethics Approval Code: SCAUAC201606-6), and was carried out in accordance with the National Research Council's Guide for the Care and Use of Laboratory Animals.

Animals, diets, and experimental design

The study was designed with three trials. Trials 1 and 2 were conducted to determine the effective dose and ratio of *S. baicalensis* (SBE) and *L. japonica* (LJE) mixed extracts (SLE) by supplementation of various proportions and doses of SLE to sow diets from the late pregnancy to weaning, with litter performance at farrowing and weaning and disease conditions being evaluated. Trial 3 was conducted to further explore the mechanisms of action of SLE as evaluated by immunity and antioxidants indices during the late gestation and lactation period. Basal diets for all sows contained corn as a cardinal energy source and soybean meal as a cardinal protein source. Diets for sows during the late pregnancy (from day 85 of gestation to farrowing) and lactation were formulated to meet or exceed the NRC (2012) recommendations (Table 1). The herbal extract from *S. baicalensis* and *L. japonica* was extracted by a combination of ultrasound and microwave systems. The SLE products were provided by Beijing Centre Biology Co., Ltd., (Beijing, China).

The objective of Trial 1 was to determine the optimal proportion of the two extracts (SBE and LJE) in the SLE mixture as evaluated by the farrowing and lactation performance. Trial 1 was conducted in a pig farm in Fujian province from July to August, with an average room temperature being 28–30°C. According to a completely randomized block design, a total of 75 Yorkshire × Landrace sows (weighing 275.55 ± 20.97 kg, mean parity 4.44 ± 1.84) on the 85th day of pregnancy were assigned based on genetic background, parity and body weight to five experiment groups: control (CON, n = 15; basal diet, Table 1), treatment group 1 (TRT1, n = 15, basal diet + 1 g/kg SLE powder product consisting of 45% SBE, 35% LJE and 20% wheat bran as carrier); treatment group 2 (TRT2, n = 15, basal diet + 1 g/kg a mixture of 50% SBE, 30% LJE, and 20% carrier); treatment group 3 (TRT3, n = 15, basal diet + 1 g/kg a mixture of 55% SBE, 25% LJE, and 20% carrier); and treatment group 4 (TRT4, basal diet +1 g/kg a mixture of 60% SBE, 20% LJE, and 20% carrier).

TABLE 1 Composition and nutrient levels of the basal diets (air-dry basis).

Items	Content
Ingredients, %	
Corn, 7.8% CP	42.11
Soybean meal, 46% CP	16.00
Barley, bark	15.0
Wheat bran	10.0
Puffed soybean, wet	8.0
Soybean oil	1.85
Fish steak powder	1.5
Calcium bicarbonate	1.27
Saccharose	1.0
Limestone	0.8
NaCl	0.4
L-Lysine sulfate, 70%	0.45
Vitamin premix[a]	0.50
Mineral premix[b]	0.30
Other	0.82
Total	100
Nutrient levels	
DE, MJ/Kg	13.70
CP, %	17.98
SID-Lysine	1.00
SID-Methionine	0.41
SID-Methionine+Cystine	0.64
SID-Threonine	0.75
SID-Tryptophan	0.2
SID-Valine	0.82
Ca, %	0.85
P, %	0.67

[a]Provided per kg of complete diet: vitamin A, 35,000 IU; vitamin D, 36,000 IU; vitamin E, 50 IU; vitamin K_3, 1.8 mg; riboflavin, 11 mg; niacin, 8 mg; D-pantothenic acid, 75 mg; biotin, 1.5 mg; folic acid, 2 mg; choline, 160 mg; vitamin B_6, 5 mg; and vitamin B_{12}, 3 mg. [b]Provided per kg of complete diet: Fe (as $FeSO_4 \cdot 7H_2O$), 90 mg; Cu (as $CuSO_4 \cdot 5H_2O$), 20 mg; Zn (as $ZnSO_4$), 100 mg; Mn (as MnO_2), 25 mg; I (as KI), 0.14 mg; and Se (as $Na_2SeO_3 \cdot 5H_2O$), 0.15 mg.

The objective of Trial 2 was to determine the optimal dietary supplementation level of the SLE mixture (50% SBE, 30% LJE, and 20% carrier) that was observed to be effective in improving sow performance in Trial 1. Trial 2 was carried out on a pig farm in Sichuan province from November to December. A total of 75 Yorkshire × Landrace sows (weighing 261.42 ± 25.74 kg, mean parity 3.13 ± 1.87) on the 85th day of pregnancy were assigned based on parity and body weight to five experiment groups: Control (CON, n = 15; basal diet, Table 1), 0.6TRT2 (basal diet + 0.6 g/kg SLE), 0.8TRT2 (basal diet + 0.8 g/kg SLE), 1.0TRT2 (basal diet + 1.0 g/kg SLE), and 1.2TRT2 (basal diet +

1.2 g/kg SLE). The basal diet was isoenergy but 1.5% lower in CP than diet shown in Table 1.

The objective of Trial 3 was to further verify the positive role of dietary SLE mixture supplementation at 1.0 g/kg diet (Table 1) on farrowing and lactation performance, and at the same time explore the mechanisms of action of SLE as evaluated by immunity and antioxidants indices based on blood and milk samples. Trial 3 was carried out on a pig farm in Sichuan province from May to July. A total of 64 Yorkshire × Landrace sows (mean weight 250.15 ± 10.35 kg, mean parity 2.70 ± 1.43) on the 85th day of pregnancy were assigned based on genetic background, parity and body weight to two experiment groups: Control (CON, $n = 32$; basal diet) and 1.0 TRT2 (SLE, $n = 32$, basal diet + 1 g/kg of SLE).

Feeding management

Sows were fed 3.0 kg/d from d 85 to 112 of pregnancy, and then fed 2.0 kg/d from d 113 of pregnancy to farrowing. After farrowing, sows were fed 2.0 kg of diet, which was increased by 1 kg/d in the following 3 days, and then sows had free access to feed from d 5 of lactation until weaning. During the gestation period, sows were housed in individual cages with concrete floors and equipped with automated drop feeders and nipple drinkers. On d 109 of gestation, sows were transported to the farrowing facility, where they were housed in individual farrowing crates (2.4 × 2.2 m) with creep area and nipple drinkers. Litters were standardized to be 12–14 piglets within 48 h after birth. Voluntary feed intake was measured daily throughout the lactation period. According to the practical situation of the pig farm in each trial, piglets were weaned at 21, 20, and 17 d postnatal, respectively, in trials 1, 2, and 3.

The feed consumption during lactation was recorded for each sow to calculate the average daily feed intake (ADFI). During the experimental period, numbers of piglets alive and dead per litter were recorded to calculate the survival ratio. Piglet body weight (BW) was recorded on d 1 (within 12 h of birth) and weaning day. In the 48th h after birth, the number of piglets in sows was adjusted so that litter piglets of sows were consistent among groups, and the number of piglets and litter weight were recorded. No creep feed was offered to piglets throughout the lactation period. The health status of sows and piglets were recorded daily during the lactation period. After weaning, the estrus interval (within 7 days) was recorded for each sow.

Sample collection

In Trial 3, blood samples of sows were collected from the ear vein using sterile vacuum tubes from 20 randomly chosen sows on d 90 of gestation and d 1, d 14 of lactation before the morning feeding. At d 14 of lactation, blood samples were taken by jugular venipuncture using sterile vacuum tubes from 40 piglets selected from the 20 sows used for sow blood collection, with one male and one female piglet selected from each sow. After blood collection, serum were separated by centrifugation at 3,000 rpm for 5 min and then serum samples were collected and stored in sterile tubes at −20°C until analysis.

Colostrum was collected from 20 randomly chosen sows on d 1 of lactation within 4 h after initiation of farrowing. Milk was collected on d 14 of lactation after intramuscular injection of 10 IU of oxytocin behind the ear. After collection, milk (5 ml) for determination of hormone and immune indices was centrifuged (3,500 rpm) for 20 min to remove the fats and then the supernatant was collected and stored at −20°C until analysis.

Chemical analysis

The glucose (Glu), triglyceride (TG), total cholesterol (TC), and free fatty acid (FFA) were measured using an auto-analyzer (BMD/Hitachi 7050 Auto Analyser; Japan). Whole milk was analyzed for dry matter, protein, fat, and lactose contents by a Milkyway-cp2 rapid milk composition analyzer (Institute of Food Science and Fermentation Engineering, Zhejiang University). The cytokines (IL-8, IL-10, TNF-α) and immunoglobulin (IgG, IgA, IgM) concentrations in colostrum, milk, and serum samples were determined using specific pig-ELISA quantification kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) according to instructions of their respective manufacturers, respectively. The serum concentrations of insulin (INS), prolactin (PRL), thyroid-stimulating hormone (TSH), leptin, and growth hormone (GH) in sows were measured by respective ELISA quantification kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). Assessment of total antioxidant capacity (T-AOC) and total superoxide dismutase (T-SOD) in serum from sows and piglets were performed using specific assay kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China).

Statistical analyses

All data were checked for normality using the univariate procedure of Statistical Analysis Software (Version 9.4, SAS Institute Inc., Cary, NC, USA) and transformed, if required. The data of litter weight at weaning, litter weight gain and the number of weaned piglets were assessed by analysis of covariance using the general linear model (GLM) procedure of SAS, and the adjusted litter weight and litter number were used as covariates. The data of piglets born alive, litter birth weight, and piglet birth weight were assessed using the total number born as covariate by covariance analysis. The data of diarrhea of piglets and estrous rate were analyzed by χ^2-test. The data of the estrus interval was analyzed by rank-sum

TABLE 2 Reproductive performance of sows (Trial 1).

Items	Treatments[1]					SEM[2]
	CON	TRT1	TRT2	TRT3	TRT4	
Average parity	4.5	4.3	4.5	4.6	4.2	2.56
Litter, no						
Total number born	12.1^{b}	12.9^{ab}	14.4^{a}	13.6^{a}	11.7^{b}	1.88
Piglets born alive	11.5^{b}	12.4^{ab}	13.9^{a}	13.1^{a}	11.6^{b}	1.80
Piglets adjusted[3]	11.1	11.0	11.3	11.4	10.9	0.55
Piglets weaned	10.6	10.7	10.9	10.3	10.4	0.66
BW, kg						
Litter birth weight	15.36^{b}	17.68^{ab}	19.00^{a}	16.99^{ab}	17.20^{ab}	2.12
Piglet birth weight	1.36	1.47	1.39	1.34	1.51	0.13
Weaning, 21 days						
Litter weight after adjusted[3]	18.33	17.22	18.85	18.63	18.80	2.00
Litter weight	55.36^{b}	59.16^{ab}	65.18^{a}	62.02^{a}	58.71^{ab}	5.37
Piglet weight	5.35^{b}	5.53^{ab}	6.02^{a}	6.03^{a}	5.67^{ab}	0.36
Litter weight gain	37.54^{b}	41.67^{ab}	46.94^{a}	45.97^{a}	40.79^{ab}	4.18
Feed intake, kg, 21 days						
Total intake	90.68^{c}	96.33^{bc}	99.99^{ab}	103.41^{a}	99.37^{ab}	4.73
ADFI	4.32^{c}	4.59^{bc}	4.76^{ab}	4.92^{a}	4.73^{ab}	0.14
Post-weaning estrus, within 7 days						
Estrus interval, d	5.89	5.31	5.58	5.67	5.50	0.24
Estrous rate, %	69.23%	86.67%	86.67%	85.71%	69.23%	0.41
Diarrhea of piglets, %	33.62%^{a}	17.68%^{b}	12.34%^{b}	12.37%^{b}	12.03%^{a}	0.04

[1]CON, n = 15; basal diet, TRT1, n = 15, control+ 1 g/kg a mixture of 45% SBE, 35% LJE, and 20% carrier (wheat bran); TRT2, n = 15, control+1 g/kg a mixture of 50% SBE, 30% LJE, and 20% carrier (wheat bran); TRT3, n = 15, control+1 g/kg a mixture of 55% SBE, 25% LJE, and 20% carrier (wheat bran); TRT4, n = 15, control+1 g/kg a mixture of 60% SBE, 20% LJE, and 20% carrier (wheat bran).
[2]Standard error of the means.
[3]In the 48th h after birth, adjusted the number of piglets and litter weight.
[a,b]Means within a row without a common superscript differ, P < 0.05.

test. The other data were analyzed with one-way analysis of variance (ANOVA) using the GLM procedure of SAS software. CONTRAST was used to compare differences with the control group. Differences were considered significant when $P \leq 0.05$, and trends were noted when $0.05 < P < 0.10$. Quadratic effects were not significant, and hence their *P*-values were omitted from the tables.

Results

Sows and piglets performance

As presented in Table 2, the performance of sows and piglets was affected by diet. Sows in the TRT2 showed significantly increased total number born, number born alive, litter birth weight, litter weight and piglet weight of weaning at 21 days, litter weight gain, total intake and ADFI of sows at day 21 of lactation, and significantly decreased piglets diarrhea compared with sows in the CON treatment ($P < 0.05$). However, the number of weaned piglets, piglet birth weight, weaning-estrous interval, and estrous rate within 7 days were not affected ($P > 0.05$) by SLE dietary supplementation. TRT3 and TRT2 had the same trend, but no difference ($P > 0.05$) in reproductive performance was observed between other treatments. Therefore, we would choose TRT2 [50% SBE, 30% LJE, and 20% carrier (wheat bran), SLE] as the optimized mixed proportion for Trial 2.

As presented in Table 3, compared with sows in the CON treatment, sows in the 1.0TRT2 significantly increase the total number born, number born alive, number of weaned piglets, litter birth weight, litter weight and piglet weight of weaning at 20 days, litter weight gain, total intake and ADFI of sows at day 20 of lactation ($P < 0.05$) and significantly decreased piglets' diarrhea ($P < 0.05$). There was no effect ($P > 0.05$) on piglet weight of birth, weaning-estrous interval, and estrous rate. In addition, 0.6TRT2 sows had higher total feed intake and ADFI (20 days) ($P < 0.05$) compared with CON sows. No difference ($P > 0.05$) in reproductive performance was observed among treatments. Therefore, the 1.0TRT2 (1.0 g/kg SLE) was selected as the optimum content for Trial 3.

TABLE 3 Reproductive performance of sows (Trial 2).

Items	Treatments[1]					SEM[2]
	CON	0.6TRT2	0.8TRT2	1.0TRT3	1.2TRT4	
Average parity	3.07	3.07	3.13	3.27	3.13	1.12
Litter, no						
Total number born	10.5^{b}	11.4^{ab}	11.2^{ab}	11.9^{a}	12.5^{a}	1.57
Piglets born alive	10.1^{b}	10.5^{ab}	10.7^{ab}	11.6^{a}	12.4^{a}	2.16
Piglets adjusted[3]	11.1	11.1	11.1	11.3	11.5	0.56
Piglets weaned	10.1^{b}	10.5^{ab}	10.7^{ab}	11.1^{a}	10.9^{ab}	0.63
BW, kg						
Litter birth weight	14.53^{b}	15.35^{ab}	15.23^{ab}	17.00^{a}	16.64^{a}	2.24
Piglet birth weight	1.45	1.52	1.48	1.48	1.37	0.16
Weaning, 20 days						
Litter weight after adjusted[3]	16.00	16.17	16.92	16.17	16.01	1.63
Litter weight	57.46^{b}	62.43^{b}	63.76^{b}	71.02^{a}	63.19^{b}	5.63
Piglet weight	5.71^{b}	5.99^{b}	6.03^{b}	6.43^{a}	5.82^{b}	0.52
Litter weight gain	43.58^{b}	47.34^{ab}	48.73^{ab}	54.99^{a}	48.29^{ab}	4.70
Feed intake, kg, 20 days						
Total intake	91.81^{b}	109.93^{a}	102.58^{ab}	107.28^{a}	101.01^{ab}	8.91
ADFI	4.59^{b}	5.50^{a}	5.13^{ab}	5.36^{a}	5.05^{ab}	0.45
Post-weaning estrus, within 7 days						
Estrus interval, d	5.42	4.91	4.77	5.15	5.07	0.60
Estrous rate, %	85.71%	78.57%	93.33%	86.67%	93.33%	0.38
Diarrhea of piglets, %	22.58%^{a}	10.32%^{b}	7.78%^{b}	3.47%^{b}	8.23%^{b}	0.10

[1]CON, n = 15, basal diet; 0.6TRT2, n = 15, control+0.6 g/kg SLE; 0.8TRT2, n = 15, control+0.8 g/kg SLE; 1.0TRT2, n = 15, control+1.0 g/kg SLE; 1.2TRT2, n = 15, control+1.2 g/kg SLE.
[2]Standard error of the means.
[3]In the 48th h after birth, adjusted the number of piglets and litter weight.
[a,b]Means within a row without a common superscript differ, P < 0.05.

As presented in Table 4, the lactation ADFI and total intake (17 days) were significantly increased ($P < 0.01$) by dietary supplementation of 1.0 g/kg SLE. In addition, compared with the CON group, the 1.0TRT2 group had significantly increased ($P < 0.05$) litter weight at weaning and litter weight gain and decreased ($P < 0.05$) diarrhea of piglets during lactation. The number of piglets born alive, litter birth weight, number of weaned piglets, and weaning-estrous interval were not affected ($P > 0.05$) by SLE supplementation.

Blood profiles of sows

As presented in Table 5, there were no differences ($P > 0.05$) in serum concentrations of GLU, TG, TC, and FFA between groups at d 90 of gestation and d 14 of lactation. Compared with the CON group, dietary SLE supplementation significantly increased serum GLU ($P < 0.01$) and TG and TC ($P < 0.05$) concentrations at the farrowing day (Table 5).

Serum hormones of sows

As presented in Table 6, there were no differences ($P > 0.05$) in the concentrations of GH, TSH, PRL, INS, and leptin in sow serum between groups at d 90 of gestation. Compared with the CON group, dietary SLE supplementation significantly increased sow serum PRL concentrations at the farrowing day and significantly increased serum PRL, leptin, and INS concentrations at d 14 of lactation ($P < 0.05$). However, the concentrations of GH and TSH in sow serum on days 1 and 14 of lactation were not affected ($P > 0.05$) by SLE diet treatments.

Serum immunization index of sows

As presented in Table 7, compared with the CON group, dietary SLE supplementation significantly increased serum IL-10 ($P < 0.05$) concentrations at farrowing (Table 7). But the humoral immunity factor contents of IgA, IgG, IgM, IL-8, and TNF-α in sow serum were not affected ($P > 0.05$) by dietary SLE supplementation.

TABLE 4 Reproductive performance of sows (Trial 3).

Items	Treatments[1]		SEM[2]
	CON	1.0TRT2	
Average parity	2.75	2.66	0.36
Litter, no			
Total number born	14.26	14.97	0.78
Piglets born alive	13.39	13.63	0.71
Piglets adjusted[3]	12.96	12.70	0.33
Piglets weaned	12.0	12.30	0.41
BW, kg			
Litter birth weight	18.53	19.01	0.95
Piglet birth weight	1.39	1.42	0.05
Weaning, 17 days			
Litter weight after adjusted[3]	18.68	18.59	0.87
Litter weight	53.72^{b}	59.05^{a}	2.54
Piglet weight	4.47	4.79	0.31
Litter weight gain	35.10^{b}	40.16^{a}	2.58
Feed intake, kg, 17 days			
Total intake	70.06^{B}	79.32^{A}	14.68
ADFI	4.16^{B}	4.67^{A}	0.27
Post-weaning estrus, within 7 days			
Estrus interval, d	4.2	4.5	0.24
Estrous rate, %	93.33%	92.31%	0.36
Diarrhea of piglets, %	9.04%^{A}	1.24%^{B}	0.05

[1]CON, n = 32, basal diet; 1.0TRT2, n = 32, control+1.0 g/kg SLE.
[2]Standard error of the means.
[3]In the 48th h after birth, adjusted the number of piglets and litter weight.
[A,B,a,b]The values with unlike superscripts differ at P < 0.05 (small letters) or P < 0.01 (capital letters).

TABLE 5 Serum biochemical indices of sows (Trial 3), mmol/L.

Items	Treatments[1]		SEM[2]
	Con	1.0TRT2	
GLU			
d 90 of gestation	4.64	4.61	0.42
d 1 of lactation	3.77^{B}	4.22^{A}	0.16
d 14 of lactation	3.82	4.26	0.35
TG			
d 90 of gestation	0.39	0.55	0.08
d 1 of lactation	0.70^{b}	0.90^{a}	0.10
d 14 of lactation	0.41	0.42	0.09
TC			
d 90 of gestation	1.57	1.56	0.12
d 1of lactation	1.71^{b}	1.93^{a}	0.09
d 14 of lactation	2.08	2.18	0.16
FFA			
d 90 of gestation	0.81	0.85	0.13
d 1of lactation	0.03	0.04	0.01
d 14 of lactation	0.44	0.57	0.15

[1]CON, n = 10, basal diet; SLE, n = 10, control+1.0 g/kg SLE.
[2]Standard error of the means.
[A,B,a,b]The values with unlike superscripts differ at P < 0.05 (small letters) or P < 0.01 (capital letters).

Colostrum and milk conventional ingredients

Colostrum and milk composition (i.e., dry matter, protein, and lactose) was not affected by dietary treatment ($P > 0.05$). Compared with the CON group, dietary SLE supplementation significantly increased fat concentrations in sow colostrum and milk on day 14 of lactation ($P < 0.05$; Table 8). As presented in Table 9, sows receiving SLE had significantly higher colostrum concentrations of IgA ($P < 0.05$) and IgG ($P < 0.01$) than the CON sows. However, no significant difference ($P > 0.05$) was observed in concentrations of IL-10, IgM, IL-8, and TNF-a in colostrum and milk on day 14 of lactation between the two groups.

Serum immunization index of piglets

As presented in Table 10, compared with the CON group, dietary SLE supplementation significantly increased concentrations of IL-10 ($P < 0.05$) and IgA ($P < 0.01$) in serum at d 14 of piglets. There were no differences ($P > 0.05$) in concentrations of IgG, IgM, IL-8, and TNF-α in serum between groups at d 14 of piglets.

Serum SOD and T-AOC activity levels of sows and piglets

The impact of dietary SLE supplementation on serum antioxidant indexes is shown in Figure 1. The T-SOD activity in sow serum on d 1 of lactation were significantly increased compared with the CON group ($P < 0.05$; Figure 1A). However, there were no differences ($P > 0.05$) in sow serum T-AOC activity between groups at farrowing and d 14 of lactation (Figure 1B). The activities of T-AOC and T-SOD in piglet serum were not affected by dietary SLE supplementation (Figures 1C,D).

Discussion

Herbal medicines have been tested extensively in swine diets as potential alternatives to antibiotics growth promoters owing to antiviral, antibacterial, and antioxidant properties, stimulation of the immune system, and improvement of digestibility and absorption of nutrients (18–20). However, the

TABLE 6 Serum hormones of sows (Trial 3).

Items	Treatments[1]		SEM[2]
	Con	1.0TRT2	
GH, ng/ml			
d 90 of gestation	6.35	6.62	1.34
d 1 of lactation	4.90	4.57	0.96
d 14 of lactation	3.99	4.60	0.65
TSH, mIU/L			
d 90 of gestation	7.00	7.18	1.99
d 1 of lactation	3.31	3.95	0.77
d 14 of lactation	2.46	4.20	1.41
PRL, ng/ml			
d 90 of gestation	2.52	2.61	1.10
d 1 of lactation	22.24^{b}	32.00^{a}	4.69
d 14 of lactation	5.25^{b}	8.63^{a}	1.40
Leptin, ng/ml			
d 90 of gestation	11.46	11.68	1.71
d 1 of lactation	10.53	12.77	1.39
d 14 of lactation	8.85^{b}	11.17^{a}	0.56
INS, mIU/L			
d 90 of gestation	63.86	64.38	3.50
d 1 of lactation	75.71	81.85	5.39
d 14 of lactation	40.99^{b}	54.37^{a}	4.45

[1]CON, n = 10, basal diet; SLE, n = 10, control+1.0 g/kg SLE.
[2]Standard error of the means.
[a,b]The values with unlike superscripts differ at $P < 0.05$ (small letters) or $P < 0.01$ (capital letters).

TABLE 7 Serum immunization index of sows (Trial 3).

Items	Treatments[1]		SEM[2]
	Con	1.0TRT2	
IgA, mg/ml			
d 1 of lactation	0.90	0.93	0.25
d 14 of lactation	1.28	1.34	0.35
IgG, mg/ml			
d 1 of lactation	10.99	13.31	2.45
d 14 of lactation	12.79	19.23	5.02
IgM, mg/ml			
d 1 of lactation	3.64	3.44	0.57
d 14 of lactation	4.95	5.17	1.19
IL-10, ng/L			
d 1 of lactation	49.98^{b}	59.60^{a}	4.48
d 14 of lactation	63.43	68.59	6.61
IL-8, ng/L			
d 1 of lactation	146.09	150.32	39.20
d 14 of lactation	190.73	183.13	46.06
TNF-a, ng/L			
d 1 of lactation	110.55	117.17	11.2
d 14 of lactation	103.39	106.15	8.75

[1]CON, n = 10, basal diet; SLE, n = 10, control+1.0 g/kg SLE.
[2]Standard error of the means.
[a,b]The values with unlike superscripts differ at $P < 0.05$ (small letters) or $P < 0.01$ (capital letters).

TABLE 8 Colostrum and milk conventional ingredients of sows (Trial 3), %.

Items	Treatments[1]		SEM[2]
	Con	1.0TRT2	
Protein			
Colostrum	6.62	6.95	0.37
Milk of d 14	3.90	3.94	0.18
Fat			
Colostrum	3.83^{b}	4.79^{a}	0.47
Milk of d 14	6.31^{B}	7.99^{A}	0.47
Lactose			
Colostrum	5.86	6.14	0.42
Milk of d 14	10.33	10.88	0.53
Dry matter			
Colostrum	18.53	18.10	1.25
Milk of d 14	10.42	10.44	0.48

[1]CON, n = 10, basal diet; SLE, n = 10, control+1.0 g/kg SLE.
[2]Standard error of the means.
[A,B,a,b]The values with unlike superscripts differ at $P < 0.05$ (small letters) or $P < 0.01$ (capital letters).

clinical efficacy has huge differences with different compatibility ratios and dosages of herbs and extracts (21). According to the "*Chinese Pharmacopeia*" records, the proportion of *L. japonica* (chlorogenic acid 12 g/tube) and *S. baicalensis* (baicalin 24 g/tube) is 1:2 in the *Yinhuang Oral Liquid*, which is beneficial for fever, cough, hemoptysis, jaundice, dysentery, acute conjunctivitis, carbuncle, and furuncle, and prevents abnormal fetal movements (22). But despite these advantages, information available in the previous literature on the response of supplementing *L. japonica* and *S. baicalensis* in swine is scarce. In the present studies, dietary herbs extract mixture supplementation exerting positive effects on production performance in sows and nursing piglets were observed in all three trials, and the effective dose of SLE was observed to be 1.0 g/kg diet with the ratio of SBE and LJE in the SLE mixture being 50 and 30%, respectively. The feed intake during lactation period is a key factor to limit the production performance of sows, and maximum sow milk output requires that feed intake was maximized (4). Previous studies have confirmed that dietary herb extract supplementation could contribute to the desired organoleptic qualities of the diets and stimulate the appetite, as well as improve digestive tract function by increasing hydrochloric acid and enzyme secretion, thus improving the feed intake and lactation yield of sows (8, 23). Likewise,

TABLE 9 Colostrum and milk immune factors of sows (Trial 3).

Items	Treatments[1]		SEM[2]
	Con	1.0TRT2	
IgA, mg/ml			
Colostrum	1.02^{b}	1.92^{a}	0.31
Milk of d 14	0.52	0.72	0.20
IgG, mg/ml			
Colostrum	18.86^{B}	28.90^{A}	2.45
Milk of d 14	0.91	1.59	0.38
IgM, mg/ml			
Colostrum	1.13	1.77	0.44
Milk of d 14	0.57	0.76	0.20
IL-10, ng/L			
Colostrum	20.51	22.10	5.70
Milk of d 14	9.88	12.39	1.91
IL-8, ng/L			
Colostrum	136.51	142.28	16.52
Milk of d 14	63.52	59.97	10.11
TNF-a, ng/L			
Colostrum	66.62	71.16	8.13
Milk of d 14	20.04	19.79	2.69

[1]CON, n = 10, basal diet; SLE, n = 10, control+1.0 g/kg SLE.
[2]Standard error of the means.
^{A,B,a,b}The values with unlike superscripts differ at $P < 0.05$ (small letters) or $P < 0.01$ (capital letters).

TABLE 10 Serum immune factors of piglets (Trial 3).

Items	Treatments[1]		SEM[2]
	Con	1.0TRT2	
IgA, mg/ml	0.58^{B}	1.02^{A}	0.09
IgG, mg/ml	3.00	3.65	0.43
IgM, mg/ml	2.16	2.04	0.48
IL-10, ng/L	30.25^{b}	42.73^{a}	5.16
IL-8, ng/L	71.34	70.88	25.86
TNF-a, ng/L	80.78	84.85	3.76

[1]CON, n = 20, basal diet; SLE, n = 20, control+1.0 g/kg SLE.
[2]Standard error of the means.
^{A,B,a,b}The values with unlike superscripts differ at $P < 0.05$ (small letters) or $P < 0.01$ (capital letters).

there were many reports on improved feed intake through herb extract additives in sows or pigs diets (15–18, 24–27). In addition, the herbal ingredients' metabolites can improve growth performance and reduce diarrhea in nursing piglets through breast milk (18, 28). Liu et al. indicated that dietary supplementation of herbal extract mixture (55% *S. baicalensis* and 25% *L. japonica*) at 0.5 and 1.0 g/d could alleviate heat stress, improve the feed intake and dry matter digestibility of sows during lactation, and reduce diarrhea and enhance daily gain in piglets (29), which were similar to observations in the current studies. However, the differences in the total number born may not be related to the SLE in this study because the SLE mixture added to the sow feed was started on the 85th day of pregnancy.

A high feed intake of lactating sows can increase piglets' growth performance and positively influence subsequent reproduction (30). Furthermore, Oliviero et al. demonstrated that a sow diet has a profound effect on the robustness of piglets (31). In the current studies, piglets weaned from SLE-supplemented sows had a greater weaning weight and overall ADG than the CON group, which suggested that SLE supplementation improved sow milk production. This improvement in milk output may be due to enhanced feed intake and nutrient digestibility. Similarly, the inclusion of herbal extract blends in lactation diet were shown to enhance piglet performance and result in higher weight at weaning (32). Zhong et al. also observed that supplementation of 0.04% phytogenic additive to sows positively affected feed intake and milk production of sows and litter performance (33). In addition, Liu et al. demonstrated that the antibacterial activity and anti-inflammatory properties of *S. baicalensis* and *L. japonica* provided a beneficial effect on the immune system and even reduced subclinical or clinical infections, subsequently benefiting the health of the pigs, which could account for enhanced growth performance (18). Watson also confirmed that the inclusion of herbs could lead to an improvement in antibody levels of colostrum and increase milk quality (34). Redoy et al. suggested that dietary supplementation with herbs (*Plantago lanceolata L.* and *Allium sativum*) could prevent the undesirable microorganisms' reproduction and stimulate the secretion of antibodies, thus positively influencing the colostral concentration of IgA and IgG, serum immunocompetence, and growth performance of animals (35). On the other hand, the beneficial properties of herbal extract such as antimicrobial, antiviral, and stimulation of the immune system could improve uterine involution and protect the sow from possible postpartum urogenital infections (36). As expected, the current study indicated that SLE supplementation decreased piglets' diarrhea, which also could account for the enhanced growth performance in this study.

Notably, relative to control sows, sows fed SLE had increased serum concentrations of triglycerides, glucose, and cholesterol on farrowing. Modern genotype sows' farrowing is a long process, and yet prolonged labor could lead to dystocia and even stillbirth. And part of the reason for the prolonged labor is insufficient energy supply (18). Glucose in serum is the main source of energy for tissue cells in the sow. In this regard, improving serum concentrations of glucose and triglycerides could help to reduce sows' stress in farrowing, which is conducive to the progress of farrowing and reduces labor. The increased triglycerides and glucose concentrations in the serum of sows receiving herbal extracts indicate the latter's homeostasis-promoting effects (18, 37). Meng et al. and Ruan

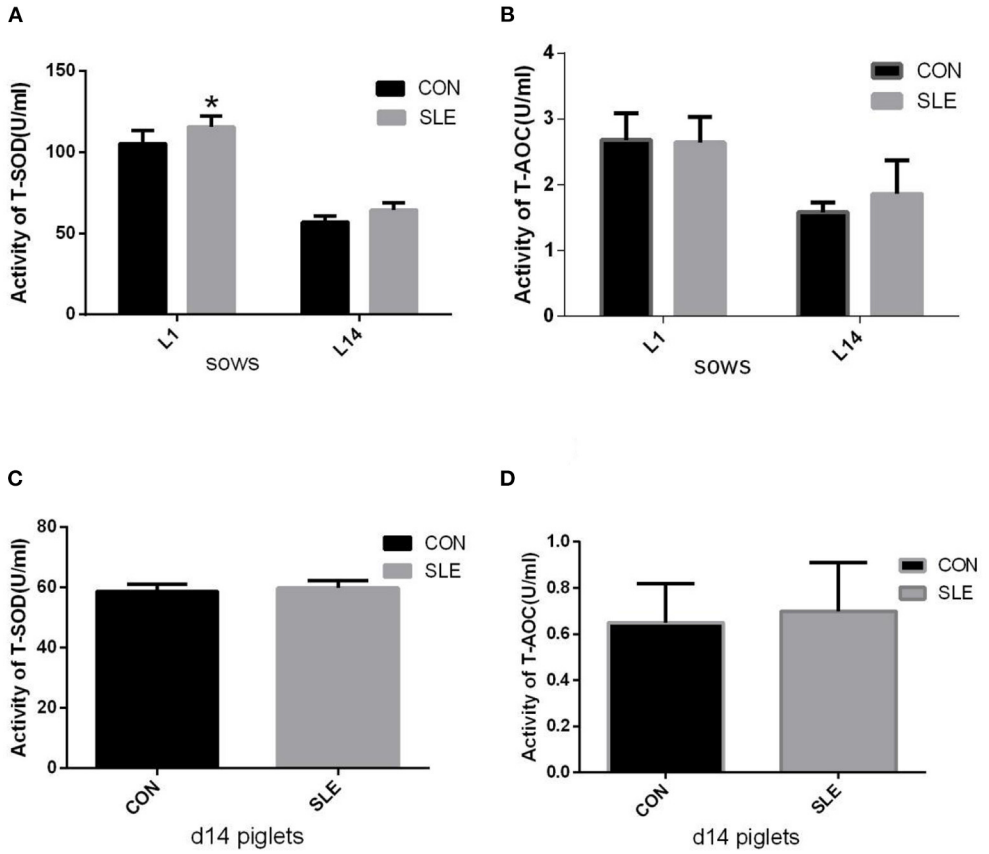

FIGURE 1
The effects of dietary *S. baicalensis* and *L. japonica* extract supplementation on serum anti-oxidative capacity of sows and piglets. **(A,B)** Total superoxide dismutase (T-SOD) and total antioxidant capacity (T-AOC) activities in serum of sows ($n = 10$); **(C,D)** T-SOD and T-AOC activities in serum of d14 piglets ($n = 20$). *Indicating a significant difference ($P < 0.05$). Data show the means ± standard deviation (SD).

et al. also indicated that chlorogenic acid could regulate glucose metabolism, and improve lipids and enzymes involved in lipid metabolism in the organism (38, 39). More than 95% of the fatty acids in the cream were in the form of triglycerides, and the ingredients needed for the synthesis of fat in the milk were derived from blood fats (1). The content of triglycerides in serum of farrowing sows was significantly increased, suggesting that more triglycerides in plasma may be absorbed by the body from the peripheral circulation of the blood and used for the synthesis of milk fat (1). However, Yan et al. suggested that the increased concentrations of serum cholesterol and triglycerides might be resulted from homeostasis and the promotion of intestinal lipid absorption, respectively (16, 27). Myer et al. indicated that the formation of fat deposits depended on the level of serum triglyceride, which is accompanied by increased triglyceride and cholesterol levels. The possible reason was the increase of fatty acid synthase and the outcome of combined action of hormone sensitive enzyme activity (40). However, contrary to the results of this experiment, chlorogenic acid and baicalein had a certain regulating effect on body fat metabolism. Chlorogenic acid seemed to be more potent for bodyweight reduction and regulation of lipid metabolism than caffeic acid (41, 42). Baicalein reduces body cholesterol content by inhibiting cholesterol acyltransferase activity and cholesterol absorption, which was also clarified in earlier studies (18, 43–45).

Interleukin 10 (IL-10) is a pleiotropic cytokine with an extensive spectrum of biological effects in immunoregulation and inflammation (46). As previously reported, chlorogenic acid had a certain regulation effect on organism immunity and adaptive immunity in the regulation of inflammation and immunity, which can regulate the number of white blood cells, the function of macrophages, expression of cytokines secretion, and immune cell activation factors (47, 48). Baicalin and baicalein not only have obvious anti-inflammatory and immunosuppressive effects but also can improve the functions of macrophages and NK cells (49, 50). In the present study, serum IL-10 levels in sows and piglets increased, showing an improvement in cellular and humoral immunity of offspring in response to SLE supplementation of sows during late gestation and nursing period.

Besides providing energy and nutrients for piglets, sow colostrum's most important function was to activate the immune system and equip piglets with specific and non-specific immunity protection functions (46, 51). Feeding lactating sows a diet supplemented with SLE increased colostrum IgG and IgA concentrations in sows and serum IgA concentrations in piglets, indicating that the active compounds in SLE were deposited in sow milk. Wang et al. reported dietary herbal extracts supplementation increased colostrum IgG and IgA concentrations (52). Thus, these findings substantiate that dietary supplementation of sows with SLE during late gestation and lactation could significantly improve serum IgA and IL-10 of piglets. These may be because active substances in SLE from colostrum and milk effectively strengthen the provision of resources for supporting cells and the immune system of piglets by regulating lipid and protein metabolism (12, 13, 38). These results indicate that dietary SLE may improve weaned piglets' immune function and resistance to pathogenic microorganisms infection and attenuate stress injury on the organism.

The antioxidant status of an organism is critical for maintaining animal health and can be affected by nutrients (53, 54). Due to the antioxidant properties contained in herbs, the use of herbs as additives is important for the antioxidant system and stress tolerance of animals. Previous studies have stated that *S. baicalensis* inhibited lipid peroxidation in rat liver homogenate (55). Su et al. also clarified the antioxidant effect of *L. japonica* extract in rats (56). Shang et al. demonstrated that SLE supplementation generated a decrease in serum cortisol that could be attributed to the anti-stress and sedative properties (11). Consistent with the antioxidant function, the current study stated that supplementation of SLE mixture in sow diets significantly increased T-SOD activities in sow serum on day one of lactation. Wang et al. and Huang et al. elucidated that the antioxidant roles of *S. baicalensis* have been traced to several of its flavones, which include wogonin, baicalin, baicalein, and the skullcap flavone (57, 58). Choi et al. clarified that the anti-oxidative activity of *L. japonica* was attributed to polyphenols, flavones, iridoids, and saponins, which exhibit various antioxidant properties (59). Taking into account that increased systemic oxidative stress is observed throughout lactation in sows and that high energy metabolic demands in the lactation process accelerate mitochondrial oxidative stress and reactive oxygen species production (60, 61), it is necessary to prevent oxidative stress by SLE supplementation during lactation.

Conclusion

In summary, this study demonstrated that supplementation of 1.0 g/kg SLE [50% *S. baicalensis*, 30% *L. japonica* extract mixture, and 20% carrier (wheat bran)] in sow diet in late gestation and during lactation was the optimum content and optimized mixed-proportion that could improve immunity and production performance of sows and nursing piglets. The SLE supplementation increased immune molecules in sows' serum and milk, which is beneficial to piglet health and growth through a transmission effect.

Data availability statement

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

Ethics statement

The animal study was reviewed and approved by Animal Care and Use Committee of Animal Nutrition Institute, Sichuan Agricultural University.

Author contributions

ZF and DW designed the study. LW and BH performed experiments. LW, BH, LH, LC, BF, YL, and SX performed data analysis. BH, LW, and ZF wrote the draft and revised the manuscript. All authors have read and approved the manuscript.

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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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Antrodia cinnamomea polysaccharide improves liver antioxidant, anti-inflammatory capacity, and cecal flora structure of slow-growing broiler breeds challenged with lipopolysaccharide

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Lipopolysaccharides (LPS) induces liver inflammatory response by activating the TLR4/NF-κB signaling pathway. *Antrodia cinnamomea* polysaccharide (ACP) is a medicinal mushroom that can protect from intoxication, liver injury, and inflammation. Nevertheless, the effect of ACP on the liver antioxidant, anti-inflammatory capacity and cecal flora structure of LPS-challenged broilers remains unclear. The aim of this experiment was to investigate the effects of ACP on the anti-oxidative and anti-inflammatory capacities of the liver, and cecal microbiota in slow-growing broilers stimulated by LPS. A total of 750 slow-growing broilers (9-day-old) were assigned to five treatments with 6 replicates of 25 chicks per replicate: a control diet, the chicks were fed a control diet and challenged with LPS. Dietary treatments 3 to 5 were the control diet supplemented with 100, 200, 400 mg/kg ACP challenged with LPS, respectively. The groups of 100 mg/kg ACP supplementation significantly increased liver index, pancreas index, and bursa of Fabricius index ($P < 0.05$). The GSH-Px content of LPS-challenged broilers was lower than that of the control group ($P < 0.001$), but the content of MDA increased ($P < 0.001$). Feeding with 100 mg/kg ACP resulted in increased the activity of T-AOC, GSH-Px, and T-SOD, and decreased MDA content ($P < 0.05$). The activity of TNF-α, IL-1β, and IL-6 of the LPS group increased, but these indicators were decreased with supplemental 100 mg/kg ACP ($P < 0.05$). Dietary application of ACP up to 100 mg/kg down-regulated ($P < 0.05$) the expression of TLR4/NF-κB pathway in the liver induced by LPS. The results of 16S rRNA demonstrated that feeding with 100 mg/kg ACP can change the diversity and composition of the gut microbiota, and restrained the decline of beneficial cecal microbiota (typically *Lactobacillus*, *Faecalibacterium*, and *Christensenellaceae R-7* group) in the

challenged LPS group ($P < 0.05$). Conclusively, feeding a diet with 100 mg/kg ACP may have beneficial effects on liver damage and the bacterial microbiota diversity and composition in the ceca of LPS-stressed slow-growing broiler breeds, probably because of its combined favorable effects on antioxidants and cytokines contents, and restoration the decline of beneficial cecal microbiota.

KEYWORDS

Antrodia cinnamomea **polysaccharide, slow-growing broiler, LPS, liver, cecal microflora**

Introduction

Antrodia cinnamomea is a medicinal distinctive mushroom, which is known in the china as "Niu-Chang-Chih" or the "Ruby of forest." It is used in folk medicine as a cure for liver problems and a number of active substances can be found in *Antrodia cinnamomea* such as polysaccharides. Natural polysaccharides have various functions such as growth promotion, anti-inflammation, maintenance of intestinal mucosal integrity, regulation of intestinal flora (1–5). It has been reported that health benefits *of Antrodia cinnamomea* have antioxidants properties due to its polysaccharide, polyphenol and triterpenoid contents (6). *Antrodia cinnamomea* polysaccharide (ACP) has various bioactivities in mice (7, 8), piglets (9), and human (10, 11). Lee et al. (12) reported that ACP could promote Heme oxygenase−1 expression and antagonize the nuclear factor kappa B (NF-κB)-dominated inflammatory pathway. Therefore, the overall growth performance can be improved due to boost the immunity and antioxidant capacity in broiler chickens.

LPS is a specific component of the Gram-negative bacteria cell wall that can cause liver inflammation in chicken (13), mice (14) and piglets (10) with activated toll-like receptor 4 (TLR4), transmitting NF-κB into nucleus that can change the abundance of inflammatory genes. Liver diseases are reported to be associated with TLR4 medicated signals (15, 16), and it is also an important target of many therapeutic agents (13, 14, 17, 18). In addition, LPS was used to cause inflammatory model in chicken (13, 19, 20).

Gut microbiota is called "the new virtual metabolic organ" (21, 22), and the gut-liver axis has attracted greater attention (23, 24). As a center of body to connect external environment, gut not only provided the first defense protect intestine from hurt, but also supplied 70% blood of liver (24, 25). Previous studies suggested that TLRs and nod-like receptors mediated LPS, peptidoglycans and flagelin activated NF-κB to produce inflammatory cytokines and chemokines to cause liver steatosis, inflammation and fibrosis (24). Therefore, intestinal flora is an important participant in the regulation of liver diseases, and it is considered the core connotation of the enteric-liver axis theory.

Yellow feather chicken, as an important slow-growing broiler breeds. It is favored by consumers for its excellent meat quality and rich meat flavor. Nowadays, there are about 4 billion yellow-feathered chickens produced every year. Recently, it has been found that supplemental polysaccharides such as *Panax ginseng* (26) and *Quinoa* polysaccharide (27) can promote richness of species and improve the structure of gut microbiota community. The application of ACP in poultry production under LPS challenge on liver and microorganisms of slow-growing broiler breeds is not fully studied. Therefore, we hypothesized that ACP may have beneficial effects on LPS-induced liver damage and the variation of gut microbiota of slow-growing broiler breeds. The aim of this study was to investigate effects of ACP on liver antioxidant, anti-inflammatory capacity, and cecal flora structure of slow-growing broiler breeds challenged with LPS.

Materials and methods

Animals, experimental design and dietary treatments

The execution of this study followed the rules drawn by the Animal Care Committee belongs to the Institute of Animal Science, Guangdong Academy of Agricultural Sciences (GAASISA-2019-036).

A total of 750 Lingnan yellow-feathered female chicks (9-day-old) were purchased from Guangdong Wiz Agricultural Science and Technology Co. Ltd. (Guangzhou, China). On d 9, chicks were weighed (body weight = 137.69 ± 1.15 g) and divided into 30 floor pens (1.3 ×3.5 m) over a 21-day experimental period. Chicks were assigned to receive one of five treatments with six replicates of 25 chicks per replicate: a control

Abbreviations: LPS, Lipopolysaccharide; ACP, *Antrodia cinnamomea* polysaccharide; GSH-Px, glutathione peroxidase; T-AOC, total antioxidant capacity; T-SOD, total superoxide dismutase; MDA, malondialdehyde; TNF-α, tumor necrosis factor α; IL-1β, interleukin 1β; TLR4, toll-like receptor (TLR)4; IL-6, interleukin 6; NF-κB, nuclear factor kappa B; COX2, Cyclooxygenase 2; Nrf2, nuclear factor erythroid-2 related factor 2; SOD1, superoxide dismutase-1; BAX, B-cell lymphoma/leukemia-2-associated X protein.

TABLE 1 Composition and nutrient levels of basal diets (as-fed basis) %.

Ingredients	%
Corn	60.50
Soybean meal	31.5
Soybean oil	1.70
L-Lys · HCl	0.16
DL-Met	0.17
Limestone	1.22
$CaHPO_4$	1.93
NaCl	0.30
Unite bran	1.52
Vitamin-mineral premix[a]	1.00
Total	100.00
Calculated nutrient composition	
ME, MJ/kg	12.12
CP	21.50
Lys	1.29
Met	0.52
Met+Cys	0.93
Thr	0.86
Trp	0.21
Ile	0.86
Ca	1.00
Total phosphorus	0.74
Non-phytate phosphorus	0.47

[a]The premix provided the following per kg of the diet: VA 15, 000 IU, VD_3 3, 300 IU, VE 10 IU, VK 0.50 mg, VB_1 1.8 mg, VB_2 3.6 mg, calcium pantothenate 10 mg, nicotinic acid 35 mg, VB_6 3.50 mg, biotin 0.15 mg, folic acid 0.55 mg, VB_{12} 0.01 mg, choline chloride 1,000 mg, Fe 80 mg, Cu 8 mg, Mn 80 mg, Zn 60 mg, I 0.35 mg, Se 0.15 mg.

diet, the chicks were fed a control diet and challenged with LPS. Dietary treatments 3 to 5 were the control diet supplemented with 100, 200, and 400 mg/kg ACP, respectively and challenged with LPS. On d 18 and 20 of age, 0.50 mL of normal saline was injected into peritoneum of birds in the control group, while 0.50 mL 500 μg/kg BW LPS was injected into peritoneum of birds in the challenged groups. The basic diet from 1 to 30 days (Table 1) was prepared according to Chinese Feeding Standard of Chicken (28).

ACP (*D-glucan*, 76.3%) was purchased from Taiwan Jia Shi Kai Biotechnology Co., Ltd. (Taibei, China). LPS generated by *E. coli* serotype O55: B5 (L4005) and was purchased from Sigma-Aldrich trading Co., Ltd. (Shanghai, China).

Samples

On d 30, after deprived of feed overnight, all birds were weighed and 2 chickens (weight close to average body weight) were selected per pen then they were slaughtered after being anesthetized with Isoflurane (CPO406V2, Fresenius Kabi, Bad Homburg, Germany). The sample of liver and digesta of the cecum were transferred into sterile tubes. Then plunged into liquid nitrogen and stored at −80°C for further study. The liver, pancreas, spleen, thymus and bursa of Fabricius were dissected and weighed. Relative weight of organs was calculated by the organ weight /live weight × 100%.

Biochemical indices in liver

The ice-cold physiologic saline (1:10, v/v) was used to homogenize samples of liver and then samples were centrifuged at 2,000 × g for 10 min. The activities of malondialdehyde (MDA), total superoxide dismutase (T-SOD), total antioxidant capacity (T-AOC) and glutathione peroxidase (GSH-Px) in liver were detected by colorimetric kits (Nanjing Jiancheng Institute of Bioengineering). Furthermore, the contents of interleukin 1β (IL-1β; Detection Range 1.00 ng/L∼20.00 ng/L), interleukin 6 (IL-6; Detection Range 1.50 ng/L∼30.00 ng/L) and tumor necrosis factor α (TNF-α, Detection Range 3.00 ng/L∼80.00 ng/L) were detected by a spectrophotometer (Biomate 5, Thermo Electron Corporation, Rochester, NY, USA) with chicken Elisa kits (Beijing Equation Biotechnology co., Ltd, Beijing, China).

The real-time quantitative PCR in liver

The method of targeted mRNA abundance detection has been described in Cui et al. (29). The gene primers were designed by Primer Premier 6.0 (Table 2), and generated by Sangon Biological Engineering Co., Ltd. (Shanghai, China). Total RNA from the liver of chickens were extracted by TRIzol Reagent (Thermo Fisher Scientific, Carlsbad, CA, USA) and checked for integrity by 1.5% agarose gel electrophoresis. Then, double-stranded DNA in RNA sample were eliminated by gDNA Remover (EZBioscience, Roseville, USA) and reacted at 25°C for 5 min. After that, 4× RT Master Mix (EZBioscience, Roseville, USA) and primers were added and reacted at 42°C for 15 min to synthesized first strand cDNA. Relative mRNA abundance was calculated using the $2^{-\Delta\Delta CT}$ with *β-actin* as the housekeeping gene.

Determination of cecal microbiota

The QIAamp PowerFecal DNA Kit (Qiagen, Hilden, Germany) was used to extract total genomic DNA of cecal digesta. The Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, United States) was used to measure DNA concentration. Then, the primer (Forward: 5'-CCTAYGGGRBGCASCAG-3' and Reverse: 5'-

TABLE 2 Primer sequences for quantitative real-time PCR.

Genes[a]	Primer sequences (5'-3')	GenBank accession number	Annealing temperature /°C	PCR product size (bp)
TLR4	F: AGTCTGAAATTGCTGAGCTCAAAT	NM_001030693.1	59	190
	R: GCGACGTTAAGCCATGGAAG			
NF-κB	F: CTACTGATTGCTGCTGGAGTTG	D13721.1	60	175
	R: CTGCTATGTGAAGAGGCGTTGT			
COX2	F: TGCAACGATATGGCTGAGAG	YP_009558655.1	58	233
	R: CTGCGATTCGGTTCTGGTAT			
Nrf2	F: ATCACCTCTTCTGCACCGAA	NM_205117.1	60	296
	R: GCTTTCTCCCGCTCTTTCTG			
SOD1	F: GGTGCTCACTTTAATCCTG	NM_205064.1	60	109
	R: CTACTTCTGCCACTCCTCC			
BAX	F: GTGATGGCATGGGACATAGCTC	CD214942.1	60	90
	R: TGGCGTAGACCTTGCGGATAA			
β-actin	F: GAGAAATTGTGCGTGACATCA	NM_205518	60	152
	R: CCTGAACCTCTCATTGCCA			

[a] *TLR4*, toll-like receptor (TLR)4; *NF-κB*, nuclear factor kappa B; *COX2*, Cyclooxygenase 2; *Nrf2*, nuclear factor erythroid-2 related factor 2; *SOD1*, superoxide dismutase-1; *BAX*, B-cell lymphoma/leukemia-2-associated X protein; β-*actin*, beta actin.

GGACTACNNGGGTATCTAAT-3') were used to amplify the bacterial 16S rRNA in the region of V3-V4. After that, the productions were purified by the AxyPrep DNA Gel Extraction Kit (Axygen Biosciences, Union City, CA, United States) and pooled in equimolar with paired-end sequenced (2 × 250) on an Illumina MiSeq platform (Shanghai BIOZERON Co., Ltd.) following standard protocols.

Based on a 97% sequence similarity cutoff by using the UPARSE software (https://drive5.com/uparse/, version 10), the operational taxonomic units (OTUs) were calculated and to identify unnormal gene sequences by UCHIME with the UCHIME software (http://drive5.com/usearch/manual/singletons.html). In each 16S rRNA gene sequence, the phylogenetic affiliation was analyzed by the Ribosomal Database Project (RDP) Classifier (https://rdp.cme.msu.edu/) against the Silva (SSU123) 16S rRNA database using a confidence threshold of 70% (30, 31). The rarefaction analysis, coverage abundance estimator, number of observed OTUs, diversity indices (Shannon and Simpson) and richness estimator (Chao 1 and ACE), which reflect bacterial diversity were calculated by mothur (version v.1.30.1) software. Based on the 97% sequence similarity, the phylum and genus levels were classified and the BLAST analysis of the OTUs against the SILVA database was used to determine Microbial species (30).

Statistical analysis

Data were analyzed by the one-way analysis of variance (ANOVA) procedure and separated by Duncan's multiple range tests in SPSS 17.0 (SPSS Inc., Chicago, IL). Each replicate mean as the experimental unit for the growth performance data analysis, the other parameters were averaged per replicate. The control group and LPS-infected group were compared by *t*-tests. Orthogonal polynomial contrasts were also used to determine linear and quadratic responses of chickens to different levels of ACP supplementation. Data are presented as the means and pooled standard errors of the means (SEM). When *P*-value is less 0.05, the differences between treatments were considered statistically significant.

Results

Viscera indices

As exhibited in Table 3, the index of liver, pancreatic, and bursa of Fabricius were significantly elevated by 100∼400 mg/kg ACP addition ($P < 0.05$). Supplemental ACP at 400 mg/kg increased the index of pancreas ($P < 0.001$) and spleen in linear trend ($P = 0.022$).

Antioxidant capacity and inflammatory factor of liver

As presented in Tables 4, 5, the activity of glutathione peroxidase (GSH-Px) in the liver of the broilers challenged with LPS was lower than that of the control group ($P < 0.001$), whereas the concentration of MDA was increased ($P < 0.001$). Supplemental 100∼400 mg/kg ACP increased

TABLE 3 Effects of *Antrodia cinnamomea* polysaccharide on organs indices of slow-growing broiler breeds challenged with LPS.

Organs, mg/g	Treatments[a]					ACP[b]			
	Control	LPS	100 mg/kg ACP+LPS	200 mg/kg ACP+LPS	400 mg/kg ACP+LPS	SEM	*P*-value	Linear	Quadratic
Liver	28.46^{A}	26.70^{Bb}	30.30^{a}	30.71^{a}	31.99^{a}	0.44	<0.001	<0.001	<0.001
Pancreas	3.25	2.92^{c}	3.50^{b}	3.83^{ab}	3.92^{a}	0.08	<0.001	<0.001	<0.001
Spleen	1.73	1.64^{b}	1.75^{ab}	1.80^{ab}	1.89^{a}	0.04	0.153	0.022	0.073
Thymus	4.91	4.80	5.39	5.32	5.26	0.12	0.275	0.212	0.176
Bursa of Fabricius	3.05	2.94^{b}	3.40^{a}	3.47^{a}	3.47^{a}	0.08	0.038	0.015	0.016

Values are means of 6 replicates per treatment with 2 samples each. LPS, Lipopolysaccharide; ACP, Antrodia cinnamomea polysaccharide; SEM, standard error.
[a]Capital letters indicate statistically significant ($P < 0.05$) differences between control group and LPS group by Student's t-test; small letters indicate statistically significant ($P < 0.05$) differences of four groups (LPS, 100 mg/kg ACP+LPS, 200 mg/kg ACP+LPS, 400 mg/kg ACP+LPS). Mean values within a row with no common superscript differ significantly ($P < 0.05$).
[b]The *P*-value are representing the ANOVA analysis of four groups (LPS, 100 mg/kg ACP+LPS, 200 mg/kg ACP+LPS, 400 mg/kg ACP+LPS) and orthogonal polynomial contrasts are used to determine linear and quadratic responses of four groups.

TABLE 4 Effects of *Antrodia cinnamomea* polysaccharide on liver antioxidant capacity of slow-growing broiler breeds challenged with LPS.

Indices	Treatments[a]					ACP[b]			
	Control	LPS	100 mg/kg ACP+LPS	200 mg/kg ACP+LPS	400 mg/kg ACP+LPS	SEM	*P*-value	Linear	Quadratic
T-SOD, U/mg prot	464.54	427.59^{b}	465.31^{a}	483.77^{a}	484.18^{a}	6.98	0.005	0.001	0.002
GSH-Px, U/mg prot	37.53^{A}	32.36^{Bb}	40.05^{a}	41.22^{a}	43.84^{a}	0.99	<0.001	<0.001	<0.001
T-AOC, U/mg prot	0.91	0.81^{b}	1.11^{a}	1.11^{a}	1.11^{a}	0.03	<0.001	0.001	<0.001
MDA, nmol/mg prot	0.38^{B}	0.78^{Aa}	0.35^{b}	0.33^{b}	0.19^{c}	0.04	<0.001	<0.001	<0.001

Values are means of 6 replicates per treatment with 2 samples each. LPS, Lipopolysaccharide; ACP, Antrodia cinnamomea polysaccharide; GSH-Px, glutathione peroxidase; T-SOD, total superoxide dismutase; MDA, malondialdehyde; T-AOC, total antioxidant capacity; Prot, protein; SEM, standard error.
[a]Capital letters indicate statistically significant ($P < 0.05$) differences between control group and LPS group by Student's t-test; small letters indicate statistically significant ($P < 0.05$) differences of four groups (LPS, 100 mg/kg ACP+LPS, 200 mg/kg ACP+LPS, 400 mg/kg ACP+LPS). Mean values within a row with no common superscript differ significantly ($P < 0.05$).
[b]The *P*-value are representing the ANOVA analysis of four groups (LPS, 100 mg/kg ACP+LPS, 200 mg/kg ACP+LPS, 400 mg/kg ACP+LPS) and orthogonal polynomial contrasts are used to determine linear and quadratic responses of four groups.

TABLE 5 Effects of *Antrodia cinnamomea* polysaccharide on liver cytokines of slow-growing broiler breeds challenged with LPS.

Indices, ng/g prot	Treatments[a]					ACP[b]			
	Control	LPS	100 mg/kg ACP+LPS	200 mg/kg ACP+LPS	400 mg/kg ACP+LPS	SEM	*P*-value	Linear	Quadratic
TNF-α	4.95^{B}	6.12^{Aa}	4.24^{b}	4.22^{b}	3.94^{b}	0.20	<0.001	<0.001	<0.001
IL-1β	1.57^{B}	2.04^{Aa}	1.33^{b}	1.32^{b}	1.11^{b}	0.08	<0.001	<0.001	<0.001
IL-6	2.47^{B}	2.83^{Aa}	2.01^{bc}	2.19^{b}	1.77^{c}	0.09	<0.001	<0.001	<0.001

Values are means of 6 replicates per treatment with 2 samples each. LPS, Lipopolysaccharide; ACP, Antrodia cinnamomea polysaccharide; TNF-α, tumor necrosis factor α; IL-1β, interleukin 1β; IL-6, interleukin 6. prot, protein; SEM, standard error.
[a]Capital letters indicate statistically significant ($P < 0.05$) differences between control group and LPS group by Student's t-test; small letters indicate statistically significant ($P < 0.05$) differences of four groups (LPS, 100 mg/kg ACP+LPS, 200 mg/kg ACP+LPS, 400 mg/kg ACP+LPS). Mean values within a row with no common superscript differ significantly ($P < 0.05$).
[b]The *P*-value are representing the ANOVA analysis of four groups (LPS, 100 mg/kg ACP+LPS, 200 mg/kg ACP+LPS, 400 mg/kg ACP+LPS) and orthogonal polynomial contrasts are used to determine linear and quadratic responses of four groups.

significantly the activities of T-AOC, GSH-Px and T-SOD and decreased the content of MDA in the liver ($P < 0.05$). The level of TNF-α, IL-1β and IL-6 were increased ($P < 0.05$) in the LPS group, but these levels were decreased by supplemental 100~400 mg/kg ACP ($P < 0.05$).

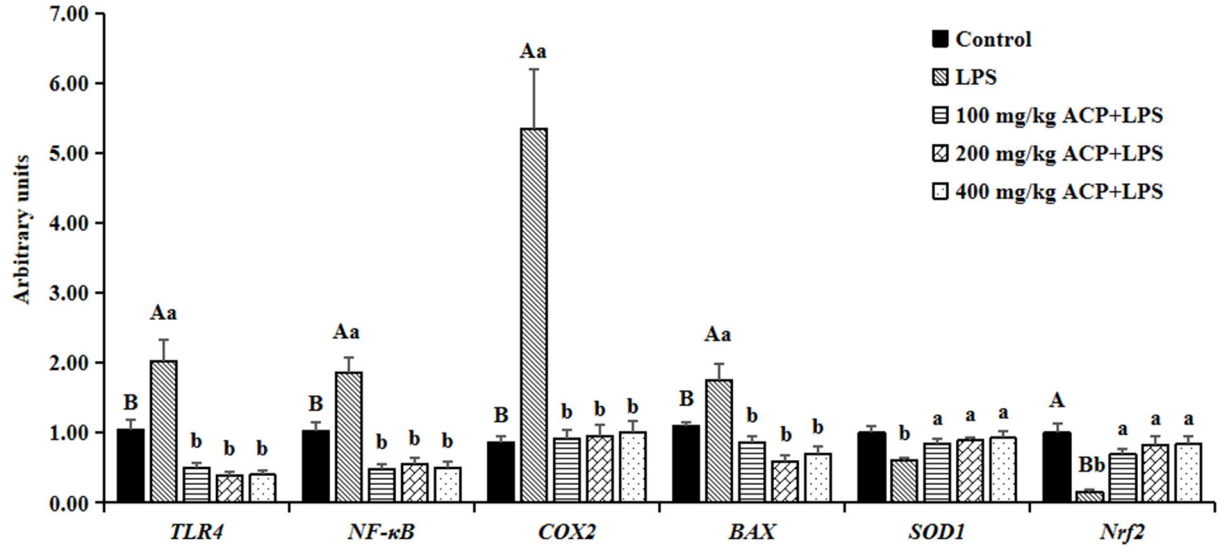

FIGURE 1
Effects of *Antrodia cinnamomea* polysaccharide on mRNA expression abundance of antioxidant and anti-damage genes in liver of slow-growing broiler breeds challenged with LPS[a]. Values are means of 6 replicates per treatment with 2 samples each. LPS, Lipopolysaccharide; ACP, *Antrodia cinnamomea* polysaccharide; *TLR4*, toll-like receptor (*TLR*)*4*; *NF-κB*, nuclear factor kappa B; *COX2*, Cyclooxygenase 2; *Nrf2*, nuclear factor erythroid-2 related factor 2; *SOD1*, superoxide dismutase-1; *BAX*, B-cell lymphoma/leukemia-2-associated X protein; Control, basal diet group; LPS, basal diet+LPS stress group. 100 mg/kg ACP+LPS, basal diet+LPS stress+100 mg/kg ACP group. 200 mg/kg ACP+LPS, basal diet+LPS stress+200 mg/kg ACP group. 400 mg/kg ACP+LPS, basal diet+LPS stress+400 mg/kg ACP group. [a]Data are expressed as means±SEM. SEM, standard error. Capital letters indicate statistically significant ($P < 0.05$) differences between control group and LPS group by Student's *t*-test; small letters indicate statistically significant ($P < 0.05$) differences of four groups (LPS, 100 mg/kg ACP+LPS, 200 mg/kg ACP+LPS, 400 mg/kg ACP+LPS). Bars with no common superscript differ significantly ($P < 0.05$).

The mRNA expression abundance of antioxidant and anti-damage genes of liver

As shown in Figure 1, LPS notably elevated the mRNA abundance of *NF-κB*, *TLR4*, *COX2* and *BAX* ($P < 0.05$), while the *Nrf2* abundance was significantly decreased ($P < 0.05$) in the liver of slow-growing broiler breeds compared with the control group. The supplemental ACP inhibited the LPS-induced *TLR4*, *NF-κB*, *COX2* and *BAX* mRNA expression in a dose-independent model ($P < 0.05$). Meanwhile, the mRNA abundance of *Nrf2* was drastically elevated and *SOD1* was slight up-regulated in the different ACP pretreatment groups ($P < 0.05$). However, there was no significantly changes on the mRNA abundance of antioxidant and anti-damage genes in the liver between the different doses of ACP group ($P > 0.05$).

Cecal microflora

A total of 2,285,846 16S rRNA effective sequences in V3-V4 region from the 48 samples, and 47,622 sequences per sample were used for further study. The average read length was 414 bp. In Table 6, the number of OTUs, sample richness and diversity are displayed. Coverage of each group was higher than 0.99, suggested that the sequencing results had a great Coverage rate and could reflect the distribution of cecal microorganisms in slow-growing broiler breeds. The Shannon index of 400 mg/kg ACP group was significantly higher than that in the LPS group. Compared with the LPS group, Simpson index of the ACP groups were all significantly lower. Additionally, compared to the LPS group, there was a significant linear effect in the Shannon of chickens that received the ACP-supplemented diet without dose dependence ($P < 0.05$). Moreover, there was a obvious linear and quadratic effect on the Simpson of chickens that fed the ACP diet in dose-independent compared to LPS stress group ($P < 0.05$).

As shown in Figure 2 and Table 7, compared with the control group, LPS reduced Firmicutes abundance ($P > 0.05$) and increased Proteobacteria abundance ($P < 0.05$). However, compared to the LPS group, significant increases of Firmicutes abundance and significant decreases of Proteobacteria abundance of slow-growing broiler breeds were observed in the different ACP groups ($P < 0.05$). Intriguingly, the Actinobacteria and Tenericutes abundance were significantly elevated in the 400 mg/kg ACP group compared to the LPS group. There was a significant linear and quadratic effect in the phylum level (except Bacteroidetes) of chickens fed with ACP diet compared with the LPS stress group ($P < 0.05$).

TABLE 6 Effects of *Antrodia cinnamomea* polysaccharide on Alpha diversity analysis of the microbiota from cecum of yellow-feathered chicken based on 97% sequence similarity.

Indices	Treatments[a]					ACP[b]			
	Control	LPS	100 mg/kg ACP+LPS	200 mg/kg ACP+LPS	400 mg/kg ACP+LPS	SEM	*P*-value	Linear	Quadratic
OTU numbers	1,228.83	1,137.83	1,258.33	1,189.67	1,260.33	31.82	0.485	0.304	0.555
Coverage, %	99.14	99.23	99.31	99.33	99.24	0.04	0.734	0.861	0.528
Richness									
Chao1	1,617.65	1,469.03	1,560.71	1,529.17	1,606.31	33.98	0.568	0.218	0.474
ACE	1,613.46	1,462.86	1,570.71	1,509.72	1,606.82	34.48	0.487	0.237	0.504
Diversity indices									
Shannon	4.34	4.12^{b}	4.54^{ab}	4.65^{ab}	4.73^{a}	0.10	0.142	0.027	0.063
Simpson	0.07	0.10^{a}	0.05^{b}	0.04^{b}	0.04^{b}	0.01	0.006	0.003	0.002

Values are means of 6 replicates per treatment. LPS, Lipopolysaccharide; ACP, Antrodia cinnamomea polysaccharide; OUT, operational taxonomic units; ACE, abundance-based coverage estimator; SEM, standard error.
[a]Capital letters indicate statistically significant ($P < 0.05$) differences between control group and LPS group by Student's t-test; small letters indicate statistically significant ($P < 0.05$) differences of four groups (LPS, 100 mg/kg ACP+LPS, 200 mg/kg ACP+LPS, 400 mg/kg ACP+LPS). Mean values within a row with no common superscript differ significantly ($P < 0.05$).
[b]The *P*-value are representing the ANOVA analysis of four groups (LPS, 100 mg/kg ACP+LPS, 200 mg/kg ACP+LPS, 400 mg/kg ACP+LPS) and orthogonal polynomial contrasts are used to determine linear and quadratic responses of four groups.

At the genus level, LPS increased the relative abundance of *Alistipes*, *Ruminiclostridium 9*, *Megamonas*, *[Ruminococcus] torques* group and *Campylobacter* ($P < 0.05$), and decreased the relative abundance of *Lactobacillus* and *Synergistes* ($P < 0.05$). Interestingly, the changes of *Christensenellaceae R-7*, *Faecalibacterium*, *Ruminiclostridium 9*, *Ruminococcus 1*, *Lactobacillus, Megamonas*, *[Ruminococcus] torques* group, *Campylobacter* and *Synergistes* were reversed by the 100∼400 mg/kg ACP-supplemented diet ($P < 0.05$).

Discussion

It is well known that that LPS induces stress response in broilers that leads to redistribute nutrients to synthesize antibodies, which reduces nutrients required for broiler growth (32, 33). LPS can damage intestinal tissue structure and intestinal mucosal barrier, increase the expression of inflammatory factors, and trigger the inflammatory response of broilers (20, 34). On the other hand, it has been found that polysaccharide can improve gut functions. For example, dietary *Algae-Derived* polysaccharide supplementation had an ameliorative effect on heat stress-induced impairment of tight junctions, antioxidant capacity and the immune response of the duodenum in broilers (35). In addition, *Ganoderma lucidum* polysaccharides and *Agaricus blazei* polysaccharides can attenuate the cadmium-induced oxidative damage by decreasing the GSH-Px and SOD activity, and decrease the level of MDA (36). Furthermore, the dietary *Algae-Derived* polysaccharides (37) and *Lycium barbarum* polysaccharides (38) supplementation can elevate the SOD activities and reduce the MDA levels in liver of broilers. In current study, the activity of anti-inflammatory cytokines and antioxidants in the liver were improved by supplemental ACP, and the index of liver and bursa of Fabricius were increased by ACP addition.

Hepatic inflammation and oxidative stress induced by LPS are closely linked with TLR4/NF-κB pathway (39–41). LPS molecule was recognized by TLR4 at the Gram-negative bacteria outer membrane and activated strong immune response via NF-κB (14, 42). It was suggested that oxidative stress activated NF-κB by altering the expression of *COX2*, *IL-1β*, *IL-6* and *TNF-α* to induce inflammation (18, 43, 44). Consistently, these results displayed that LPS decreased the level of GSH-Px and increased the content of MDA in liver. Moreover, these data were in accordance with previous publications of Han et al. (39) and Mei et al. (45) as they found that LPS increased mRNA level of *TLR4*, *TNF-α*, *NF-κB*, *IL-1β*, *COX-2*, *IL-6* and *BAX*, and decreased the expression of *Nrf2* in the liver of broilers. In the present study, it has been showed that LPS caused negative affect on liver health, which was consistent with the finding by Cheng et al. (13) on Beijing white chickens.

Dietary supplemented with *Artemisia ordosica* polysaccharide obviously alleviated LPS-caused oxidative stress through TLR4/NF-κB pathway (41). Recent discoveries suggested that *Ganoderma lucidum* polysaccharides and *Agaricus blazei* polysaccharides inhibited the TLR4 signaling pathway and weakened the damage caused by cadmium in chickens (36). Dietary *Algae-Derived* polysaccharide ameliorated the impairment of histology, cell apoptosis and immune balance in bursa of Fabricius of heat stressed broilers, which is involved in modulation of NF-κB signaling pathway (5). Interestingly, liver damage was significantly alleviated by ACP. All data showed that ACP may have positive effects on LPS-caused liver inflammation by rescue

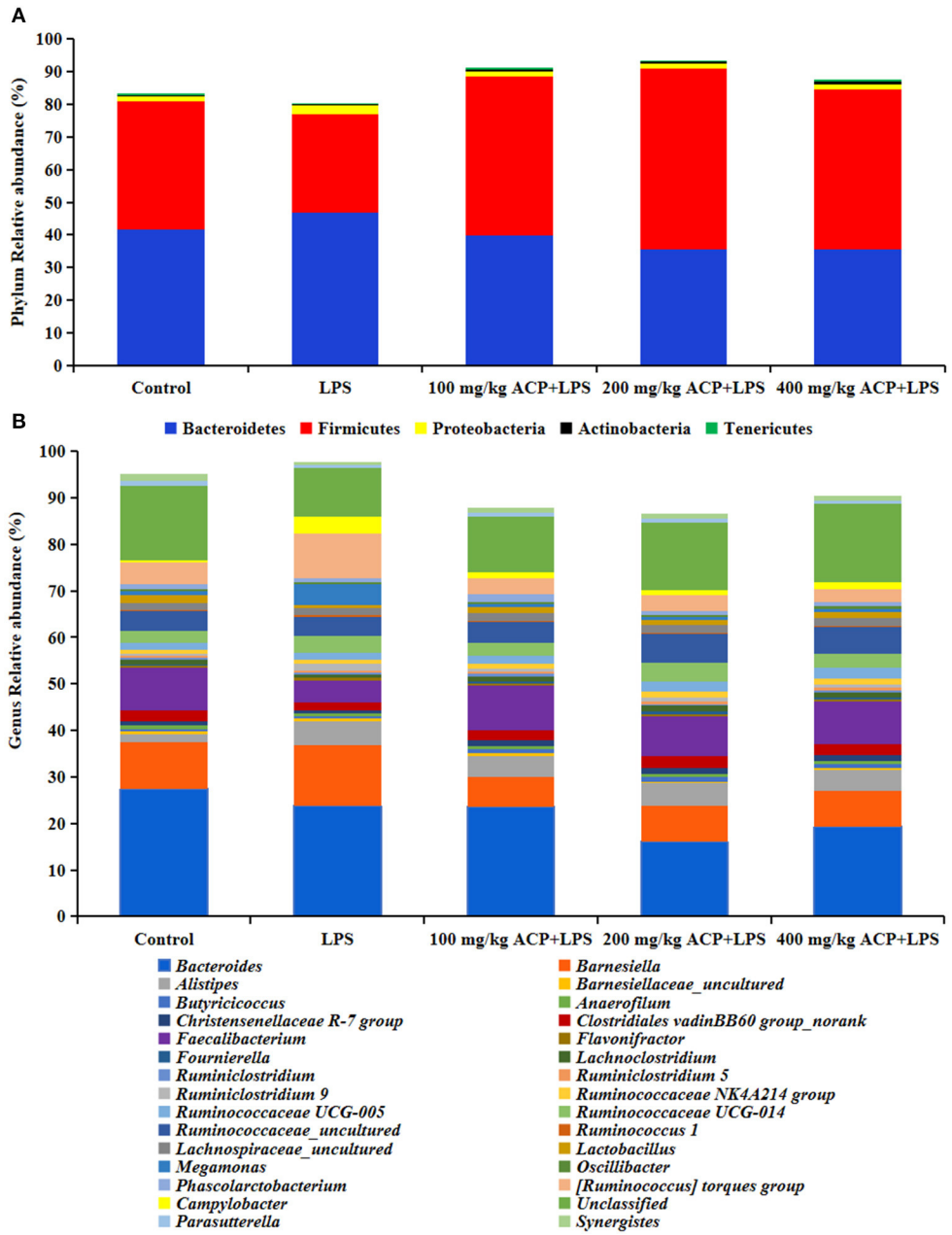

FIGURE 2
Effects of *Antrodia cinnamomea* polysaccharide on microbiome composition in the cecum of slow-growing broilers challenged with LPS. **(A)** Composition and distribution of the microbiota at the phylum level. **(B)** Composition and distribution of the microbiota at the genus level. Values are means of 6 replicates per treatment. LPS, Lipopolysaccharide; ACP, *Antrodia cinnamomea* polysaccharide; Control, basal diet group; LPS, basal diet+LPS stress group; 100 mg/kg ACP+LPS, basal diet+LPS stress+100 mg/kg ACP group. 200 mg/kg ACP+LPS, basal diet+LPS stress+200 mg/kg ACP group. 400 mg/kg ACP+LPS, basal diet+LPS stress+400 mg/kg ACP group.

the oxidative stress *via* activation of the TLR4/NF-κB pathway in chicken.

Liver diseases are linked to intestinal dysbiosis. Several studies claimed that gut microbiota affected liver pathophysiology directly or indirectly through a series of signaling pathways (23–25). Polysaccharides can regulate the composition of intestinal flora and have an effect on the level of the host intestinal flora (46). As an important link for the interaction between the body and polysaccharides, the study of intestinal flora should not be ignored. After glycolysis by intestinal microorganisms, polysaccharides can promote the growth of probiotics and intestinal biodiversity (47, 48). In the current study, dietary supplemented with 100 mg/kg ACP increased species richness and diversity indices, which were

TABLE 7 Effects of *Antrodia cinnamomea* polysaccharide on microbiome composition in the cecum of slow-growing broiler breeds challenged with LPS.

Parameter	Treatments[a]					ACP[b]			
	Control	LPS	100 mg/kg ACP+LPS	200 mg/kg ACP+LPS	400 mg/kg ACP+LPS	SEM	*P*-value	Linear	Quadratic
Phylum relative abundance (%)									
Bacteroidetes	41.78	46.69	39.95	35.42	35.53	2.20	0.225	0.047	0.106
Firmicutes	39.14	30.29^{b}	48.41^{a}	55.61^{a}	49.00^{a}	2.52	<0.001	0.002	<0.001
Proteobacteria	1.34^{B}	2.56^{Aa}	1.73^{b}	1.41^{b}	1.64^{b}	0.14	0.003	0.003	0.001
Actinobacteria	0.52	0.42^{b}	0.46^{b}	0.53^{ab}	0.77^{a}	0.05	0.042	0.007	0.015
Tenericutes	0.14	0.15^{b}	0.23^{ab}	0.19^{ab}	0.28^{a}	0.02	0.032	0.012	0.048
Genus relative abundance (%)									
Alistipes	1.79^{B}	5.08^{A}	4.62	4.92	4.60	0.24	0.896	0.644	0.895
Christensenellaceae R-7 group	0.74	0.60^{b}	1.18^{a}	1.25^{a}	1.17^{a}	0.09	0.013	0.019	0.004
Clostridiales vadinBB60 group_norank	2.43	1.54	2.30	2.49	2.27	0.16	0.228	0.164	0.105
Faecalibacterium	9.21	4.88^{b}	9.46^{a}	8.65^{a}	9.20^{a}	0.54	0.003	0.013	0.006
Ruminiclostridium 9	0.43^{B}	1.55^{Aa}	0.80^{b}	0.80^{b}	0.82^{b}	0.10	0.004	0.011	0.002
Ruminococcus 1	0.12	0.38^{a}	0.22^{b}	0.21^{b}	0.20^{b}	0.03	0.034	0.017	0.016
Lactobacillus	1.86^{A}	0.62^{Bc}	1.14^{ab}	1.09^{b}	1.46^{a}	0.09	0.002	0.001	0.003
Megamonas	0.67^{B}	4.39^{Aa}	0.64^{b}	0.49^{b}	0.54^{b}	0.51	0.035	0.023	0.016
[Ruminococcus] torques group	4.61^{B}	9.76^{Aa}	3.47^{b}	3.32^{b}	2.91^{b}	0.82	<0.001	0.001	0.000
Campylobacter	0.41^{B}	3.53^{Aa}	1.32^{b}	1.16^{b}	1.37^{b}	0.29	<0.001	0.005	0.000
Synergistes	1.49^{A}	0.57^{Bb}	1.07^{a}	1.14^{a}	0.97^{ab}	0.08	0.058	0.118	0.020
Unclassified	16.12	10.49^{b}	11.96^{b}	14.50^{ab}	16.83^{a}	0.88	0.026	0.001	0.008

Values are means of 6 replicates per treatment. LPS, Lipopolysaccharide; ACP, Antrodia cinnamomea polysaccharide; SEM, standard error.
[a]Capital letters indicate statistically significant ($P < 0.05$) differences between control group and LPS group by Student's t-test; small letters indicate statistically significant ($P < 0.05$) differences of four groups (LPS, 100 mg/kg ACP+LPS, 200 mg/kg ACP+LPS, 400 mg/kg ACP+LPS). Mean values within a row with no common superscript differ significantly ($P < 0.05$).
[b]The *P*-value are representing the ANOVA analysis of four groups (LPS, 100 mg/kg ACP+LPS, 200 mg/kg ACP+LPS, 400 mg/kg ACP+LPS) and orthogonal polynomial contrasts are used to determine linear and quadratic responses of four groups.

decreased by LPS in the present study. This was reflected by the Shannon index and Simpson index with statistical differences.

It has been reported that *Panax ginseng* polysaccharides changed the diversity and composition of the gut microbiota with antibiotic-associated diarrhea in mice (26). *Panax ginseng* polysaccharides can increase phylum Firmicutes abundance and decrease phyla Bacteroidetes, Proteobacteria and Actinobacteria abundance (26). *Quinoa* polysaccharide enhanced species richness by regulating the community structure of gut microbiota, reducing the ratio of Firmicutes to Bacteroides and Proteobacteria abundance in rats (27). This study showed that Firmicutes and Bacteroidetes were the greatest predominant phylum in caeca of slow-growing broilers, which was inconsistent with previous reports (49, 50). Meanwhile, our results revealed that ACP was inhibited the decline of *Faecalibacterium* (typically Firmicutes) and the rise of Proteobacteria in LPS-induced group. Accordingly, Lee et al. (12) reported that a linear decrease was detected in the cecum coliform (Proteobacteria) count by ACP supplementation in comparison with the control group. Notably, ACP restrained beneficial cecal microbiota reduction (typically *Lactobacillus* and *Christensenellaceae R-7* group) in LPS-induced chickens. Consistently, the lactic acid bacteria showed an increased tendency by ACP supplementation (12). Moreover, Zhang et al. (48) has concluded that plant bioactive substances produced metabolites by gut microbiota in the distal gastrointestinal. The results of this study also support the function of ACP in the rescue of a normal microbial environment exerting a prebiotic promotion of beneficial bacteria.

The liver is an important metabolic organ and it is a multi-purpose organ including, clearing excess-production of ROS to maintain oxidative balance by decreasing MDA observed after LPS-challenge herein. In addition, it has been reported that polysaccharide can reach the liver *via* the portal vein after absorption along the gastrointestinal tract (51). Therefore, the potential beneficial effect of ACP as an antioxidant would be expected primarily in liver. On the other side, gut microbiota dysbiosis is associated with oxidative stress (52). In addition, the relationship between oxidative stress and gut microbiota has been illustrated (53). It has been found that oxidative

stress alters the oxygen gradient and shifts the equilibrium of microbiota from anaerobic to facultative anaerobic groups (54). Therefore, the gut health (microbiome) can interact tightly with the liver that called a "gut-liver axis". This axis offers liver specific interacts with a substantial amount of microbiota derived signals (24). Accordingly, it can be inferred that feeding a diet with ACP may help to alleviate the oxidative stress in the liver and regulate the microbiota composition in ceca after LPS-challenge in the present study.

Conclusions

Feeding a diet with 100 mg/kg ACP may have beneficial effects on liver damage and the bacterial microbiota diversity and composition in the ceca of LPS-stressed slow-growing broiler breeds, probably because of its combined favorable effects on antioxidants and cytokines contents, and inhibition of the expression of TLR4/NF-κB signaling pathway in the liver, and restoration the decline of beneficial cecal microbiota (typically *Lactobacillus*, *Faecalibacterium*, and *Christensenellaceae* R-7 group).

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/supplementary materials.

Ethics statement

The animal study was reviewed and approved by Animal Care Committee of the Institute of Animal Science, Guangdong Academy of Agricultural Sciences.

Author contributions

JY, CZ, and MA performed experiments, analyzed data, and wrote the manuscript. QF, XL, and YW performed experiments. RA, AA, and SJ supervised the project, developed the study concept, and wrote and edited the manuscript. All authors read and approved the final manuscript.

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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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Tannic acid-chelated zinc supplementation alleviates intestinal injury in piglets challenged by porcine epidemic diarrhea virus

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Porcine epidemic diarrhea virus (PEDV) has become a challenging problem in pig industry all over the world, causing significant profit losses. Tannins and organic zinc have been shown to exert protective effects on the intestinal dysfunction caused by endotoxins. However, there is little information on tannic acid-chelated zinc (TAZ) supplementation in the diet of newborn piglets. This study was conducted to determine the effects of TAZ on the intestinal function of piglets infected with PEDV. Thirty-two 7-day-old piglets were randomly allocated to 1 of 4 treatments in a 2 × 2 factorial design consisting of 2 diets (0 or 50 mg/kg BW TAZ) and challenge (saline or PEDV). On day 9 of the trial, 8 pigs per treatment received either sterile saline or PEDV solution at 10^6 $TCID_{50}$ (50% tissue culture infectious dose) per pig. Pigs infected with PEDV had greater diarrhea rate and lower average daily gain (ADG) ($P < 0.05$). PEDV infection decreased plasma D-xylose concentration, most antioxidative enzyme activities in plasma and intestine, as well as the small intestinal villus height ($P < 0.05$). Plasma diamine oxidase and blood parameters were also affected by PEDV infection. Dietary supplementation with TAZ could ameliorate the PEDV-induced changes in all measured variables ($P < 0.05$). Moreover, TAZ decreased the concentration of malondialdehyde in plasma, duodenum, jejunum, and colon ($P < 0.05$). Collectively, our results indicated that dietary TAZ could alleviate PEDV induced damage on intestinal mucosa and antioxidative capacity, and improve the absorptive function and growth in piglets. Therefore, our novel findings also suggest that TAZ, as a new feed additive for neonatal and weaning piglets, has the potential to be an alternative to ZnO.

KEYWORDS

antioxidant capacity, intestinal functions, piglets, porcine epidemic diarrhea virus, tannic acid-chelated zinc

Introduction

Porcine epidemic diarrhea virus (PEDV) spreads through feed and fecal oral route, which is a main pathogen that causes enteric diseases in swine industry (1). The virus induces apoptosis and necrosis of intestinal epithelium, mainly in the jejunum and ileum, which causes watery malabsorptive diarrhea, vomiting, and high mortality in pigs at all ages, especially during the neonatal and weanling periods (2–5). It has been demonstrated that feed additives, such as organic acids (6), organic trace minerals, medium-chain fatty acids (7), plant extracts (8), amino acid derivates (9) could ameliorate PEDV-infected intestinal injury. However, the mechanism of functional feed additives for prevention and treatment of PEDV-infected intestinal are still lacking.

In current practices, dietary supplementation with pharmacological zinc oxide (ZnO, 1600–2500 mg/kg zinc) in piglets during the first 2 weeks after weaning could prevent diarrhea (10, 11). A previous study conducted by our research team also reported that 100 mg/kg BW ZnO could improve growth performance, intestinal function, and antioxidant capacity in PEDV-infected piglets (12). However, medicinal ZnO in pig production will be disused by 2022 in Europe because of the environmental pollution and antibiotic-resistant issue (13). Therefore, hydrolyzed tannins have been widely used in piglet diets to decrease diarrhea rate, modulate intestinal health, and enhance growth performance (11, 14–16).

Studies have shown that tannic acid has various biological functions such as antioxidative, antibacterial, and antiviral property (17). However, there is still controversy on the effect of tannins in piglets (14, 18). Therefore, in the present study, we evaluated the effect of a new form of organic zinc, which is chelated with tannic acid, on the growth, antioxidative status, intestinal morphology in PEDV-infected piglets. Our findings are expected to explore an alternative to ZnO, and determine the mechanism of tannic acid-chelated zinc (TAZ) in alleviating the negative effects of PEDV in neonatal piglets.

Materials and methods

Animal care and diets

All animal procedures used in this study were approved by the Institutional Animal Care and Use Committee of Wuhan Polytechnic University (Number: 20161121). A total of 32 healthy 7-day-old piglets (Duroc × Landrace × Yorkshire, BW = 2.46 ± 0.21 kg) were used in this experiment. Pigs were housed individually with strict control of cross-infection in two environment-controlled nursery rooms (30 ± 2°C) and given *ad libitum* access to water throughout the study. The TAZ was obtained from the Animal Nutrition and Intestinal Health Research Group of Wuhan Polytechnic University, which contained ≥80% tannin, 6–7% zinc, crude fiber <2.00%, ash <2.50%, and moisture <8.00%. Piglets were provided a basal diet (liquid milk replacer), which was formulated to meet or exceed the nutrient requirements of suckling piglets. The milk replacer was purchased from Wuhan Anyou Feed Co., Ltd (Wuhan, China). Before feeding, the milk replacer was dissolved in warm water (45–55°C) to form a liquid feed (dry matter content of 20%) (9). Pigs were fed the liquid feed every 3 h between 8:00 am and 8:00 pm.

Experiment design

Pigs were fed the control liquid diet or TAZ-supplemented liquid diet for 9 days before the PEDV challenge (16 pigs per group). Immediately after PEDV challenge, pigs were divided into four treatments in a 2 × 2 factorial design. The main factors consisted of diet (0 or 50 mg/kg BW TAZ supplementation in diet; +TAZ or –TAZ) and challenge (PEDV or saline administration; +PEDV or –PEDV). On day 9 of the experiment, eight pigs in each dietary treatment were orally administered with either PEDV at a dose of 10^6 $TCID_{50}$ (50% tissue culture infectious dose) per pig or the same volume of sterile saline (Control). On day 12 of the trial, 10% D-xylose (1 mL/kg BW) was orally administrated to piglets to determine the intestinal absorption capacity and mucosal integrity (9). One hour later, all piglets were weighed and blood samples were collected from the anterior vena cava, and then all pigs were sacrificed under sodium pentobarbital anesthesia (50 mg/kg BW, iv) to obtain intestinal samples (12).

Collection of blood and intestinal samples

As mentioned previously, all blood samples were collected from anterior vena cava of piglets into heparinized vacuum tubes (Becton-Dickinson Vacutainer System, Franklin Lake, NJ, USA) at 1 h post D-xylose administration on day 12 of the trial (19). Blood samples were centrifuged at 3000 rpm for 15 min at 4°C to obtain plasma, which was then stored at −20°C until analysis.

After slaughtering, the pig abdomen was opened immediately and the whole gastrointestinal tract was exposed. The intestine was dissected free of the mesentery and placed on a chilled stainless steel tray. The 1- and 10-cm segments were obtained from the distal duodenum, mid-jejunum, mid-ileum and mid-colon, respectively (19, 20). The 5 cm intestinal segments were flushed gently with ice-cold phosphate buffered saline (PBS, pH = 7.4) and then placed in 4% fresh, chilled formalin solution for histological measurements. The 10-cm segments were opened longitudinally and the contents were flushed with ice-cold PBS. Mucosa was collected by scraping using a sterile glass microscope slide at 4°C, rapidly frozen in

liquid nitrogen, and stored at −80°C until analysis. All samples were collected within 15 min after killing.

Growth performance and diarrhea rate

Piglets were weighted on d 0, 9, and 12 of the experiment to calculate the average daily gain (ADG). Health status and diarrhea score were recorded throughout the experimental period. The fecal score was classified into four levels: 0 = strip or granular feces, 1 = soft stool feces, 2 = thick and water feces, and 3 = water feces. Score ≥ 2 was considered diarrhea. The formula of diarrhea rate was given as follows: diarrhea rate (DR) = total number of pigs with diarrhea/(total number of test piglets × test days) × 100% (21).

Blood parameters

The concentrations of blood biochemical parameters, such as total protein (TP), albumin (ALB), aspartate transaminase (AST), alanine transaminase (ALT), γ-glutamyltransferase (GGT), alkaline phosphatase (ALP), lactate dehydrogenase (LDH), total cholesterol (TC), triacylglycerol (TG), glucose (GLU), calcium (Ca), phosphorus (P), creatinine (CREA), high density lipoprotein (HDL), and low density lipoprotein (LDL) were measured with Wako kits (Wako Pure Chemical Industries, Ltd., Osaka, Japan) using a Hitachi 7060 Automatic Biochemical Analyzer (Hitachi, Tokyo, Japan).

Determination of D-xylose and diamine oxidase activity in plasma

Plasma D-xylose concentration and DAO activity were determined by colorimetric method using commercial kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). All assays were performed according to the instructions of manufacturer.

Antioxidant capacity in plasma and intestinal mucosa

Plasma, mucosa of duodenum, jejunum, ileum, and colon were used for analysis of antioxidative enzymes and related products. The activities of glutathione peroxidase (GSH-Px), catalase (CAT), total superoxide dismutase (T-SOD), total antioxidant capacity (T-AOC), as well as the concentration of hydrogen peroxide (H_2O_2) and malondialdehyde (MDA) were determined by using commercially available kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) according to the protocols of manufacturer (22). Assays were performed in triplicate.

Intestinal histomorphology

Intestinal histomorphology were examined according to the method of Yi et al. (21). Briefly, the fixed intestinal segments were embedded in paraffin. Consecutive 5 μm sections were cut and then stained with haematoxylin and eosin. Intestinal morphology was determined using a light microscope (Leica Microsystems, Wetzlar, Germany) with Leica Application Suite image analysis software (Leica Microsystems, Wetzlar, Germany). The villus height, villus width at half-height, and crypt depth were measured from 10 randomly selected villi and associated crypts on each section at 40 × magnification. Villus height was measured from the tip of villus to the crypt opening and crypt depth was measured from the base of crypt to the level of crypt opening. The villus height/crypt depth ratio and villous surface area were then calculated from these measurements.

Statistical analyses

All data were analyzed by one-way ANOVA using the GLM procedure of SPSS 20.0 software appropriate for a 2 × 2 factorial design (SPSS Inc. Chicago, IL, USA). The statistical model consisted of the effects of diet (+TAZ vs. –TAZ) and challenge (saline vs. PEDV) and their interactions. Data were expressed as means and pooled SEMs. In cases where the differences were significant, the means were compared by Duncan's multiple range test. A value of $P < 0.05$ were considered significant, and $0.05 \leq P < 0.10$ as trends.

Results

Average daily gain and diarrhea rate

The effect of TAZ on ADG and diarrhea rate in PEDV-infected piglets is shown in Table 1. During days 0–9 (pre-infection), there was no difference in the ADG and DR of pigs fed the control and TAZ-supplemented diets ($P > 0.05$). During days 9–12 of the trial (post-infection), PEDV infection decreased the ADG, and increased the diarrhea rate ($P < 0.05$). There were interactive effects between TAZ and PEDV, the TAZ administration mitigated diarrhea and increased the ADG induced by PEDV infection ($P < 0.05$).

Blood parameters

The effect of TAZ on blood parameters in PEDV-infected piglets is shown in Table 2. Compared with non-infected pigs,

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TABLE 1 The effect of tannic acid-chelated zinc on ADG and diarrhea rate in PEDV-infected piglets.

Items	–PEDV		+PEDV		SEM	*P*-values		
	–TAZ	+TAZ	–TAZ	+TAZ		PEDV	TAZ	PEDV × TAZ
Days 0–9								
ADG (g)	83.5	88.6	85.6	90.6	8.65	0.845	0.762	0.895
Diarrhea rate (%)	4.3	3.5	4.8	3.2	0.98	0.345	0.421	0.598
Days 9–12								
ADG (g)	120.2^{a}	143.4^{a}	31.9^{b}	112.7^{a}	9.58	<0.001	<0.001	0.014
Diarrhea rate (%)	0^{c}	0^{c}	83.8^{a}	58.3^{b}	2.35	<0.001	<0.001	0.012

Values are mean and pooled SEM, n = 8. PEDV, porcine epidemic diarrhea virus; TAZ, tannic acid-chelated zinc. ^{a,b,c}Within a row, means with different superscripts differ, $P < 0.05$. ADG, average daily gain.

TABLE 2 The effect of tannic acid-chelated zinc on blood parameters in PEDV-infected piglets.

Items	–PEDV		+PEDV		SEM	*P*-values		
	–TAZ	+TAZ	–TAZ	+TAZ		PEDV	TAZ	PEDV × TAZ
TP (g/L)	6.02	6.17	6.19	6.29	0.104	0.520	0.579	0.895
ALB (g/L)	2.96^{ab}	2.74^{b}	2.70^{b}	3.13^{a}	0.056	0.142	0.937	0.048
AST (U/L)	38.83	37.17	32.30	35.29	1.761	0.262	0.859	0.532
ALT (U/L)	54.83	53.33	65.40	55.14	1.574	0.009	0.019	0.157
ALP (U/L)	780.17	865.33	625.70	546.71	42.385	0.005	0.968	0.299
TC (mg/dL)	201.62	241.42	107.25	106.40	13.233	<0.001	0.238	0.219
TG (mg/dL)	40.49	27.05	60.83	47.25	4.371	0.017	0.102	0.993
GLU (mg/dL)	73.45	80.03	90.03	92.57	3.539	0.049	0.522	0.776
Ca (mg/dL)	10.62	10.53	11.23	22.95	3.027	0.303	0.357	0.350
P (mg/dL)	9.60^{a}	9.15^{ab}	7.89^{c}	8.67^{b}	0.161	<0.001	0.471	0.011
CREA (mg/dL)	0.972	0.755	0.635	0.779	0.074	0.310	0.811	0.245
HDL (mg/dL)	99.89	120.95	48.57	45.12	6.966	<0.001	0.254	0.116
LDL (mg/dL)	155.22	196.78	67.53	62.79	13.547	<0.001	0.324	0.217
GGT (U/L)	38.17	39.00	30.60	45.00	4.253	0.858	0.090	0.129
LDH (U/L)	832.03	864.70	728.01	752.94	18.746	<0.001	0.398	0.909

Values are mean and pooled SEM, n = 8. PEDV, porcine epidemic diarrhea virus; TAZ, tannic acid-chelated zinc. ^{a,b,c}Within a row, means with different superscripts differ, $P < 0.05$. TB, total bilirubin; TP, total protein; ALB, albumin; AST, aspartate transaminase; ALT, alanine transaminase; GGT, γ-glutamyltransferase; ALP, alkaline phosphatase; LDH, lactate dehydrogenase; TC, total cholesterol; TG, triacylglycerol; GLU, glucose; Ca, calcium; P, phosphorus; CREA, creatinine; HDL, high density lipoprotein; LDL, low density lipoprotein.

PEDV-infected piglets had lower concentrations of ALP, TC, P, HDL, LDL, and LDH in plasma, and had greater concentration of ALT, TG and GLU ($P < 0.05$). Pigs fed the TAZ diet had a lower plasma ALT level, and tended to have greater plasma GGT than the control pigs ($P = 0.090$). There were interactive effects between PEDV and TAZ on plasma ALB and P concentrations ($P < 0.05$). The concentrations of ALB and P was increased in pigs infected with PEDV fed the TAZ diet compared with the PEDV-infected pigs fed a diet without TAZ (–TAZ) ($P < 0.05$), whereas there was no difference in these parameters in saline (–PEDV) treatments ($P > 0.05$).

Diamine oxidase activity and D-xylose concentration

Data on plasma DAO activity and D-xylose concentration are summarized in Table 3. The PEDV-infected pigs had greater activity of DAO and lower D-xylose concentration in plasma than non-infected pigs ($P < 0.05$). Pigs fed the TAZ diet showed lower plasma DAO activity and greater D-xylose concentration than pigs in control group ($P < 0.05$).

TABLE 3 The effect of tannic acid-chelated zinc on DAO activity and D-xylose concentration in PEDV-infected piglets.

Items	−PEDV		+PEDV		SEM	*P*-values		
	−TAZ	+TAZ	−TAZ	+TAZ		PEDV	TAZ	PEDV × TAZ
DAO (U/L)	18.86	18.28	22.58	19.78	0.466	0.001	0.021	0.115
D-xylose (mmol/L)	3.23	3.63	1.83	2.08	0.141	<0.001	<0.001	0.293

Values are mean and pooled SEM, n = 8. PEDV, porcine epidemic diarrhea virus; TAZ, tannic acid-chelated zinc; DAO, Diamine oxidase.

Plasma antioxidant capacity

The effect of TAZ on plasma antioxidant capacity in PEDV-infected piglets is shown in Table 4. Compared with non-infected pigs, PEDV-infected pigs had lower GSH-Px and T-SOD activity in plasma, and greater H_2O_2 concentration than those in the control treatment ($P < 0.05$). The activity of CAT in plasma was increased in TAZ, and the plasma H_2O_2 and MDA concentration was decreased compared with the control group ($P < 0.05$). There was PEDV × TAZ interaction on the plasma CAT activity ($P < 0.05$). The data showed that TAZ supplementation was more effective to increase the activity of CAT in plasma of PEDV-infected pigs than non-infected pigs ($P < 0.05$). However, the T-AOC in plasma was not affected either by PEDV or dietary TAZ ($P > 0.05$).

Intestinal antioxidant capacity

The effect of TAZ on the intestinal antioxidant capacity in PEDV-infected piglets is shown in Table 5. Compared with non-infected pigs, PEDV-infected pigs had lower GSH-Px and CAT activities in duodenum and jejunum, and greater MDA concentration in duodenum than those in the control treatment ($P < 0.05$). The concentration of H_2O_2 in colon was tended to increase in PEDV treatments compared with the non-infected pigs ($P = 0.076$). Pigs fed the TAZ diet had greater T-AOC and GSH-Px in duodenum, jejunum, and colon ($P < 0.05$), T-SOD in duodenum and jejunum ($P < 0.05$), tended to have higher CAT in jejunum ($P = 0.075$), and lower MDA concentration in duodenum, jejunum, and colon ($P < 0.05$). The colon H_2O_2 concentration was also decreased in the TAZ treatment compared with the control ($P < 0.05$). There were PEDV × TAZ interactions on the GSH-Px in duodenum and jejunum, jejunal T-SOD, as well as MDA and H_2O_2 concentration in colon ($P < 0.05$).

Intestinal morphology

Data on the small intestinal histomorphology are summarized in Table 6. PEDV infection decreased villus height, villus height/crypt depth ratio, and villous surface area in all small intestinal segments ($P < 0.05$), and increased the crypt depth in small intestine and colon ($P < 0.05$). There were PEDV × TAZ interactions in villus height in the jejunum and villus height/crypt depth ratio in duodenum and jejunum, as well as the crypt depth in the small intestine and colon ($P < 0.05$). Data indicated that TAZ supplementation could increase the duodenal and jejunal villus height, villus height/crypt depth ratio and villous surface area, and decrease the crypt depth in duodenum, jejunum and colon ($P < 0.05$), as compared to the control (−TAZ).

Discussion

In the last decades, PEDV outbreaks all over the world induced huge economic loses in swine industry (23). Although a series of feed additive were evaluated to prevent PEDV, the results were inconsistent. As a potential alternative to inorganic ZnO to alleviate diarrhea, we set up a PEDV infection model to investigate the protective effect of TAZ on growth, antioxidant capacity and intestinal morphology in piglets.

In the present study, infected pigs exhibited the symptoms of PEDV, such as diarrhea, vomiting and thin intestinal wall. PEDV infection decreased the ADG, and increased the diarrhea rate of piglets, which was consistent with previous studies (24–26). Our previous studies also showed that oral administration of $10^{4.5}$ $TCID_{50}$ resulted in retarded growth and sever diarrhea in piglets (9, 12). Dietary TAZ alleviated the ADG reduction caused by infection. These results may be related to the interference effect on the integrity of enveloped structure of PEDV by tannin and zinc, and inhibition of the reproduction of pathogens (27). In agreement with our studies, lots of studies also showed that tannin and zinc (ZnO and organic zinc) improved the growth performance of piglets (11, 14–16). In addition, dietary administration of ZnO decreased the fecal score in a previous study, thus alleviating the ADG reduction caused by PEDV (12). These results indicated that TAZ could be a potential substitute of ZnO to prevent diarrhea and promote growth in neonatal and weaning piglets.

In our study, we found that PEDV increased the concentrations of ALT, TG and GLU in plasma, which indicated that PEDV infection already resulted in inflammatory reaction in piglets. It was reported that injury of gastrointestinal

TABLE 4 The effect of tannic acid-chelated zinc on plasma antioxidant capacity in PEDV-infected piglets.

Items	−PEDV		+PEDV		SEM	*P*-values		
	−TAZ	+TAZ	−TAZ	+TAZ		PEDV	TAZ	PEDV × TAZ
T-AOC (mM)	3.077	2.902	3.315	2.913	0.102	0.550	0.176	0.588
GSH-Px (U/ml)	363.9	370.1	329.1	359.4	5.95	0.045	0.102	0.273
T-SOD (U/ml)	86.33	86.69	80.74	82.25	0.923	0.006	0.567	0.727
CAT (U/ml)	3.68^{c}	3.99^{b}	3.09^{d}	5.03^{a}	0.151	0.006	<0.001	<0.001
H_2O_2 (nmol/L)	67.25	63.05	95.43	79.66	5.98	0.001	0.012	0.251
MDA (nmol/mL)	0.255	0.215	0.262	0.210	0.007	0.938	<0.001	0.587

Values are mean and pooled SEM, n = 8. PEDV, porcine epidemic diarrhea virus; TAZ, tannic acid-chelated zinc. ^{a,b,c,d}Within a row, means with different superscripts differ, $P < 0.05$. T-AOC, total antioxidant capacity; GSH-Px, glutathione peroxidase; CAT, catalase; T-SOD, total superoxide and dismutase; H_2O_2, hydrogen peroxide; MDA, malondialdehyde.

TABLE 5 The effect of tannic acid-chelated zinc on intestinal antioxidant capacity in PEDV-infected piglets.

Items	−PEDV		+PEDV		SEM	*P*-values		
	−TAZ	+TAZ	−TAZ	+TAZ		PEDV	TAZ	PEDV × TAZ
Duodenum								
T-AOC (mmol/g protein)	0.179	0.655	0.202	0.542	0.046	0.197	<0.001	0.058
GSH-Px (U/mg protein)	31.98^{a}	30.43^{a}	19.96^{b}	33.44^{a}	1.333	<0.001	0.001	0.011
T-SOD (U/mg protein)	204.04	288.93	229.10	247.07	10.665	0.649	0.010	0.081
CAT (U/mg protein)	6.86	8.09	4.80	6.09	0.434	0.015	0.113	0.966
MDA (nmol/mg protein)	0.544	0.172	0.640	0.287	0.045	0.040	<0.001	0.839
H_2O_2 (μmol/g protein)	3.739	3.702	3.745	3.778	0.153	0.901	0.996	0.916
Jejunum								
T-AOC (mmol/g protein)	0.175	0.257	0.168	0.218	0.011	0.120	<0.001	0.267
GSH-Px (U/mg protein)	28.96^{b}	46.37^{a}	22.87^{c}	44.18^{a}	2.134	<0.001	0.013	<0.001
T-SOD (U/mg protein)	173.26^{b}	357.59^{a}	213.70^{b}	194.31^{b}	21.193	0.099	0.031	0.009
CAT (U/mg protein)	19.83	23.78	15.51	17.32	0.964	0.002	0.075	0.493
MDA (nmol/mg protein)	0.498	0.355	0.605	0.350	0.028	0.182	<0.001	0.149
H_2O_2 (μmol/g protein)	3.182	6.894	2.985	2.084	0.987	0.214	0.481	0.252
Ileum								
T-AOC (mmol/g protein)	0.977	0.980	0.984	0.981	0.006	0.739	0.990	0.782
GSH-Px (U/mg protein)	29.95	32.00	23.74	31.42	2.060	0.187	0.065	0.270
T-SOD (U/mg protein)	161.72	170.77	175.39	184.17	3.651	0.065	0.214	0.984
CAT (U/mg protein)	1.86	1.79	1.91	1.78	0.034	0.779	0.157	0.702
MDA (nmol/mg protein)	0.655	0.679	0.645	0.677	0.019	0.892	0.498	0.914
H_2O_2 (μmol/g protein)	0.843	0.969	0.875	1.002	0.068	0.820	0.384	0.999
Colon								
T-AOC (mmol/g protein)	1.071	1.086	1.091	1.112	0.005	0.012	0.044	0.715
GSH-Px (U/mg protein)	4.99	6.37	4.76	7.68	0.303	0.175	<0.001	0.058
T-SOD (U/mg protein)	216.09	212.23	223.35	226.71	6.519	0.438	0.986	0.795
CAT (U/mg protein)	3.80	4.38	3.52	3.60	0.235	0.285	0.499	0.610
MDA (nmol/mg protein)	1.389^{a}	1.398^{a}	1.404^{a}	0.966^{b}	0.062	0.055	0.049	0.041
H_2O_2 (μmol/g protein)	1.151^{b}	1.210^{b}	1.552^{a}	1.045^{b}	0.049	0.076	0.002	<0.001

Values are mean and pooled SEM, n = 8. PEDV, porcine epidemic diarrhea virus; TAZ, tannic acid-chelated zinc. ^{a,b}Within a row, means with different superscripts differ, $P < 0.05$. T-AOC, total antioxidant capacity; GSH-Px, glutathione peroxidase; CAT, catalase; T-SOD, total superoxide and dismutase; H_2O_2, hydrogen peroxide; MDA, malondialdehyde.

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TABLE 6 The effect of tannic acid-chelated zinc on the intestinal morphology in PEDV-infected piglets.

Items	−PEDV		+PEDV		SEM	*P*-values		
	−TAZ	+TAZ	−TAZ	+TAZ		PEDV	TAZ	PEDV × TAZ
Villus height (μm)								
Duodenum	205.4	311.4	141.5	196.6	15.27	<0.001	0.001	0.198
Jejunum	198.2^{b}	274.2^{a}	75.9^{c}	94.2^{c}	17.90	<0.001	0.001	0.020
Ileum	183.6	188.1	93.8	104.2	9.68	<0.001	0.307	0.687
Crypt depth (μm)								
Duodenum	80.6^{b}	90.1^{b}	147.4^{a}	98.8^{b}	6.64	0.040	0.001	0.007
Jejunum	98.4^{b}	95.9^{b}	135.7^{a}	46.8^{c}	7.99	<0.001	0.004	0.001
Ileum	91.0	94.1	155.5	154.9	4.27	<0.001	0.734	0.623
Colon	180.38^{b}	178.57^{b}	244.23^{a}	188.92^{b}	7.021	<0.001	0.020	0.030
Villus height/Crypt depth								
Duodenum	2.548^{a}	3.456^{b}	0.960^{c}	1.990^{bc}	0.060	<0.001	<0.001	0.024
Jejunum	2.014^{a}	2.859^{a}	0.559^{b}	2.013^{a}	0.038	0.023	0.026	0.013
Ileum	2.018	1.999	0.603	0.673	0.025	<0.001	0.112	0.557
Villous surface area (μm^2)								
Duodenum	37635.7	49987.4	17689.6	26744.8	2975.00	<0.001	0.011	0.660
Jejunum	23158.1	38531.7	20353.7	24418.4	2583.74	0.004	0.010	0.557
Ileum	25161.3	24670.8	12298.0	13853.4	1424.92	<0.001	0.727	0.504

Values are mean and pooled SEM, n = 8. PEDV, porcine epidemic diarrhea virus; TAZ, tannic acid-chelated zinc. ^{a,b,c}Within a row, means with different superscripts differ, $P < 0.05$.

tract and liver could cause the increment of blood ALT and AST, which can sensitively reflect the function of the liver (28, 29). In this study, it was observed that the TAZ groups reduced plasma ALT, indicating that TAZ supplementation may have therapeutic effects on the hepatic architecture and function damage induced by PEDV infection (9). Moreover, the blood P concentration was increased in pigs infected with PEDV fed the TAZ diet, which may indicate that TAZ improve the integrity of intestinal epithelium, leading higher nutrient digestibility.

Plasma DAO activity and D-xylose can be used as indicators for the integrity of intestinal barrier, which is the basis for preventing pathogenic bacteria, virus, and other harmful substances (30, 31). Impaired intestine is a major cause of diarrhea, and the concentration of D-xylose in blood and urine will decrease because of malabsorption, and the activity of DAO will increase after the damage of the intestine mucosa (9, 12, 32). In consistent with previous studies, the plasma D-xylose content was decreased, and the DAO activity was increased after PEDV infection, indicating that PEDV induced intestinal epithelial cell apoptosis and impaired intestinal function. Interestingly, dietary supplementation of TAZ reduced the DAO activity and increased D-xylose concentration in plasma of piglets, indicating that TAZ is beneficial to reduce intestinal permeability, which also further explains the decreased diarrhea rate in the present study.

Oxidative damage of cell and tissues by weanling stress, mycotoxin, and virus is well documented (33–35). In the present study, PEDV challenge decreased plasma and intestinal mucosal GSH-Px, T-SOD, and CAT activities, while increased MDA and H_2O_2, indicating that PEDV successfully induced humoral and intestinal mucosal oxidative injury in piglets. Interestingly, supplementation with TAZ mitigated these series of oxidative damage. The hydroxyl groups of phenol rings are responsible for a strong antioxidant function of TAZ (36). A series of studies have reported that tannin rich diets could improve the antioxidative capacity in pigs. Furthermore, it was reported that polyphenols extracted from grape seeds, gallnut and chestnut improved the antioxidant status of pigs in challenge models (33, 34, 37). Different forms of zinc, especially the organic zinc sources were also reported to enhance the endogenous antioxidant defenses by acting on antioxidant enzymes and the synthesis of the metallothionein proteins, which are able to scavenge free radicals, such as hydroxyl radicals and reactive oxygen species (38, 39).

Intestinal health is the basis for meet the growth potential of piglets. Villus height, crypt depth and villous surface area are strongly related to the absorptive function, which are well accepted as indicators to reflect the morphological integrity of small intestine in animals (40, 41). In this study, PEDV infection decreased villus height and villus height/crypt depth ratios, and increased crypt depth in all segments of the small intestine, suggesting that PEDV induced intestinal structural damage and increased mucosal permeability, which was in agreement with our previous studies (9, 12). Notably, we found that TAZ supplementation increased villus height, villous surface area, and villus height/crypt depth ratios, as well as the crypt depth

in colon. In consistent with our study, some studies on pigs also found that fruits and Chinese medicinal herb original tannins, and zinc could improve these intestinal morphological parameters (12, 33, 42–44). These results could also explain the better growth performance in +TAZ treatments in our study.

Conclusion

In conclusion, we provide significant evidence for the effect of TAZ on growth performance, antioxidant capacity, intestinal morphology in piglets. Supplementation with TAZ could alleviate PEDV-induced growth retardation, oxidative stress, intestinal integrity damage in the neonatal piglets model. Our novel findings also suggest that TAZ, as a new feed additive for neonatal and weaning piglets, has the potential to be an alternative to ZnO.

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 Wuhan Polytechnic University (Number: 20161121).

Author contributions

ZZ analyzed the data and wrote the manuscript. SW and LZheng conducted the animal experiment and analyzed the data. SG read and revised the manuscript. LZhu, CD, TW, and DY also performed the experiment work. YH and BD designed the study and acquired funding. All authors contributed to the article and approved the submitted version of the manuscript.

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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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The effects of fucoidan as a dairy substitute on diarrhea rate and intestinal barrier function of the large intestine in weaned lambs

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This paper explores the effects of fucoidan on the frequency of diarrhea, colon morphology, colon antioxidant status, cytokine content, short-chain fatty acids, and microflora of cecal contents in early weaned lambs in order to provide a reference for the intestinal health of young ruminants. Fucoidan is a natural active polysaccharide extracted from kelp and other large brown algae. It has many biological effects, such as improving immunity, nourishing the stomach and intestines, and anti-tumor properties. This study investigated the effects of fucoidan supplementation in milk replacer on the large intestine's ability to act as an intestinal barrier in weaned lambs. With six duplicate pens and one lamb per pen, a total of 24 weaned lambs (average starting body weight of 7.32 ± 0.37 kg) were randomly assigned to one of four milk replacer treatments. Four concentrations of fucoidan supplementation (0, 0.1, 0.3, and 0.6% dry matter intake) were employed to investigate the effects of fucoidan on cecal fermentation and colon microbial organization. The test period lasted 37 days (1 week before the test and 1 month after the test), and lamb cecal contents and colon organization were collected for examination. In addition, the fecal status of all lambs was observed and recorded daily, allowing us to calculate the incidence of diarrhea in weaned lambs. The findings demonstrated that fucoidan may significantly increase the concentration of short-chain fatty acids (propionic acid and butyric acid) in the cecal digesta of weaned lambs. In weaned lambs, 16S rDNA testing showed that fucoidan at 0.3–0.6% (dry matter intake) was beneficial for boosting the variety of the intestinal bacteria and modifying the relative abundance of a few bacterial strains. In addition, fucoidan enhanced colon antioxidant and immune functions and decreased the diarrhea rate to relieve weaning stress. This result demonstrates that milk replacer supplementation with fucoidan contributes to the improvement in the large intestinal health of weaned lambs.

KEYWORDS

cecal short-chain fatty acids, cecal microbes, colon histomorphology, colon antioxidants, colon immunity, colon cytokines

Introduction

The number of sheep and goats in China ranks among the highest in the world, with an estimated 307 million domestic sheep and goats (1). However, goat husbandry in southern China is influenced by factors such as geographic location, climatic conditions, and breed. Early weaning is a crucial technological advancement. It can lead to the growth of the lamb and shorten the ewes' breeding and estrous cycles (2). Unfortunately, early lamb weaning technology remains underexploited because the digestive systems of lambs are immature and cannot take maximal advantage of plant-based solid feed. In addition, weaning is a critical phase of well-marked physiological change and evokes psychological and physiologic stress responses, especially post-weaning diarrhea (PWD), which is a common problem in lambs (3–5). These challenges restrict the further development of the industry. Therefore, novel nutritional approaches to alleviate weaning stress have become a major focus of industry interest in young animal health, breeding, and feed.

In recent years, biologically active substances extracted from plants have attracted increasing attention for the prevention and treatment of diseases. Seaweed polysaccharides have been shown to improve immune responses, redox status, and gut health in animals (6–10). Fucoidan refers to a group of complex sulfated polysaccharides derived from brown seaweed species. Its core components are fucose and sulfate groups, which mediate a variety of significant biological effects with antioxidant (11), anti-inflammatory (12, 13), and immune-enhancing (14–18) properties. These properties can alleviate diarrhea symptoms and improve growth (19). Fucoidan, as a natural strong antioxidant, can effectively remove free radicals, reduce the production of inflammatory factors, and alleviate inflammatory reactions (11–13). At the same time, the alternating (1 → 3) and (1 → 4) glycosidic bonds on the main chain of fucoidan have a regulatory effect on intestinal immunity. The intestinal immune balance is maintained by stimulating a variety of mononuclear cells, macrophages, and auxiliary T-cells, as well as regulating the secretion of cytokines such as TNF-α (14–18). O'Shea (19) found that after feeding 240 mg/kg fucoidan to piglets affected by chronic colitis, diarrhea symptoms were significantly improved and weight gain was obvious.

We previously showed that lambs fed fucoidan had an average daily weight gain that was increased by over 34% over 30 days compared to the control group that did not receive fucoidan. We found that feeding fucoidan at concentrations of 0.3 and 0.6% improved antioxidant enzyme activity, raised the amount of anti-inflammatory substances in blood samples from weaned lambs, and decreased the amount of pro-inflammatory substances (20). However, there is no evidence to explain the mechanism by which fucoidan alleviates PWD in lambs. Therefore, this paper explores the effects of fucoidan on the frequency of diarrhea, colon morphology, colon antioxidant status, cytokine content, short-chain fatty acids, and microflora of cecal contents in early weaned lambs in order to provide a reference for the intestinal health of young ruminants.

Materials and methods

Materials

The fucoidan was purchased from Mingyue Hailin Fucoidan Biotechnology Co., Ltd. (Qingdao City, Shandong Province, China). The fucoidan was produced on January 5, 2021, with the following characteristics: purity: 98%, appearance: light yellow powder, composition: 66.3% sugar, 24.9% fucose, and 28.9% sulfate, and moisture level: 7.87%. The milk replacer power was obtained commercially (Beijing Precision Animal Nutrition Research Center, Beijing, China). The starter was formulated to meet or exceed the recommendations for lamb nutrition under China's Agricultural Industry Standard (Concentrate:Roughage = 80:20; NY/T 816-2004, Table 1).

Animals, experimental design, and management

The experiments were performed under the supervision of Guangdong Ocean University's Animal Care and Use Committee. 24 healthy Chuanzhong black male lambs (30 days old) with equal body weights of 7.32 ± 0.37 kg were weaned and provided with unrestricted access to water, starter food, and milk replacer powder for at least 7 days. Lambs were randomized into four groups (control, low-dose, middle-dose, and high-dose; $n = 6$ each). The lambs in the low-dose group (FL) received fucoidan at 0.1% of their daily dry matter intake (DMI). For the middle-dose group (FM), lambs were given fucoidan at a daily dose of 0.3% DMI. The lambs in the high-dose group (FH) were given fucoidan at a daily dose of 0.6% DMI.

All lambs were freely fed starters, and an equal amount of milk replacer powder (1.2%) was provided on a daily basis at 08:00, 11:00, 14:00, and 17:00 h. The milk replacer powder containing fucoidan was reconstituted with boiling water (weight of milk replacer powder: volume of water = 1:6). All lambs were single-housed. The total test period was 37 days. Lamb housing was cleaned daily, disinfected, and ventilated regularly. On the last day of the study, the lambs were sacrificed by jugular exsanguination. The cecum contents and colon tissues were then removed separately and immediately stored at −80°C until needed.

TABLE 1 Composition and nutrient composition of the starter (dry matter basis) %.

Ingredient	Content	Nutrition level	Starter content	Milk replacer power content
Alfalfa meal	23.16	DM	89.25	97.58
Expanded soybean	32.97	DE[2]/(MJ/kg)	13.45	11.14
Corn	22.49	CP	23.20	15.40
Rapeseed meal	7.33	EE	8.14	15.43
Bran	3.71	Ash	5.06	14.52
Cottonseed cake	7.33	Ca	0.92	1.02
Limestone	0.50	P	0.58	0.66
NaCl	0.52	NDF	17.15	
$CaHPO_4$	1.00	ADF	5.45	
Premix[1]	1.00			
Total	100.00			

[1]The premix provided the following per kg of diet: Fe 1.10 g, Cu 0.73 g, Mn 0.31 g, Zn 0.26 g, I 0.01 g, Se 0.02 g, Co 0.22 g, vitamin A 76 190 IU, vitamin D 3 429 IU, vitamin E 170 IU, vitamin B_1 23.32 mg, vitamin B_2 28.00 mg, vitamin B_6 22.63 mg, vitamin B_{12} 137.13 mg, vitamin B_5 91.42 mg, nicotinic acid 181.01 mg. [2]Digestive energy was a calculated value, while the others were measured values.

Fecal index and diarrhea rate

The fecal status of all lambs was observed and recorded on a daily basis at 08:00, 13:00, and 18:00 h. A fecal score of > 3 was considered diarrhea. Figure 1: Fecal scoring standards were: 1, hard and well-formed pellets; 2, normal and formed pellets; 3, pasty and semi-formed pellets; 4, soft and pasty stools; 5, watery diarrhea. The fecal index and diarrhea rate were calculated as follows: Fecal index = total fecal score / all lambs; diarrhea rate = frequency of diarrhea in each group / (test days × total animal numbers in each group) (21).

Colon histomorphology

Colons were postfixed for 24 h in 4% paraformaldehyde and processed in paraffin. The wax blocks were then divided into 4 μm chunks using a microtome, then dewaxed. The sections were deparaffinized in two changes of xylene before being rehydrated in ethanol. Hematoxylin-eosin staining (HE) and diastase-periodic acid-Schiff (D-PAS) staining were performed with kits (Servicebio, Wuhan, China) in accordance with the manufacturer's instructions. Histologic structures were observed using an inverted fluorescence microscope. Villus height, crypt depth, mucosal thickness, and muscular thickness were determined.

Colon antioxidant and immune function

The colon tissues were removed from the −80°C freezer and slowly thawed at 4°C. Colon tissues of each group were accurately weighed to achieve a weight (g):volume (mL) ratio of 1:9. An homogenizer was used to homogenize and centrifuge the samples using 0.9% normal saline as the diluent. Biochemical assays were performed according to the manufacturers' kit instructions. Assay kits for the total antioxidant capacity (T-AOC), activities of superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GSH-Px), and malondialdehyde (MDA) content were purchased from the Nanjing Jiancheng Bioengineering Institute (Nanjing, China). Enzyme-linked immunosorbent assay (ELISA) kits for IL-10, IL-1β, TNF-α, IFN-γ, IgA, IgG, IgM, and SIgA were obtained from Jiangsu Meimian Industrial Co., Ltd. (Nanjing, China).

Cecum microflora 16S rDNA sequencing and analysis

The manufacturer's procedure was followed to obtain genomic DNA. After quality and purity assessments, the extracted DNA samples were used as PCR templates. The obtained DNA bands were visualized by 1.8% agarose gel electrophoresis. The 16S rDNA V3 + V4 region of the sample was then amplified using primers 338F (5'-ACTCCTACGGGAGGCAGCA-3') and 806R (5'-GGACTACHVGGGTWTCTAAT-3') and enriched by PCR amplification to form a final library. All sequencing was conducted by Biomarker Technology Co., Ltd. (Beijing, China).

Cecum short-chain fatty acids

The content and composition of cecal SCFA were determined using gas chromatography-mass spectrometry (Trace 1310 and ISQLT, Thermo, USA) following previously published protocols (22–24). The following chromatographic settings were utilized: injection volume of 1 μL; inlet

temperature of 250°C; split ratio of 4:1; ion source temperature of 300°C, and transfer line temperature of 250°C. Oven temperature program: starting temperature at 90°C, then 10°C/min to 120°C and 5°C/min to 150°C, followed by a 25°C/min, 2 min climb to 250°C. Helium was used as the carrier gas, at a flow rate of 1.0 mL/min. The following mass spectrometry conditions were used: the electron ionization source of the instruments was operated with an electron energy of 70 eV and a SIM scanning mode was adopted. Recoveries and SCFA content were calculated following the method described by Giera et al. (25).

Statistical analysis

The data were subjected to one-way ANOVA using SPSS 23.0 (SPSS, Inc., Chicago, IL, United States) to analyze the effects of fucoidan. Significant differences between means were compared using Duncan's multiple comparisons test. The standard error of the mean (SEM) was used to represent the variation. Prism 8 was used to plot diarrhea rates, colon tissue shape, antioxidant and immunological markers, and cecal short-chain fatty acid concentrations of the lambs, which were deemed significant at $P < 0.05$ (GraphPad Software, San Diego, CA, USA).

FIGURE 1
Fecal consistency score [**(left–right)**: score of 1, 2, 3, 4, and 5].

Results

The effect of fucoidan on diarrhea rate and fecal index in weaned lambs

The fecal index and diarrhea rate reflect the health of the intestines, which impacts the growth of the weaned animals (Figure 2). Compared to the control group, the fucoidan-treated lambs had considerably lower fecal indexes and diarrhea rates ($P < 0.05$). The fecal index and diarrhea rate in groups FL, FM, and FH were significantly lower than those in the control by 16.38, 26.21, and 21.36% ($P < 0.05$) and 30.89, 64.85, and 54.42% ($P < 0.001$), respectively. However, the fecal index was not decreased in a dose-dependent manner when the weaned lambs were fed fucoidan.

The effects of fucoidan on the colon histomorphology of weaned lambs

To assess the histological changes in the goblet cells of the colon in the lambs, we performed HE staining (Figure 3A). Meanwhile, the status of mucin-producing epithelial goblet cells was evaluated through D-PAS staining (Figure 3B). The results were confirmed *via* software analysis (Figure 3C). Compared with feeding 0.3∼0.6% fucoidan, the villus height and mucosal thickness in the CON and FL groups were markedly decreased (Figure 3C) ($P < 0.05$), and the villus epithelial cells were

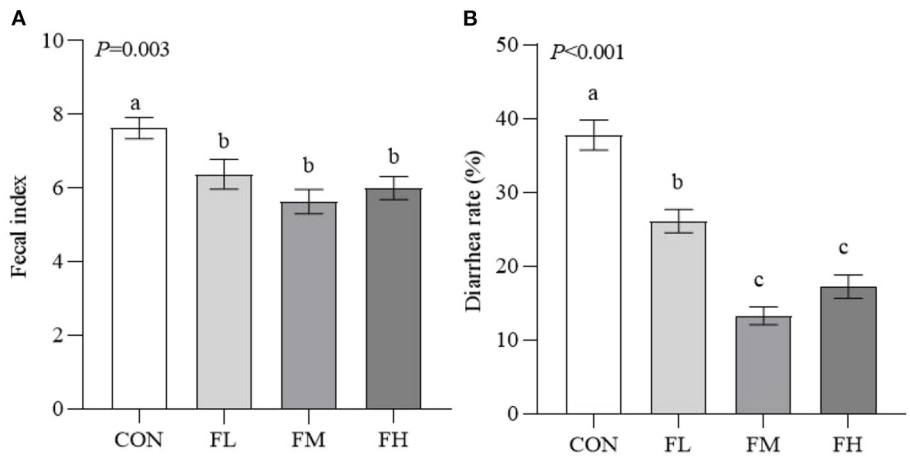

FIGURE 2
Effects of fucoidan on the fecal index and diarrhea rate of weaned lambs. **(A)** Fecal index. **(B)** Diarrhea rate. CON, control; FL, 0.1% fucoidan (DMI); FM, 0.3% fucoidan (DMI); FH, 0.6% fucoidan (DMI). Data are denoted as mean ± standard error of the mean (SEM) ($n = 6$). ^{a,b,c}Demonstrate a statistically significant distinction, $P < 0.05$.

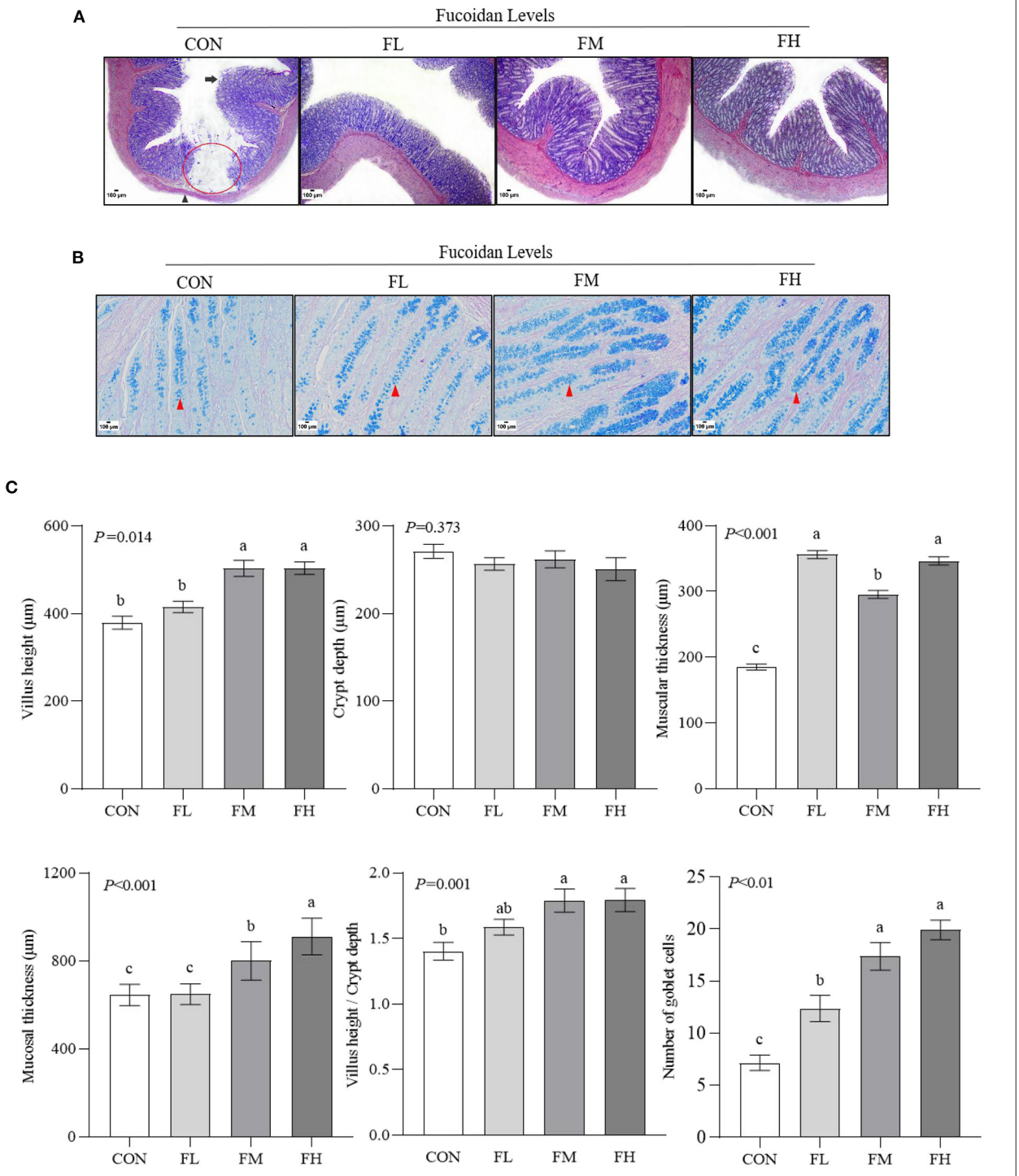

FIGURE 3
The effects of fucoidan on the colon morphology and goblet cell count of weaned lambs ($n = 3$). **(A)** HE staining results of colon paraffin sections (40×). The red circle indicates the absence of intestinal mucosa; the long black arrow shows the shedding of mucosal epithelial cells; the black arrowhead shows muscular atrophy. **(B)** D-PAS staining results of colon paraffin sections (200×). The red arrowhead shows the goblet cell (blue). **(C)** Morphology and goblet cell number in lamb colons were analyzed. CON, control; FL, 0.1% fucoidan (DMI); FM, 0.3% fucoidan (DMI); FH, 0.6% fucoidan (DMI). Data are denoted as mean ± SEM ($n = 3$). ^{a,b,c}Demonstrate a statistically significant distinction, $P < 0.05$.

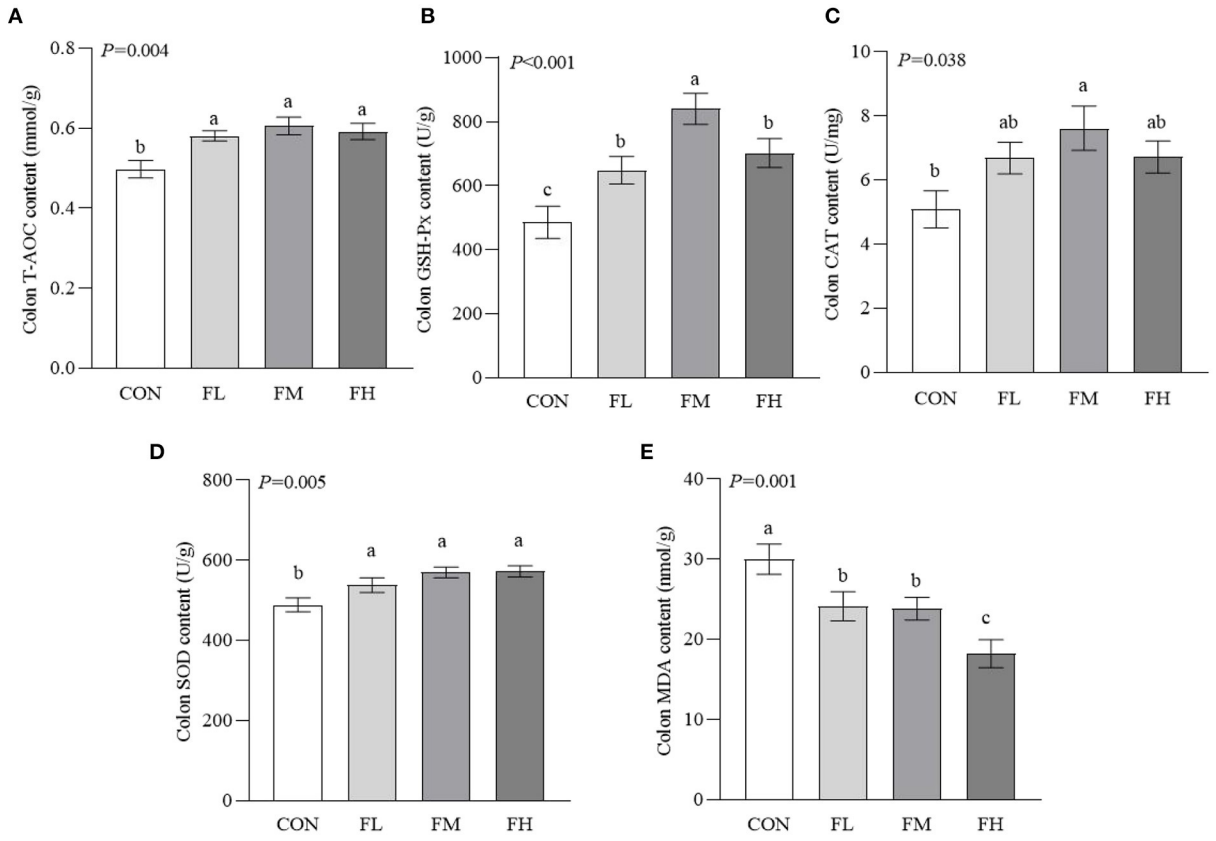

FIGURE 4
Effects of fucoidan on the colon antioxidant indexes of weaned lambs. **(A)** T-AOC. **(B)** GSH-Px. **(C)** CAT. **(D)** SOD. **(E)** MDA. CON, control; FL, 0.1% fucoidan (DMI); FM, 0.3% fucoidan (DMI); FH, 0.6% fucoidan (DMI). Data are denoted as mean ± SEM ($n = 3$). ^{a,b,c}Demonstrate a statistically significant distinction, $P < 0.05$.

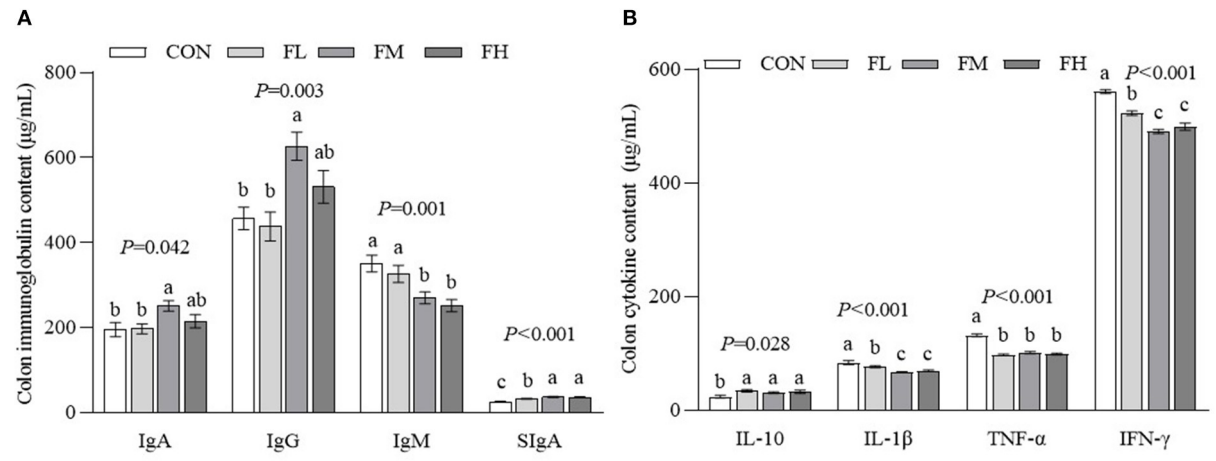

FIGURE 5
The effects of fucoidan on colon immunoglobulin and cytokine content in weaned lambs. **(A)** Colon immunoglobulin concentrations. **(B)** Colon cytokine concentrations. CON, control; FL, 0.1% fucoidan (DMI); FM, 0.3% fucoidan (DMI); FH, 0.6% fucoidan (DMI). Data are denoted as mean ± SEM ($n = 3$). ^{a,b,c}Demonstrate a statistically significant distinction, $P < 0.05$.

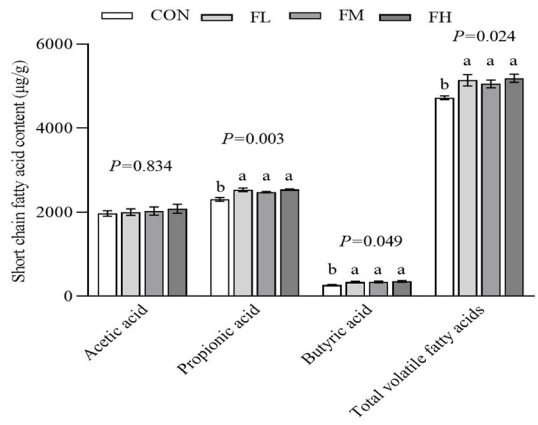

FIGURE 6
The effects of fucoidan on cecum SCFA in weaned lambs. CON, control; FL, 0.1% fucoidan (DMI); FM, 0.3% fucoidan (DMI); FH, 0.6% fucoidan (DMI). Data are denoted as mean ± SEM ($n = 3$). [a,b]Demonstrate a statistically significant distinction, $P < 0.05$.

shedding in the CON (the long black arrows show the shedding of mucosal epithelial cells; Figure 3A). In contrast to the control group, fucoidan-fed lambs had increased numbers of colon goblet cells (the red arrowheads show the goblet cells; Figure 3B), increased ratio of villous height to crypt depth (Figure 3C), and increased thickness of the colon muscular walls, especially in the 0.3∼0.6% fucoidan groups (Figures 3A,C; $P < 0.05$).

The effects of fucoidan on colon antioxidant in weaned lambs

To determine how fucoidan affects the colon antioxidant system, antioxidant enzyme activities were measured (Figure 4). The T-AOC and the activities of GSH-Px and SOD were higher in the fucoidan-treated groups than in the CON group ($P < 0.05$), particularly the FM group. The CAT activity of the FM group was significantly higher than in the CON group ($P = 0.005$). Meanwhile, the MDA content of the CON group was greater than those of the groups that were fed fucoidan ($P = 0.001$).

The effects of fucoidan on colon immunity in weaned lambs

The gut is the largest immune organ in the body. We evaluated the colon immunoglobulin and cytokine contents through ELISA (Figure 5). Compared with the CON and FL groups, the contents of IgA and IgG in the FM group were higher ($P < 0.05$). The SIgA contents of the fucoidan-fed groups were considerably greater ($P < 0.05$) compared to controls, particularly the FM and FH groups. The CON and FL groups had more IgM than the FM and FH groups ($P < 0.05$). The CON group also had a greater proinflammatory factor content than the fucoidan-fed groups ($P < 0.05$), especially the FM and FH groups. Although the IL-10 concentration in the CON group was greater compared to the fucoidan-fed groups ($P < 0.05$), there was no obvious dose-dependent effect ($P > 0.05$).

The effects of fucoidan on cecum SCFA content in weaned lambs

The content and composition of cecum SCFA were determined using gas chromatography-mass spectrometry (Figure 6). The fucoidan-fed groups had greater levels of propionic acid, butyric acid, and total volatile fatty acids than the CON group ($P < 0.05$), but there was no obvious dose-dependent impact ($P > 0.05$).

The effects of fucoidan on gut bacterial composition in weaned lambs

By sequencing the bacterial 16S rDNA V3 + V4 region, the microbiota of the cecal contents in the four groups of weaned lambs were examined. High-throughput sequencing was conducted on three random cecum samples from each group. 18 samples produced a total of 956,804 clean reads, with 79,734 clean reads on average ($n = 3$). These sequences were assigned to 28 phyla, 52 classes, 116 orders, 216 families, 459 genera, and 528 species based on a 97% similarity definition of an operational taxonomic unit (OTU).

The quantity of common and unique OTUs amongst the four groups is summarized in the Venn diagram of the OTUs (Figure 7A). In the four treatment groups, given fucoidan at 0–0.6%, the number of unique OTUs was 4, 4, 18, and 6, respectively, showing a trend of first increasing and then decreasing. This is consistent with trends in the total OTUs.

As shown in Figure 7B, the curve began to plateau when the number of effective sequences reached 10,000. This implies that the quantity of sequencing did not result in an increase in the cecum microbiota and that there was sufficient coverage of almost all microbiota species in all samples, which indicates robust microbiological data. Moreover, the rank abundance curves displayed a smooth trend. A flatter slope indicates a more homogeneous distribution of species (Figure 7C). The abscissa span increased first and then decreased as the concentration of fucoidan was increased, and this trend showed that the species abundances increased first and then decreased as the fucoidan dose increased.

The Ace, Chao, Simpson, and Shannon indexes were used to determine the diversity and richness of the microbial

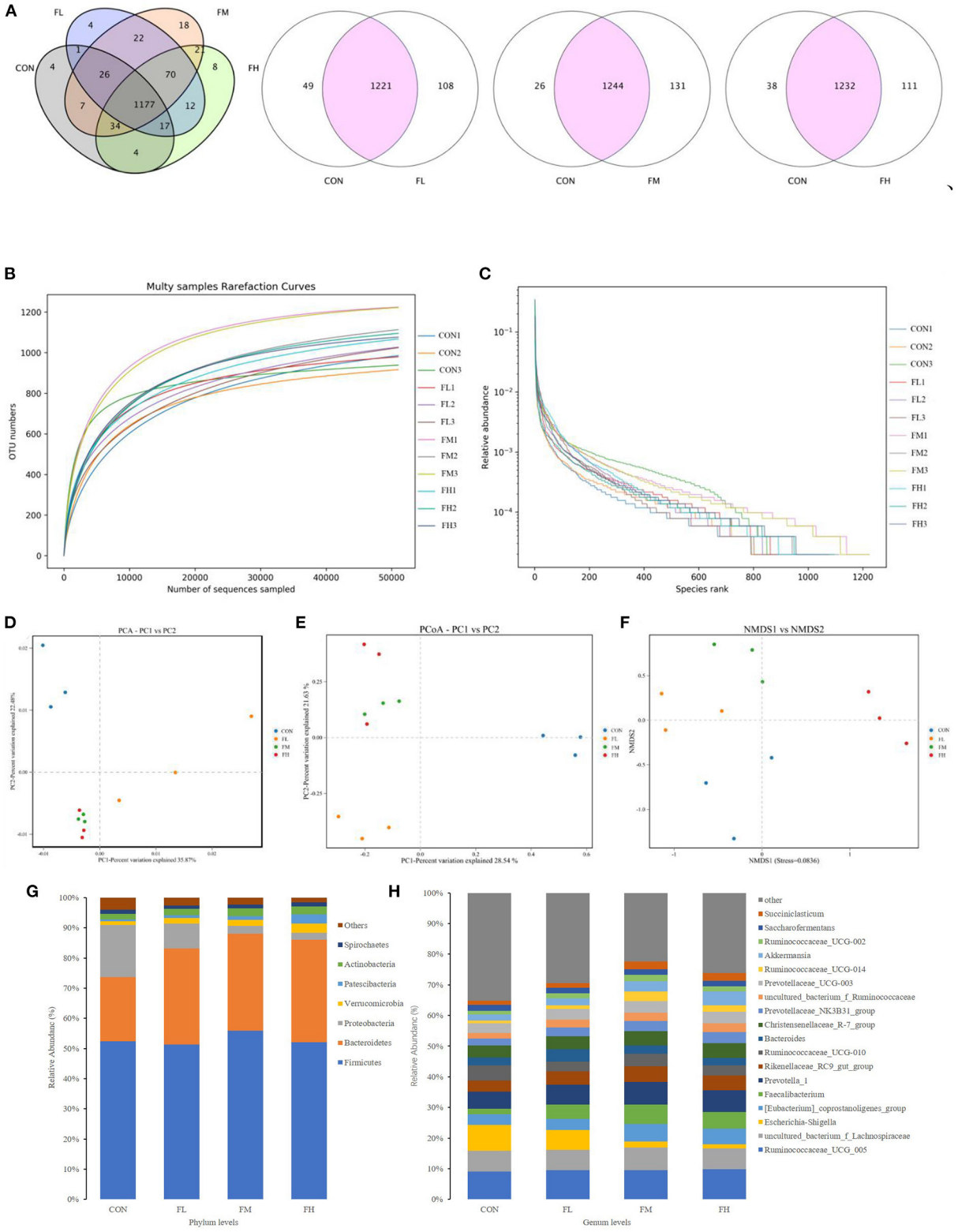

FIGURE 7
Effects of fucoidan on the cecal microbiota of weaned lambs ($n = 3$). **(A)** Venn diagram. **(B,C)** Correlation curves of species diversity. Multi-sample rarefaction curves **(B)** and the Rank-Abundance curves **(C)**. **(D–F)** Bate diversity analysis. **(D)** MetaStats analysis-based PCA analysis; **(E)** PCoA analysis using weighted UniFrac distances; **(F)** NMDS analysis. **(G,H)** Histograms showing the abundance of microbiota in the cecum at the phylum **(left)** and genus **(right)** levels. CON, control; FL, 0.1% fucoidan (DMI); FM, 0.3% fucoidan (DMI); FH, 0.6% fucoidan (DMI).

TABLE 2 The effects of fucoidan on the microbial alpha diversity index in the ceca of weaned lambs.

Items	Fucoidan levels				SEM	*P*-value
	CON	FL	FM	FH		
ACE	1082.24	1140.47	1099.44	1239.05	25.746	0.113
Chao1	1096.44	1144.36	1184.35	1244.07	21.763	0.072
Simpson	0.84^{b}	0.95^{a}	0.97^{a}	0.95^{a}	0.018	0.027
Shannon	4.95^{b}	6.82^{a}	7.15^{a}	7.21^{a}	0.339	0.023
Coverage/%	99.70	99.68	99.73	99.77	0.023	0.555

^{a,b,c}Demonstrate a statistically significant distinction, $P < 0.05$.

TABLE 3 The effects of fucoidan on the major bacterial phyla in the compositions of the ceca (average relative abundance ≥1% in at least one group) (%).

Items	Fucoidan levels				SEM	*P*-value
	CON	FL	FM	FH		
Firmicutes	52.35	51.25	55.86	52.05	1.131	0.546
Bacteroidetes	21.24^{b}	31.91^{a}	32.17^{a}	33.96^{a}	1.762	0.011
Proteobacteria	17.39^{a}	8.29^{b}	2.62^{c}	2.31^{c}	2.509	0.009
Actinobacteria	1.88^{b}	2.01^{b}	2.59^{a}	2.69^{a}	1.300	0.025
Verrucomicrobia	1.12	1.81	1.90	3.07	0.457	0.567
Patescibacteria	0.69	0.95	1.30	3.01	0.374	0.090
Spirochaetes	1.35	1.10	1.24	1.35	0.101	0.844

^{a,b,c}Demonstrate a statistically significant distinction, $P < 0.05$.

communities in cecal samples (Table 2). Chao1 increased significantly with increasing fucoidan levels ($P = 0.072$). Fucoidan-fed groups also considerably outperformed the CON group in terms of Shannon and Simpson diversity indices ($P < 0.05$), but no other discrepancies were observed (fucoidan groups not shown). The gut microbial alpha-diversity of the weaned lambs was improved by supplementation with fucoidan, especially in the FM group.

To pinpoint the main microorganisms that significantly influenced the PCA findings, we computed the PCA values. The first three (PC1, PC2, and PC3) components accounted for 35.87, 22.48, and 7.95%, respectively, and the cumulative contribution rate reached 66.30% ($P < 0.03$) (Figure 7D). Compared with the control group, fucoidan-fed groups were clustered more closely together and had greater similarities. However, the dissimilarity in cecal microbial communities of the FL group was greater compared with the other groups. This suggests that fucoidan may introduce dramatic changes to the overall structure of the gut microbial community, but no dose effects were found. The PCoA was very consistent with the results of the PCA (Figure 7E). Non-metric multidimensional scaling (NMDS) analysis also demonstrated that the gut flora of the samples differed. NMDS analysis indicated that the microbiome composition among the four groups was considerably different (Stress = 0.0836) (Figure 7F). Collectively, these results showed that the fucoidan treatments affected the diversity and composition of the cecal microbiota of the weaned lambs.

Fucoidan added to milk replacers enhanced the relative abundance of Bacteroidetes and Actinobacteria in the cecum ($P = 0.011$ and 0.025, respectively) while decreasing the relative richness of Proteobacteria ($P = 0.009$) (Table 3 and Figure 7G). Fucoidan also raised the relative abundance of Rikenellaceae_RC9_gut_group, Prevotella_1, and Faecalibacterium in the cecum ($P < 0.05$), while decreasing the relative abundance of Escherichia-Shigella ($P < 0.001$). In this investigation, adding milk replacer supplemented with 0.3–0.6% fucoidan led to an increase in the relative abundance of Ruminococcaceae_UCG-014, Succiniclasticum, and Akkermansia in the cecum ($P < 0.05$) (Table 4 and Figure 7H).

Discussion

Previous studies on lambs with non-specific pathogenic diarrhea found that body weight and respiratory frequency of diarrhea were significantly reduced after weaning compared to before weaning, and the serum cytokines IL-4, IL-6, and IL-8 were significantly higher than in healthy lambs (26). Hence,

TABLE 4 The effects of fucoidan on the major bacterial genera in the compositions of the ceca (average relative abundance ≥1% in at least one group) (%).

Items	Fucoidan levels				SEM	*P*-value
	CON	FL	FM	FH		
Ruminococcaceae_UCG-005	8.59	9.17	9.22	9.46	0.290	0.807
Uncultured_bacterium_f_Lachnospiraceae	6.33	6.46	7.17	6.62	0.365	0.893
Escherichia-Shigella	7.87^{a}	6.14^{b}	1.87^{c}	1.30^{c}	0.859	<0.001
(Eubacterium)_coprostanoligenes_group	3.32	3.50	5.62	4.91	0.382	0.063
Faecalibacterium	1.71^{b}	4.51^{a}	6.16^{a}	5.28^{a}	0.552	0.002
Prevotella_1	5.26^{c}	6.26^{b}	7.06^{a}	6.91^{a}	0.231	0.001
Rikenellaceae_RC9_gut_group	3.42^{b}	4.25^{a}	4.96^{a}	4.69^{a}	0.198	0.004
Ruminococcaceae_UCG-010	4.59	3.02	3.96	3.18	0.291	0.196
Bacteroides	2.46	3.85	2.72	2.38	0.253	0.130
Christensenellaceae_R-7_group	3.64	4.09	4.44	4.56	0.145	0.077
Prevotellaceae_NK3B31_group	2.09	2.80	3.29	3.51	0.213	0.057
Uncultured_bacterium_f_Ruminococcaceae	1.78	2.48	2.57	2.79	0.152	0.073
Prevotellaceae_UCG-003	2.98	3.32	3.64	3.65	0.112	0.081
Ruminococcaceae_UCG-014	0.87^{c}	1.22^{c}	3.06^{a}	2.05^{b}	0.273	0.001
Akkermansia	1.81^{c}	2.09^{c}	3.23^{b}	4.40^{a}	0.341	0.002
Ruminococcaceae_UCG-002	1.12	1.65	2.06	1.61	0.152	0.177
Saccharofermentans	1.86	1.77	1.74	1.71	0.024	0.116
Succiniclasticum	1.22^{b}	1.33^{b}	2.45^{a}	2.52^{a}	0.191	<0.001

^{a,b,c}Demonstrate a statistically significant distinction, $P < 0.05$.

diarrhea after weaning can easily induce an inflammatory response in lambs. This is in line with the findings of other investigations (20). Our findings demonstrated that fucoidan improved the PWD of weaned lambs. Specifically, 0.3∼0.6% fucoidan has an obvious inhibitory effect on diarrhea in early-weaned lambs, with the diarrhea rate reduced by more than 50%. Fucoidan is composed of fucose and sulfate groups, which are responsible for a variety of biological effects (11–18). Hence, we speculate that there are three possible reasons why fucoidan reduces the rate of diarrhea in lambs. First, due to the action of the esophageal groove, plant polysaccharide that has not been degraded by the rumen microorganisms flows through the hindgut, which stimulates the secretion of mucous substances by intestinal cells. For example, the secretion of digestive enzymes in the small intestine helps lambs digest (27) and absorb nutrients and the goblet cells in the colon secrete mucin (28, 29). This can prevent bacteria from directly contacting the intestinal epithelial cells and reduce the possibility of non-infectious diarrhea (30). Second, fucoidan increases the activity and secretion of intestinal antioxidant enzymes, which scavenge excessive intestinal free radicals and prevent free radical-related disorders of metabolism (31, 32). Studies have shown that fucoidan's antioxidant properties depend on the composition of its monosaccharides, the location and quantity of its sulfate groups, as well as its molecular weight (33–35). Additionally, there are three main mechanisms explaining its mode of action. ① Reactive oxygen species (ROS) are captured by lipid peroxidation, combining with hydroxyl ions on the hydrocarbon chain of the polysaccharide to create water molecules. Peroxy radicals are further oxidized to form compounds that the body can tolerate by the reaction of single-electron carbon atoms. In addition, single-electron carbon atoms are further oxidized into peroxy radicals, which are then transformed into biocompatible compounds (36). ② the -OH from polysaccharide rings complexes with metal ions to generate free radicals and indirectly scavenges ROS (37). ③ Conventional antioxidant functions involve increasing the activities of antioxidant enzymes, such as SOD and GSH-Px, and terminating free radical chain reactions (38). Meanwhile, fucoidan promotes the maturation of intestinal mucosal immunity and reduces chronic intestinal inflammation and repeated immune responses, thus reducing intestinal tissue damage (14). Finally, fucoidan promotes the proliferation and attachment of beneficial bacteria and forms a microorganism barrier, thereby inhibiting the colonization and reproduction of some pathogenic bacteria (39, 40). Therefore, this study further elaborates on the relieving effect of fucoidan on diarrhea in early-weaned lambs from the aspects of colon tissue morphology, antioxidant enzymes, cytokines, and cecal microbes.

The colon consists of the mucosa, submucosa, muscularis, and serosa. Important indexes for assessing the colon's absorption capacity are the villus height, crypt depth, and

villus height/crypt depth ratio. Goblet cells are one of the intestinal mucosal epithelial cells. The number of goblet cells gradually increases from the small intestine to the large intestine. The mucus secreted by these goblet cells has lubricating and protective effects on the intestine, participates in intestinal mucosal immunity and intestinal injury surface reconstruction, and maintains the integrity of the mucosal barrier (41). According to our findings, the colonic mucosas in the fucoidan groups were thicker and the mucosal epithelium was more intact compared to the group that did not get fucoidan (Figure 3A). In the lamb colon, it was discovered that 0.3–0.6% fucoidan increased the number of goblet cells, villus height, and mucosal thickness. Additionally, the ratio of villus height to crypt depth and muscle layer thickness both significantly increased (Figures 3B,C), suggesting that fucoidan can treat intestinal health issues brought on by weaning stress. This is another plausible explanation for the decreased rate of diarrhea.

Both non-infectious and infectious diarrhea are sources of harmful stimuli, prompting intestinal tissue to produce a large amount of ROS and their metabolites, leading to a redox imbalance in the body. This will exacerbate potential hazards such as lipid peroxidation, DNA damage, and inflammatory cytokine overexpression, inducing the apoptosis of mucosal cells. Chronic inflammation can also lead to metabolic disorders of the body and cause diseases (42–44). Therefore, the increase in lipid oxidation products and immune activity may explain the increased diarrhea rate and colonic mucosal damage in early-weaned lambs. Our results showed that feeding fucoidan increased T-AOC and the activities of SOD and GSH-Px in the colon, and decreased MDA content. Fucoidan may also stimulate the Nrf2/ARE signaling pathway in the small intestine to improve its cytoprotective effects (16, 45). Hence, fucoidan promotes the synthesis and secretion of antioxidant enzymes, effectively removes excessive ROS, and relieves the damage to tissues caused by oxidative stress (46, 47). In contrast, feeding 0.3–0.6% fucoidan lowered colonic IgM content in comparison to the control group, which was compatible with serum immunity results (20). Meanwhile, fucoidan treatment decreased the content of colonic pro-inflammatory factors (IL-1β, TNF-α, and INF-γ) and increased the content of the anti-inflammatory factor IL-10, compared with the control group without fucoidan. In particular, feeding 0.3% fucoidan increased colonic IgA, IgG, and SIgA contents. This is in line with findings by Walsh et al. (48) and Aikahtane et al. (49). The changes in these immunoglobulin contents indicated that the weaned lambs had been recently infected by pathogenic bacteria, resulting in the increased immune response. Many studies have shown that fucoidan maintains the intestinal immune balance by regulating the ratio of Th1/Th2 and the expression of immunoglobulins in intestinal helper T-cells (14, 16, 18). By activating the NF-κB, MAPK, and AP-1 signaling pathways, fucoidan induces the production of NO, TNF-α, IL-1β, and other cytokines to regulate intestinal immunity (50, 51). Similarly, Liying et al. (52) showed that the lipopolysaccharide-induced production of TNF-α and IL-1β in RAW 264.7 macrophages was significantly inhibited by fucoidan extracted from *Laminaria japonica* (without sulfate groups at the C-4 and C-2 sites), resulting in less cell death. In short, fucoidan increased the levels of intestinal antibodies in early-weaned lambs, improved the integrity of the intestinal barrier, and alleviated weaning stress and intestinal inflammation. However, most studies that have hinted at the mechanisms by which fucoidan affects the immune system have been unable to explain how its unusual structure and chemical make-up serve a specific functional purpose (53, 54). In the future, our research team plans to investigate the role of fucoidan in the immune system and antioxidant defenses of goat intestinal epithelial cells.

Prior to weaning, the gastrointestinal tract (GIT) of young ruminants plays a vital role in nutrient absorption, similar to piglets. However, symptoms of weaning stress are often accompanied by disorders of the gut microbiota (55, 56). The GIT microbiota is an important "invisible organ" in the body (57). Antagonism is a crucial mechanism for maintaining the microecological balance in the gut, including attachment space and/or nutrients, producing antibacterial substances, and induced immunity (56). Our 16S rDNA gene sequencing results found that the Simpson and ACE indexes were significantly increased in the fucoidan-fed groups compared with the control group. Therefore, the addition of fucoidan in milk substitute increased the bacterial richness and diversity of the colon in weaning lambs. The diversity of microorganisms helps to maintain the stability and resistance of the intestinal ecosystem, which in turn assists in the maintenance of intestinal health and reduces the risk of disease (58). Firmicutes, Proteobacteria, Bacteroidetes, and Actinobacteria were the dominant bacterial phyla. Fucoidan (0.3∼0.6%) supplementation significantly increased the relative abundance of Bacteroidetes and Actinobacteria, but decreased that of Proteobacteria. Furthermore, there was a significant increase in the relative abundances of 9 genera (Firmicutes: Romboutsia, Ruminococcus_1, Ruminococcaceae_NK4A214_group, Ruminocaceae_UCG-014, Faecalibacterium, and Succiniclasticum; Bacteroidetes: Prevotella_1, Rikenellaceae_RC9_gut_group, and Prevotellaceae). These genera selectively promote the degradation of saccharides (59, 60) and crude fiber (61). By reducing intestinal pH and thus limiting the colonization of harmful bacteria that are sensitive to low pH, organic acids created by the fermentation of feed can prevent pathogenic infection (62). Meanwhile, in the gut, SCFA acts as a signal molecule that regulates a versatile class of intestinal immune cells (63, 64). Crucially, SCFA can maintain the metabolic equilibrium of colon-forming cells and protect them from outside harm (65). Our results demonstrated that cecal propionic acid, butyric acid, and total volatile fatty acid content were enhanced by fucoidan feeding. Fucoidan increased the abundance of SCFA-producing bacteria in the gut, thereby promoting the production of SCFA in the cecum, in line

with the findings of Xue et al. (39). In addition, fucoidan reduced the abundance of Proteobacteria phyla, especially Escherichia-Shigella abundance. According to Liu Mengjian et al. (21) and Liu et al. (66), low molecular-weight fucoidan improves the composition and diversity of the gut microbiota in weaned lambs (39). In particular, fucoidan efficiently inhibits Escherichia-Shigella growth (66). Fucoidan also ameliorates intestinal damage in weaned lambs by boosting neutrophil and macrophage numbers (67) and lowering inflammatory cytokine expression (68). This could also partly explain the above-mentioned results of the decreased fecal index, diarrhea rate, and cytokine content in the colons of lambs fed fucoidan in the current study. The relative abundance of Succiniclasticum and Akkermansia in the cecum was also raised by adding milk replacer supplemented with 0.3–0.6% fucoidan. The relative abundance of Succiniclasticum and Akkermansia in the cecum is much lower than that of other bacteria, but the steady-state effect on the gastrointestinal environment is far greater than the numerical difference (69–72).

Conclusion

Collectively, the results presented herein suggest that milk replacement supplementation with 0.3–0.6% (DMI) fucoidan could decrease diarrhea rates. Fucoidan may be closely linked with improvements in the colon antioxidant capacity and immunity, as well as the gut microbiota community composition in weaned lambs. The results of this study provide a scientific basis for the application of fucoidan as a feed additive for protecting intestinal health and performance in weaned lambs.

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 authors.

Ethics statement

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

Author contributions

FY and GG: conceptualization. GG: data curation and editing the manuscript. FY, RL, and HY: methodology and writing—review and editing. ZW and CF: software. WY: investigation. FY, SG, and ZG: resources. FY: project administration and funding acquisition. All authors have read and agreed to the published version of the manuscript.

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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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Effects of tannic acid on growth performance, relative organ weight, antioxidative status, and intestinal histomorphology in broilers exposed to aflatoxin B_1

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A total of 480 one-day-old AA broiler chicks were randomly allocated to one of four treatments in a 2 × 2 factorial to investigate the effects of tannic acid (TA) on growth performance, relative organ weight, antioxidant capacity, and intestinal health in broilers dietary exposed to aflatoxin B_1 (AFB_1). Treatments were as follows: (1) CON, control diet; (2) TA, CON + 250 mg/kg TA; (3) AFB_1, CON + 500 μg/kg AFB_1; and (4) TA+AFB_1, CON + 250 mg/kg TA + 500 μg/kg AFB_1. There were 10 replicate pens with 12 broilers per replicate. Dietary AFB_1 challenge increased the feed conversion ratio during days 1 to 21 ($P < 0.05$). The TA in the diet did not show significant effects on the growth performance of broilers during the whole experiment period ($P > 0.05$). The liver and kidney relative weight was increased in the AF challenge groups compared with the CON ($P < 0.05$). The addition of TA could alleviate the relative weight increase of liver and kidney caused by AFB_1 ($P < 0.05$). Broilers fed the AFB_1 diets had lower activity of glutathione peroxidase, catalase, total superoxide dismutase, S-transferase, and total antioxidant capacity in plasma, liver and jejunum, and greater malondialdehyde content ($P < 0.05$). Dietary supplemented with 250 mg/kg TA increased the activities of antioxidative enzymes, and decreased malondialdehyde content ($P < 0.05$). In addition, AFB_1 significantly reduced the villus height and crypt depth ratio in the ileum on day 42 ($P < 0.05$). In conclusion, supplementation with 250 mg/kg TA could partially protect the antioxidant capacity and prevent the enlargement of liver in broilers dietary challenged with 500 μg/kg AFB_1.

KEYWORDS

Aflatoxin B1, antioxidant capacity, broiler, growth performance, intestinal health, tannic acid

Introduction

Aflatoxins are mainly produced by *Aspergillus flavus* and *Aspergillus parasiticus*, and widely exist in food and feed that are frequently caused health and economic problems in many countries (1). Among the 18 types of aflatoxin derivatives, aflatoxin B_1 (AFB_1) is the most common and toxic in the poultry feed industry (2). Poultry is extremely sensitive to AFB_1, and long-term exposure to AFB_1 may cause growth retardation, immunosuppression, hepatotoxic, and even death (3–5). Oxidative stress has been reported to play a significant role in the toxicity mechanism caused by AFB_1 (6, 7). FDA (8) refines the maximum concentration of aflatoxin in poultry is 100 μg/kg of feed, whereas 500 μg/kg can be a practical testing concentration in feedstuff in the USA.

Chinese gallnut tannic acid (TA) belongs to the hydrolyzed tannin family, and is a polyphenolic compound of high molecular weight (500–3,000 Da), which can remove free radicals and prevent lipid oxidation (9). Because of the polyphenolic hydroxyl structure, the TA has various biological activities, such as antimicrobial, anti-inflammatory, anticancer, and immunomodulatory effects (10–12). Moreover, studies have shown that dietary supplementation with antioxidants, including plant extracts and tannins can protect broilers from AFB_1-induced toxicity by enhancing the antioxidant capacity and immunity (6, 13–16). Nevertheless, it remains unclear whether dietary supplementation with TA could alleviate acute aflatoxicosis by improving the antioxidant capacity of broilers fed AFB_1 contaminated diets.

Therefore, the aim of this study was to determine the effects of the TA on growth performance, antioxidative status, and intestinal histomorphology of broilers exposed to feed contaminated with 500 μg/kg AFB_1.

Materials and methods

All animal procedures used in this study were performed in the experimental farm of Wuhan Polytechnic University, and were approved by the Institutional Animal Care and Use Committee of Wuhan Polytechnic University (Number: 20161121).

AFB_1 and TA

The AFB_1 (purity ≥98%, HPLC) was produced from *Aspergillus flavus* provided by Qingdao Pribolab Biological Engineering Company Limited (Shandong, China), and the AFB_1 concentration in the feed was designed to 500 μg/kg in AFB_1 treatments. Dietary AFB_1 concentrations were confirmed by analysis (17). Briefly, feed samples were extracted with acetonitrile:water (86:14), and an aliquot of the extract was passed through a puriTox TC-M160 cleanup column (Trilogy Analytical Laboratory Inc., Washington, MO, USA) and suitably diluted with water before analysis using HPLC with Kobra cell postcolumn derivatization with fluorescence detection at 365 nm excitation and 440 nm emission.

The hydrolysable TA was extracted from Chinese gallnut by the Wufeng Chicheng Biotechnology Company Limited (Yichang, China), which contained ≥80% tannin, crude fiber <2.00%, ash <2.50%, and moisture <8.00%.

Dietary treatments and animal management

A 2 × 2 factorial complete randomized block design was employed and 480 one-day-old sex-mixed AA broilers were randomly assigned to 4 treatment groups, each with 10 replicates of 12 birds per pen. Experimental diets were as follows: (1) CON, basal diet; (2) TA, CON + 250 mg/kg A; (3) AFB_1, CON + 500 μg/kg TA; and (4) TA+AFB_1, CON + 250 mg/kg TA + 500 μg/kg AFB_1. The basal diet was formulated to meet or exceed the nutrient requirements of AA broilers. Diets were fed in 2 phases: phase 1 (from days 1 to 21) and phase 2 (from days 22 to 42). The composition and nutrient levels of the basal diets are presented in Table 1.

All broiler chicks were reared in stainless steel pens (1.4 m × 1.4 m) in an environmentally controlled room at the Animal Research Center of Wuhan Polytechnic University and given *ad libitum* access to diets and water throughout the study. The room temperature was maintained at 33 ± 2°C for the first week and then gradually decreased to 24°C until the end of the experiment, and broilers were maintained on a 23 h constant light and 1 h darkness every day throughout the whole trial.

Growth performance

Broilers and feed were weighed on the beginning, days 21 and 42 of the trial, and calculated the average daily gain (ADG), average daily feed intake (ADFI), and feed conversion ratio (FCR).

Sample collection

On days 21 and 42, two broilers from each replicate (20 broilers per group) were randomly selected and blood samples were aseptically collected from the wing vein into vacuum blood vessels. Plasma was obtained by centrifuging (3,000 × g for 15 min at 4°C) the whole blood and stored at −20°C for the assay of antioxidative parameters.

Then, the same broilers were weighed individually and euthanized by cervical dislocation. The liver, spleen, bursa

TABLE 1 Composition of experimental diets (as-fed basal).

Ingredients (%)	Days 1–21	Days 22–42
Corn	51.45	51.49
Soybean meal	40.73	37.40
Soybean oil	3.36	7.18
Dicalcium phosphate	1.92	1.64
Limestone	1.16	1.06
Trace mineral premix[a]	0.20	0.20
Vitamin premix[b]	0.04	0.03
Sodium chloride	0.35	0.31
l-Lysine (99%)	0.28	0.22
dl-methionine (98%)	0.26	0.32
Choline chloride	0.25	0.25
Calculated composition		
ME (MJ/kg)	12.55	13.18
Analyzed composition		
Crude protein (%)	21.50	20.50
Lys (%)	1.30	1.20
Met + Cys (%)	0.90	0.70
Thr (%)	0.82	0.74
Calcium (%)	1.00	0.90
Available phosphorus (%)	0.45	0.40

[a] Provided per kg of complete diet: 10 mg Mn ($MnSO_4$), 80 mg Zn ($ZnSO_4$), 5 mg Cu ($CuSO_4$), 0.5 mg I ($Ca(IO_3)_2$), and 0.3 mg Se (Na_2SeO_3).
[b] Provided per kg of complete diet: 10,000 IU vitamin A (transretinyl acetate), 3,000 IU vitamin D_3 (cholecalciferol), 30 IU vitamin E (all-rac-α-tocopherol acetate), 2.4 mg menadione, 6.0 mg riboflavin, 2.5 mg pyridoxine HCl, 13 mg calcium pantothenate, 23.5 mg niacin, and 0.04 mg biotin.

of Fabricius, thymus, and kidney were removed cleaned of the adhering tissue by trained personnel and weighed. Relative organ weights were calculated as follows: Relative weight = (Organ weight)/(Final body weight) × 1,000. The small intestine was removed and gently cleaned with ice-cold saline. Intestinal segments (1–2 cm) taken from the mid-region of the duodenum, jejunum, and ileum were immediately fixed in 4% paraformaldehyde for the examination of morphological parameters. Additionally, the portion of liver and jejunum were sampled and stored at −80°C for analysis of antioxidant status.

Antioxidative status

Approximately 1 g of liver or jejunum was homogenized in 10 mL of ice-cold saline and centrifuged at 2,500 × g, 4°C for 10 min. The supernatants were collected for further analysis. The activities of glutathione peroxidase (GSH-Px), total superoxide dismutase (T-SOD), total antioxidant capacity (T-AOC), glutathione S-transferase (GST), catalase (CAT), and the content of malondialdehyde (MDA) in the plasma and supernatants were measured using purchased assay kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China), according to the instructions of the manufacturer (18).

Intestinal histomorphology

The intestinal histomorphology was measured as described by Guo et al. (19). Briefly, the fixed intestinal segments were embedded in paraffin. Consecutive sections (5 μm) were stained with hematoxylin and eosin and were observed for histomorphological examination. The measurements were performed with an Olympus optical microscope using ProgRes CapturePro software (Jenoptik, Jena, Germany). The villus height and crypt depth were measured from 10 randomly selected villi and associated crypts on each section at 40× magnification. Villus height was measured from the tip of the villus to the crypt opening and crypt depth was measured from the base of the crypt to the level of the crypt opening. The villus height to crypt depth ratio (V/C) was then calculated from these measurements.

Statistical analyses

All experiment data were analyzed by a two-way ANOVA analysis using the GLM procedure of SPSS 26.0 software. In cases where the differences were significant, the means were compared by Duncan's multiple range test. The results are shown as mean and the standard error of mean (SEM). Significance was considered at $P < 0.05$, and $0.05 \leq P < 0.10$ was considered to have a trend of difference.

Results

Dietary analyses of AFB_1

Biochemical tests indicated that the CON and TA diets were negative for AFB1 throughout the experiment. The analyzed concentration of AFB1 in AFB1 and AFB1+TA diets were 505.9 vs. 503.2 μg/kg during days 1 to 21, and 520.3 vs. 521.3 μg/kg during days 22–42, respectively.

Growth performance

As shown in Table 2, AFB_1 challenge increased the FCR during days 1–21 ($P < 0.05$). The addition of TA in the diet did not show significant effects on the ADG, ADFI, and FCR of broilers during the whole experiment period ($P > 0.05$). No interaction effect was observed between AFB_1 and TA on the growth performance ($P > 0.05$).

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TABLE 2 Effects of tannic acid on growth performance of broilers challenged with AFB_1[a].

Items	CON	TA	AFB_1	TA + AFB_1	SEM	*P*-value		
						AFB_1	TA	AFB_1 × TA
Days 1–21								
ADG (g)	32.92	32.76	33.41	32.30	0.23	0.968	0.195	0.325
ADFI (g)	46.34	46.20	47.57	46.55	0.31	0.227	0.371	0.497
FCR	1.41	1.41	1.42	1.44	0.01	0.022	0.322	0.444
Days 22–42								
ADG (g)	63.63	61.89	62.27	61.19	0.68	0.474	0.328	0.817
ADFI (g)	114.11	112.70	114.67	112.12	1.05	0.997	0.387	0.802
FCR	1.80	1.82	1.84	1.83	0.02	0.454	0.801	0.637
Days 1–42								
ADG (g)	49.06	47.68	48.72	47.51	0.42	0.774	0.153	0.923
ADFI (g)	81.33	79.59	81.30	80.44	0.60	0.751	0.323	0.733
FCR	1.66	1.67	1.67	1.69	0.01	0.475	0.460	0.757

[a] Each mean represents 10 replications with 12 broilers per replication. CON, control diet; AFB_1, 500 μg/kg aflatoxin B_1 of feed; TA, 250 mg/kg tannic acid of feed; TA + AFB_1, 250 mg/kg TA + 500 μg/kg AFB_1.

TABLE 3 Effects of tannic acid on relative organ weight of broilers challenged with AFB_1[a].

Items (g/kg)	CON	TA	AFB_1	TA + AFB_1	SEM	*P*-value		
						AFB_1	TA	AFB_1 × TA
Day 21								
Liver	20.00[b]	20.54[b]	23.67[a]	20.90[b]	0.28	<0.001	0.002	<0.001
Spleen	0.78	0.76	0.92	0.84	0.03	0.049	0.356	0.608
Bursa of Fabricius	2.89	2.93	3.10	2.98	0.07	0.391	0.781	0.596
Thymus	3.51	3.70	3.38	3.45	0.11	0.394	0.553	0.795
Kidney	7.75[b]	7.98[b]	9.27[a]	7.96[b]	0.13	<0.001	<0.001	<0.001
Day 42								
Liver	18.20[b]	18.41[b]	22.18[a]	18.91[a]	0.33	<0.001	0.001	<0.001
Spleen	0.93	1.18	1.22	1.09	0.06	0.376	0.592	0.117
Bursa of Fabricius	2.39	2.26	2.31	2.47	0.11	0.781	0.955	0.529
Thymus	3.06	3.46	3.44	3.27	0.13	0.716	0.670	0.302
Kidney	5.78[b]	5.53[b]	7.23[a]	5.65[b]	0.13	<0.001	<0.001	<0.001

[a] Each mean represents 10 replications with 2 broilers per replication. CON, control diet; AFB_1, 500 μg/kg aflatoxin B_1 of feed; TA, 250 mg/kg tannic acid of feed; TA + AFB_1, 250 mg/kg TA + 500 μg/kg AFB_1.
[a,b,c]Means in the same row with no common superscripts differ significantly ($P < 0.05$).

Relative organ weight

As shown in Table 3, on days 21 and 42, AFB_1 and TA exhibited significant interactive effects on the relative weight of the liver and kidney in broilers ($P < 0.05$). The liver and kidney relative weight was increased in the AFB_1 treatments compared with the CON ($P < 0.05$), while supplementation with TA into AFB_1 contaminated diet decreased liver and kidney relative weight ($P < 0.05$). The relative weights of the spleen, bursa of Fabricius, and thymus were unaffected by AFB_1 challenge and TA treatment on days 21 and 42 ($P > 0.05$).

Intestinal histomorphology

As presented in Table 4, on day 42, AFB_1 challenge reduced the villus height and crypt depth ratio in the ileum ($P < 0.05$). The ileal villus height tended to decrease ($P = 0.079$), and the crypt depth of the jejunum tended to increase ($P = 0.082$) in AFB_1 treatments compared with non-contaminated diets. The TA did not show significant effects on the intestinal histomorphology of broilers ($P > 0.05$). However, the villus height ($P = 0.059$) and villus height/crypt depth ($P = 0.052$) ratio were tended to increase in TA treatments. No interaction

TABLE 4 Effects of tannic acid on intestinal histomorphology of broilers challenged with AFB_1[a].

Items[b]		CON	TA	AFB_1	TA + AFB_1	SEM	*P*-value		
							AFB_1	TA	AFB_1 × TA
Day 21									
Duodenum	VH (μm)	1144.03	1212.71	1014.64	1133.42	32.65	0.119	0.159	0.700
	CD (μm)	89.57	92.50	86.41	86.29	2.94	0.464	0.825	0.811
	V/C (μm/μm)	13.04	13.63	12.36	14.35	0.62	0.989	0.334	0.597
Jejunum	VH (μm)	814.13	883.12	715.60	861.82	28.00	0.281	0.059	0.485
	CD (μm)	65.81	68.59	68.64	66.80	1.35	0.856	0.869	0.426
	V/C (μm/μm)	12.33	12.87	10.44	12.83	0.38	0.195	0.052	0.209
Ileum	VH (μm)	668.47	696.48	597.15	628.85	18.96	0.079	0.440	0.962
	CD (μm)	80.93	74.12	76.11	85.60	2.89	0.583	0.825	0.184
	V/C (μm/μm)	8.90	9.57	8.34	7.90	0.32	0.093	0.859	0.396
Day 42									
Duodenum	VH (μm)	1417.00	1369.67	1261.61	1320.79	46.49	0.298	0.951	0.584
	CD (μm)	139.15	113.19	108.09	114.06	7.08	0.298	0.489	0.272
	V/C (μm/μm)	12.01	13.90	12.91	12.28	0.70	0.809	0.672	0.401
Jejunum	VH (μm)	962.97	915.73	896.66	940.08	27.25	0.715	0.973	0.431
	CD (μm)	93.60	98.79	99.91	116.26	3.47	0.082	0.114	0.404
	V/C (μm/μm)	9.45	9.87	9.72	8.73	0.31	0.501	0.654	0.277
Ileum	VH (μm)	687.38	655.45	630.79	600.11	25.53	0.298	0.558	0.991
	CD (μm)	80.06	81.49	87.07	90.84	2.50	0.116	0.612	0.818
	V/C (μm/μm)	8.90	8.41	7.61	6.96	0.31	0.028	0.346	0.895

[a] Each mean represents 10 replications with 2 broilers per replication. CON, control diet; AFB_1, 500 μg/kg aflatoxin B_1 of feed; TA, 250 mg/kg tannic acid of feed; TA + AFB_1, 250 mg/kg TA + 500 μg/kg AFB_1.
[b] CD, crypt depth; V/C, villus height and crypt depth ratio; VH, villus height.

TABLE 5 Effects of tannic acid on plasma antioxidant capacity of broilers challenged with AFB_1[a].

Items[b]	CON	TA	AFB_1	TA + AFB_1	SEM	*P*-value		
						AFB_1	TA	AFB_1 × TA
Day 21								
T-AOC (mmol/L)	0.52[a]	0.48[ab]	0.42[b]	0.49[ab]	0.01	0.103	0.723	0.034
CAT (U/mL)	3.35	3.60	2.64	2.85	0.12	0.002	0.304	0.936
GST (U/mL)	19.22	20.32	18.44	20.74	0.32	0.760	0.006	0.312
GSH-Px (U/mL)	1624.26	1698.25	1535.91	1550.99	20.09	0.002	0.222	0.417
T-SOD (U/mL)	105.51	105.02	99.98	103.11	1.25	0.147	0.603	0.475
MDA (nmol/mL)	4.22	4.05	4.52	4.27	0.10	0.198	0.293	0.865
Day 42								
T-AOC (mmol/L)	0.43	0.40	0.43	0.46	0.01	0.368	0.831	0.351
CAT (U/mL)	3.82	3.89	3.69	3.99	0.04	0.826	0.014	0.118
GST (U/mL)	20.85[a]	21.31[a]	17.29[b]	20.62[a]	0.37	0.001	0.002	0.015
GSH-Px (U/mL)	1782.43[a]	1888.16[a]	1559.46[b]	1894.93[a]	31.21	0.026	<0.001	0.019
T-SOD (U/mL)	108.19	107.23	105.91	110.43	1.60	0.889	0.591	0.409
MDA (nmol/mL)	3.34[b]	3.21[b]	3.92[a]	3.16[b]	0.07	0.030	0.001	0.011

[a] Each mean represents 10 replications with 2 broilers per replication. CON, control diet; AFB_1, 500 μg/kg aflatoxin B_1 of feed; TA, 250 mg/kg tannic acid of feed; TA + AFB_1, 250 mg/kg TA + 500 μg/kg AFB_1.
[b] T-SOD, total superoxide dismutase; GSH-Px, glutathione peroxidase; GST, glutathione S-transferase; T-AOC, total antioxidant capacity; MDA, malondialdehyde.
[a,b,c]Means in the same row with no common superscripts differ significantly ($P < 0.05$).

TABLE 6 Effects of tannic acid on liver antioxidant capacity of broilers challenged with AFB_1.

Items	CON	TA	AFB_1	TA + AFB_1	SEM	*P*-value		
						AFB_1	TA	AFB_1 × TA
Day 21								
T-AOC (nmol/mgprot)	207.24	235.86	184.09	180.90	5.96	<0.001	0.211	0.120
CAT (U/mgprot)	15.28	18.58	15.81	18.66	0.66	0.813	0.021	0.861
GST (U/mgprot)	23.35	26.88	16.69	20.32	0.84	<0.001	0.006	0.969
GSH-Px (U/mgprot)	63.38	69.81	31.68	44.88	3.11	<0.001	0.022	0.413
T-SOD (U/mgprot)	1692.04[b]	1885.59[a]	1443.62[c]	1413.18[c]	38.77	<0.001	0.103	0.027
MDA (nmol/mgprot)	1.71	1.65	1.73	1.58	0.06	0.864	0.404	0.730
Day 42								
T-AOC (nmol/mgprot)	143.34	147.58	125.06	126.11	3.78	0.008	0.713	0.824
CAT (U/mgprot)	19.32	18.96	19.49	19.84	0.60	0.676	0.998	0.778
GST (U/mgprot)	52.71	54.63	47.56	44.78	1.27	0.002	0.851	0.315
GSH-Px (U/mgprot)	58.87	56.95	51.96	60.32	1.51	0.552	0.282	0.090
T-SOD (U/mgprot)	1766.21	1723.15	1606.53	1802.90	37.69	0.595	0.310	0.117
MDA (nmol/mgprot)	2.01	1.86	2.00	1.70	0.09	0.642	0.202	0.674

[a]Each mean represents 10 replications with 2 broilers per replication. CON, control diet; AFB_1, 500 μg/kg aflatoxin B_1 of feed; TA, 250 mg/kg tannic acid of feed; TA + AFB_1, 250 mg/kg TA + 500 μg/kg AFB_1.
[b] T-SOD, total superoxide dismutase; GSH-Px, glutathione peroxidase; GST, glutathione S-transferase; T-AOC, total antioxidant capacity; MDA, malondialdehyde.
[a,b,c]Means in the same row with no common superscripts differ significantly ($P < 0.05$).

TABLE 7 Effects of tannic acid on jejunum antioxidant capacity of broilers challenged with AFB_1[a].

Items[b]	CON	TA	AFB_1	TA + AFB_1	SEM	*P*-value		
						AFB_1	TA	AFB_1 × TA
Day 21								
CAT (U/mgprot)	7.80	9.85	7.58	7.94	0.48	0.275	0.219	0.386
GST (U/mgprot)	21.87	23.25	21.08	21.06	0.35	0.033	0.321	0.308
GSH-Px (U/mgprot)	20.72	21.19	18.31	19.07	0.44	0.009	0.460	0.860
T-SOD (U/mgprot)	275.90	277.80	266.84	277.97	3.07	0.479	0.301	0.462
MDA (nmol/mgprot)	5.02	3.90	4.94	4.47	0.16	0.407	0.010	0.264
Day 42								
CAT (U/mgprot)	7.10	8.50	8.50	6.73	0.31	0.072	0.225	0.277
GST (U/mgprot)	24.99	27.75	24.47	27.75	0.88	0.883	0.094	0.883
GSH-Px (U/mgprot)	19.93	21.09	17.89	19.37	0.58	0.112	0.261	0.892
T-SOD (U/mgprot)	300.37	330.85	295.25	329.45	5.49	0.747	0.003	0.854
MDA (nmol/mgprot)	4.37	3.99	4.51	4.21	0.12	0.451	0.166	0.876

[a] Each mean represents 10 replications with 2 broilers per replication. CON, control diet; AFB_1, 500 μg/kg aflatoxin B_1 of feed; TA, 250 mg/kg tannic acid of feed; TA + AFB_1, 250 mg/kg TA + 500 μg/kg AFB_1.
[b] T-SOD, total superoxide dismutase; GSH-Px, glutathione peroxidase; GST, glutathione S-transferase; T-AOC, total antioxidant capacity; MDA, malondialdehyde.

was found between AFB_1 and TA in intestinal histomorphology ($P > 0.05$).

Antioxidant capacity

The results of the antioxidant capacity in the plasma are shown in Table 5, AFB_1 challenge decreased plasma CAT and GSH-Px activities on day 21 ($P < 0.05$). Compared with the diet without TA, TA supplementation increased CAT activity in plasma on day 42 ($P < 0.05$). The AFB_1 and TA exhibited interactive effects on the T-AOC, GST, GSH-Px, and MDA ($P < 0.05$). Compared with the CON, dietary expose to AFB_1 decreased the T-AOC, GSH-Px, and GST activities on days 21 and 42, and increased the MDA content on day 42, respectively ($P < 0.05$). The addition of TA to AFB_1 contaminated diet

significantly improved the CAT, GSH-Px, and, GST activities, and decreased the MDA content on day 42 ($P < 0.05$).

As presented in Table 6, AFB_1 challenge decreased the GST and T-AOC in the liver on days 21 and 42, as well as GSH-Px and T-SOD activity on day 21 ($P < 0.05$). Broilers fed the TA diet had greater hepatic CAT, GST, and GSH-Px activities on day 21 ($P < 0.05$). Furthermore, on day 21, AFB_1 and TA showed interactive effects on the T-SOD in the liver ($P < 0.05$).

In Table 7, AFB_1 challenge decreased the GST and GSH-Px activities in the jejunum on day 21 ($P < 0.05$). Dietary supplemented with TA increased the T-SOD activity in jejunum on day 42. The MDA content of jejunum was also decreased in the TA treatments compared with other treatments ($P < 0.05$).

Discussion

Dietary exposure to AFB_1 can cause tremendous economic losses by reducing growth performance, feed efficiency, and increasing mortality in the poultry industry (20–24). In our study, we found that the administration of 500 μg/kg AFB_1 diets increased FCR during days 1–21 in broilers. These results are in alignment with several studies, which demonstrated the detriment of broiler health and performance by feeding diets contaminated with 0.1–1 mg/kg AFB_1 (25, 26). These adverse effects can be explained as AFB_1 could inhibit protein synthesis and lipogenesis, reduce the activity of digestive enzymes, and change the energy metabolism of the cell (15, 27). We hypothesized that a commercially relevant concentration of AFB1 (500 μg/kg) during 42 days could decrease the growth rate in broilers. Unfortunately, the ADG and ADFI were not affected by the AFB_1 challenge in the present study. Slizewska et al. (28) also reported that fed 1 mg/kg AFB_1 of diet did not affect the ADG and ADFI of broilers. Likewise, Chen et al. (29) and Mesgar et al. (30) noted that feed intake, body weight gain, and feed efficiency were not affected by the 500 and 1,000 μg/kg of AFB_1. Therefore, the toxic effects of AFB_1 may be acute or chronic, influenced by the age, dose, diet composition, and duration of exposure (31).

In a previous study, we found that 250 and 500 mg/kg TA increased growth performance of broilers (32). In the contrary, supplementation with 250 mg/kg TA had no beneficial effect on the growth performance of broilers. Similar to the current results, Jamroz et al. (33) found that 250–500 mg/kg sweet chestnut tannin had no effect on performance, whereas 1,000 mg/kg TA reduced the final body weight in broilers. In addition, Choi et al. (34) reported that dietary supplementation of 500–5,000 mg/kg TA linearly decreased body weight of boilers infected with *Eimeria Maxima*. On the contrary, Liu et al. (35) found that 1,000 mg/kg chestnut tannins did not affect the body weight gain and feed intake in broilers. Cengiz et al. (36) also indicated that supplemented with 2,000 mg/kg chestnut tannin in broiler diets did not affect the performance. The dosage effect of TA on the growth performance of broilers seems to be unclear. However, it is reported that high dose of TA has negative effects on the growth of broilers, and biological effects are strongly dose-dependent (37, 38). Redondo et al. (39) hypothesized that the addition of excessive TA to the diet may increase the astringency and bitterness of the feed, thereby reducing the feed intake. Based on the different results, the inconsistency might be attributed to the source of tannic acid, administration dosage, diet composition, and age of the bird (40).

Aflatoxin has been known to mainly accumulated and metabolized in the liver and kidney after absorption, causing impairment of the liver and kidney (41, 42). In the present study, we observed that 500 μg/kg AFB_1 caused a significant increase in the relative weight of liver and kidney, which is consistent with other studies (43–45). The enlargement of organ weight is attributed to disorders of lipid metabolism, and the inhibition of lipid transportation, leading to lipid deposition, which results in hepatomegaly (15, 46). Many studies describe the role of plant extract could ameliorate the adverse effect of AFB_1 in broilers (47–49). In our previous study, we also found that the increase in liver and kidney relative weight in the AFB_1 group was ameliorated by the supplementation of 250 and 500 mg/kg TA (32). Therefore, these results confirmed that TA has a protective effect on the liver and kidney damage caused by AFB_1.

Intestinal villus height, crypt depth, and villus height/crypt depth ratio are important indexes to evaluate intestinal nutrient digestion and absorption capacity of poultry (50). These parameters especially the villus height/crypt depth ratio was positively related to the absorptive efficiency of the intestine (51). In the present study, intestinal histomorphology result revealed that dietary AFB_1 exposure decreased the villus height and crypt depth ratio in the ileum of 42-day-old broilers. Similar to the results by Tavangar et al. (22), who reported that 1 mg/kg AFB_1 decreased small intestine villus height and villus height to crypt depth ratio of broilers. These results showed that AFB_1 could decrease the capacity of intestinal mucosa to digest and absorb nutrients by depressing intestinal development. Brus et al. (52) found that tannin extract could promote the proliferation of intestinal epithelial cells to promote intestinal development *in vitro*. Therefore, further studies need to be conducted to confirm the positive effect of TA on intestinal morphology in broilers.

It has been demonstrated that AFB_1 could induce the production of reactive oxygen species (ROS) and oxidative stress, thereby inducing cell and DNA damage (53). The antioxidant system of organism can eliminate the adverse effects of ROS, and the GST, T-SOD, CAT, and GSH-Px are important endogenous antioxidant enzymes, which play a key role in scavenging free radicals and maintaining the intracellular redox equilibrium (15). In the present study, AFB_1 significantly increased the concentrations of MDA

and decreased the antioxidant enzyme activities of T-SOD, GSH-Px, GST, and CAT in the liver, jejunum, and the plasma of broilers when compared with the CON. These results are in consistent with previous studies, which demonstrated that different dosage of AFB_1 decreased the activity of antioxidant enzymes, increased the lipid peroxidation, and inhibited the antioxidant capacity of broilers (14, 15, 54, 55). Recently, researchers have been interested in the usage of antioxidants to counter the toxic effects of aflatoxins (56). Our present results confirmed that 250 mg/kg TA could enhance antioxidative capacity, and alleviate the adverse effects of AFB_1 on oxidative stress in the liver, jejunum, and plasma, which is similar to previous studies (57–59). Consequently, these results indicated that TA could play an important role in preventing the AFB_1-induced oxidative damage in broilers.

Conclusion

In conclusion, supplementation with 250 mg/kg TA could alleviate the oxidative damage, and prevent the enlargement of liver in broilers dietary challenge with 500 μg/kg AFB1. Therefore, Chinese gallnut TA may be used as a feed additive in the prevention of aflatoxicosis and improve the health of poultry.

Data availability statement

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

Ethics statement

The animal study was reviewed and approved by Institutional Animal Care and Use Committee of Wuhan Polytechnic University (Number: 20161121).

Author contributions

ZZ and BD conceived and designed the experiment. YX, JC, SG, SW, ZL, and LL performed the experiment. YX, ZL, and YQ analyzed the data. YX and ZZ wrote the manuscript. All authors read and approved the final manuscript.

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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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Supplementation with honeysuckle extract improves growth performance, immune performance, gut morphology, and cecal microbes in geese

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The study aimed to investigate the effects of honeysuckle extract (HE) on growth performance, serum biochemical indexes, immune organ indexes, gut morphology, and gut microbes in geese. A total of 180 28-day-old Holdobaki geese were randomly divided into three groups. Each group contained 6 replicates (10 geese, with 5 males and 5 females). The BD group was fed the basal diet, the HE1 group was fed the basal diet supplemented with 1 g/kg of HE, and the HE2 group was fed the basal diet supplemented with 2 g/kg of HE. The experiment lasted for 42 days. The results showed that, compared with the BD group, the average daily gain (ADG) of the HE1 and HE2 groups tended to increase ($0.05 < P < 0.10$), but the average daily feed intake (ADFI) and final body weight (BW) did not differ significantly, and the feed/gain ratio (F/G) was significantly lower ($P < 0.01$). The bursa index and the thymus index tended to increase ($0.05 < P < 0.10$), and serum immunoglobulin A (IgA) and immunoglobulin G (IgG) levels increased significantly ($P < 0.05$). In the HE1 and HE2 groups, the crypt depth (CD) in the jejunum tended to decrease ($0.05 < P < 0.10$), and the villus height/crypt depth ratio (V/C) increased significantly in the jejunum and the ileum ($P < 0.05$). According to 16sRNA microbial community diversity analysis, *Firmicutes*, *Bacteroidetes*, *Proteobacteria*, and *Actinobacteria* were the dominant phyla. The abundance of *Firmicutes* was significantly decreased ($P < 0.01$), while that of *Bacteroidetes* was significantly increased ($P < 0.01$), in the HE1 and HE2 groups compared with the BD group. *Bacteroides barnesiae, Subdoligranulum variabile, Bacteroides plebeius,* and *Faecalibacterium prausnitzii* were the dominant species, and the abundance of *B. plebeius* and *F. prausnitzii* was significantly increased ($P < 0.05$). According to the LEfSe analysis, BD enriched *g_Dorea* and *g_Dehalobacterium*; HE1 enriched g_*Faecalibacterium, g_Dialister, g_Prevotella, g_Megamonas, g_Phascolarctobacterium, g_Paraprevotella, g_Anaerostipes, g_Staphylococcus, g_Odoribacter, g_Succinivibrio,* and *g_Sutterella*; and HE2 enriched g_*Parabacteroides*, *g_Olsenella, g_human,* and *g_Rikenella*. According to the Spearman correlation analysis, *Bacteroides plebeius* was positively correlated with final BW, ADG, IgA, IgG, VH (ileum), and V/C (ileum) and was negatively correlated with F/G and CD (ileum);

Ruminococcus gnavus was negatively correlated with final BW, ADG, IgA, and IgG. HE supplementation at 1 g/kg improved growth performance, immune performance, gut morphology, and cecal microbes.

KEYWORDS

goose, growth performance, immune, intestinal morphology, cecal microbiome

Introduction

Intensive poultry farming causes environmental pollution problems, which will affect poultry immunity, resulting in higher mortality and economic losses. Supplementing antibiotics can improve poultry performance and reduce mortality. Therefore, antibiotics will be supplemented during the breeding process to enhance immunity. However, the abuse of antibiotics will lead to the problem of drug resistance and antibiotic residues. As using antibiotics will affect the health of humans and animals and destroy the microbial ecosystem, many countries have completely banned the use of antibiotics in animal husbandry (1, 2). Therefore, it has become a trend to replace antibiotics with natural and environment-friendly alternative substances. In the current research, organic acids, plant extracts, yeasts, and probiotics are potential alternative products (3, 4). Plant extracts are widely used in agriculture and play an important role in sustainable agriculture (5). Honeysuckle is a traditional medicine that has been widely used since ancient China. It has high nutrition and is rich in carbohydrates, protein, crude fat, crude fiber, vitamins, minerals, and other nutrients; it also contains phenols, ketones, and other substances, which have various effects, such as lowering blood sugar, improving anti-oxidation, and improving immunity (6–8). However, honeysuckle also contains anti-nutritional factors such as tannins, and excessive supplementation will be detrimental to animals. Separating and purifying honeysuckle and processing HE can effectively preserve the beneficial substances while removing harmful substances. Meng et al. found that supplementing HE could reduce the mRNA expression of fat synthesis-related genes, strengthen the intestinal mucosal barrier of grass carp, improve lipid metabolism and immune function, and reshape the intestinal flora (9, 10). Zhao et al. found that supplementing HE during the perinatal period improved dry matter intake, lactation capacity, anti-inflammatory properties, and antioxidant capacity of dairy cows (11). Its effect on geese has not been reported so far. Therefore, in this experiment, 28-day-old Holdobaki geese were used as a sample, and different proportions of HE were supplemented into the diet to explore its effects on growth performance, immune performance, intestinal morphology, and cecal microbes.

TABLE 1 Ingredients and composition of basal diets (DM basis) %.

Ingredients, g/kg		Nutrition levels[b], g/kg	
Corn	58.16	ME(MJ/kg)	2.70
Soybean meal	25.60	Crude protein	19.49
Bran	10.10	Crude fiber	7.00
Soybean oil	1.50	Ca	0.46
Stone power	1.00	Total P	0.32
Methionine	0.18	Methionine	0.43
Threonine	0.09	Lysine	1.056
Lysine	0.37	Threonine	0.742
3%Premix[a]	3.00	Methionine + Cystine	0.688

[a]One kilogram of the premix contained the following: Fe 100 mg, Cu 8 mg, Mn 120 mg, Zn 100 mg, Se 0.4 mg, Co 1.0 mg, I 0.4 mg, VA 8330 IU, VB1 2.0 mg, VB 2.8 mg, VB6 1.2 mg, VB12 0.03mg, VD3 1440IU,VE 30 IU,biotin 0.2 mg, folic acid 2.0 mg, pantothenic acid 20 mg, niacin acid 40 mg.
[b]Nutrient levels were all calculated values.

TABLE 2 Effect of HE on growth performance[a].

Items	Treatment				*P*-value
	BD	HE1	HE2	SEM	
Initial BW, g	2000.25	1982.65	1976.48	19.187	0.873
Final BW, g	4305.70	4457.52	4459.77	38.758	0.178
ADG,g/d	47.05	50.51	50.68	0.738	0.076
ADFI,g/d	258.06[b]	259.35[a]	259.52[a]	0.344	0.168
F/G	5.48[a]	5.13[b]	5.12[b]	0.051	0.001

Different lowercase letters with the same column date meant significant difference ($P \leq 0.05$).
[a]Each value represents the mean of 6 replicate pens.
The a and b indicates the content of the form indicate that the difference is significant.

Materials and methods

Experimental design, diets, and birds

The experimental protocol was approved by the Shanghai Academy of Agricultural Sciences, and the experimental methods and ethics complied with relevant regulations.

Holdobaki goose is an excellent variety cultivated by the Hungarian Holdobaki Goose Co., Ltd. It has the characteristics of fast growth and delicious meat. It is marketed as a product

TABLE 3 Effect of HE on immune organ index[a].

Items	Treatment				
	BD	HE1	HE2	SEM	*P*-value
Thymus index	0.73	0.89	0.80	0.032	0.054
Spleen Index	0.59	0.66	0.58	0.054	0.822
Bursa of Fabricius index	0.43	0.54	0.56	0.027	0.052

Different lowercase letters with the same column date meant significant difference ($P \leq 0.05$).
[a]Each value represents the mean of 6 replicate pens.

TABLE 4 Effect of HE on serum biochemical indexes[a].

Items	Treatment				
	BD	HE1	HE2	SEM	*P*-value
TP, g/L	47.45	45.50	43.45	0.961	0.247
ALB, g/L	11.30	10.90	10.77	0.173	0.452
ALP, U/L	207.17	201.33	201.17	10.040	0.966
GLU, mmol/L	10.88	11.18	11.73	0.235	0.346
CHOL, mmol/L	3.52	3.77	3.36	0.102	0.287
HDL-C, mmol/L	2.01	2.23	1.95	0.649	0.174
LDL-C, mmol/L	1.17	1.03	1.17	0.053	0.512
TGF-β, pg/ml	226.31	170.03	360.33	48.546	0.273
IL-10, pg/ml	140.79	107.97	141.50	9.865	0.301
IgA, mg/ml	12.84^{b}	20.45^{a}	21.63^{a}	1.443	0.015
IgG, mg/ml	32.50^{b}	46.94^{a}	52.68^{a}	3.455	0.037

Different lowercase letters with the same column date meant significant difference ($P \leq 0.05$).
[a]Each value represents the mean of 6 replicate pens.
The a and b indicates the content of the form indicate that the difference is significant.

at 60–70 days of age, with an average weight of about 4,500 g. The geese were purchased from Anhui Xiangtiange Goose Industry Professional Cooperative (28 days old, a total of 180 males and females), and the experimental site was Zhuanghang Experimental Station of Shanghai Academy of Agricultural Sciences. Each goose was weighed before the start of the experiment, and its initial average body weight was taken. They were divided into 3 groups, with each group having 6 cages, and the sizes of each cage were 200 cm/200 cm/50 cm. There was no significant difference between the groups. The first group was fed with a basal diet (BD), and the second and third groups were fed with a basal diet supplemented with 1 g/kg of HE (HE1) and 2 g/kg of HE (HE2), respectively (produced by Shaanxi Tangmen Biotechnology Co., Ltd., China). The basal diet formula (Table 1) refers to the nutrient requirements recommended by the NCR Standard (12) and according to the actual design of China's current goose production.

TABLE 5 Effect of HE on intestinal morphometry[a].

Items	Treatment				
	BD	HE1	HE2	SEM	*P*-value
Jejunum					
VH,um	499.30	503.21	509.96	5.441	0.723
CD,um	103.90	93.89	94.93	1.960	0.072
V/C	5.12^{c}	5.51^{b}	5.98^{a}	0.128	0.021
Ileum					
VH,um	444.55	452.92	457.62	4.203	0.440
CD,um	92.97	87.56	87.09	1.387	0.157
V/C	4.94^{b}	5.67^{a}	5.42^{a}	0.205	0.034

Different lowercase letters with the same column date meant significant difference ($P \leq 0.05$).
[a]Each value represents the mean of 6 replicate pens.
The a and b indicates the content of the form indicate that the difference is significant.

Growth performance

At the beginning (day 28) and end (day 70) of the experiment, the geese in each cage were weighed, and the average daily weight gain (ADG), average daily feed intake (ADFI), and feed/gain ratio (F/G) of each cage were calculated. The geese were fasted for 8 h before weighing.

Sample collection

At the end of the experiment, one goose (male) with a weight close to the mean was selected from each cage. Blood was collected from the fin vein and placed in a vacuum blood collection tube. After standing at room temperature for 5 h, centrifugation at 3,000 rpm for 10 min was performed to collect serum. Then, the jugular vein was bled and slaughtered, the abdominal cavity was opened, the bursa, the spleen, and the liver were taken out first, and the adhering fat was removed immediately after weighing and recording, and the immune organ index was calculated (immune organ index = immune organ weight, g/live weight, kg). The jejunum and the ileum were removed, the contents of the digestive tract of the jejunum were gently squeezed out, the residue was rinsed with normal saline, the residual water in the digestive organs was dried with a filter paper, the jejunum and ileum anterior segments were cut (one-fourth of the front end) at approximately 2 cm, and normal saline was used. The contents were rinsed, fixed with 10% formaldehyde, sectioned with paraffin, stained with hematoxylin-eosin, and observed under a light microscope.Villus height (VH) was measured from the tip (with a lamina propria) of the villus to the base (villus-crypt junction), crypt depth (CD) was measured from the villus-crypt junction to the distal limit of the crypt, and villus height/crypt

depth ratio (V/C) was calculated. In supplementation, cecal chyme samples were taken into 2 ml EP tubes, which were snap-frozen in liquid nitrogen and stored at 80°C for later use.

Serum biochemical indicators

The collected serum samples were sent to Shanghai Pinyi Biotechnology Co., LTD to detect transforming total protein (TP), albumin (ALB), glucose (GLU), total cholesterol (CHOL), low-density lipoprotein cholesterol (LDL-C), high-density lipoprotein cholesterol (HDL-C), growth factor-β (TGF-β), interleukin 10 (IL-10), immunoglobulin A (IgA), and immunoglobulin G (IgG).

16sRNA microbial community diversity analysis

For 16S rRNA sequencing analysis and data processing, Shanghai Personal Technology Co., Ltd. used gut microbes. Using the method of Divisive Amplicon Denoising Algorithm (13), noise reduction was used to obtain biological sequence ASVs (amplicon sequence variants) that did not contain amplification, sequencing errors, or chimeras. To comprehensively assess the α-diversity of microbial communities, we used the Chao1 index and observed species indices to characterize richness, Shannon and Simpson indices to characterize diversity, Faith's PD index to characterize evolution-based diversity, and Pielou's evenness index to characterize evenness.

Statistical analysis

The experimental data were analyzed using a one-way ANOVA in SPSS software (SPSS 26.0, Abacus Concepts, Berkeley, CA, United States), and Duncan's multiple comparison method was used to test whether there were significant differences between groups. The results were expressed as the mean and standard error of the mean (SEM), with a *Pi*-value < 0.05 indicating a significant difference. The Spearman correlation analysis method was used to determine the relationship between the cecal microbial communities and the measured parameters.

Results

Growth performance

The growth performance is shown in Table 2. Compared with the BD group, the final BW of the HE1 and HE2 groups increased, but the difference was not significant ($P = 0.178$). The ADG increased ($P = 0.076$), and the ADFI decreased ($P = 0.168$). The F/G ratio increased significantly ($P = 0.001$).

TABLE 6 Effect of HE on α-diversity[a].

Items	Treatment			SEM	*P*-value
	BD	HE1	HE2		
Chao1	2646.26	2901.59	2890.06	119.27	0.642
Observed_species	2397.80	2674.15	2592.62	110.59	0.606
Shannon	8.19	8.62	8.65	0.161	0.460
Simpson	0.97	0.98	0.99	0.004	0.356
Pielou_e	0.73	0.76	0.76	0.011	0.444
Faith_pd	130.11	148.46	141.11	4.672	0.287
Goods_coverage	0.99	0.99	0.99	0.001	0.646

Different lowercase letters with the same column date meant significant difference ($P \leq 0.05$).
[a]Each value represents the mean of 6 replicate pens.

Immune organ indexes

The immune organ index is shown in Table 3. There was no significant difference in the spleen index, but the thymus index and the bursa of the Fabricius index increased in the HE2 group ($P = 0.052$).

Serum immune index

The effect of HE on goose serum immune indexes is shown in Table 4. The serum IgA and IgG were significantly increased in the HE1 and HE2 groups compared with the BD group ($P = 0.015$, $P = 0.037$).

Intestinal morphology

Table 5 shows that, compared with the BD group, there was no difference in VH between the HE1 and HE2 groups ($P = 0.723$) in the jejunum, the CD had a decreasing trend in the HE1 and HE2 groups ($P = 0.072$), and the V/C ratio of the HE1 and HE2 groups significantly improved ($P = 0.021$). In the ileum, compared with the BD, there was no difference in VH between the groups ($P = 0.440$), the CD had a decreasing trend in the HE1 and HE2 groups ($P = 0.157$), and the V/C ratio of the HE1 and HE2 groups significantly improved ($P = 0.034$).

Gut microbial diversity and composition

α-Diversity refers to the indicators of richness, diversity, and evenness of species in a locally homogeneous habitat. The differences in each index of α-diversity are shown in Table 6,

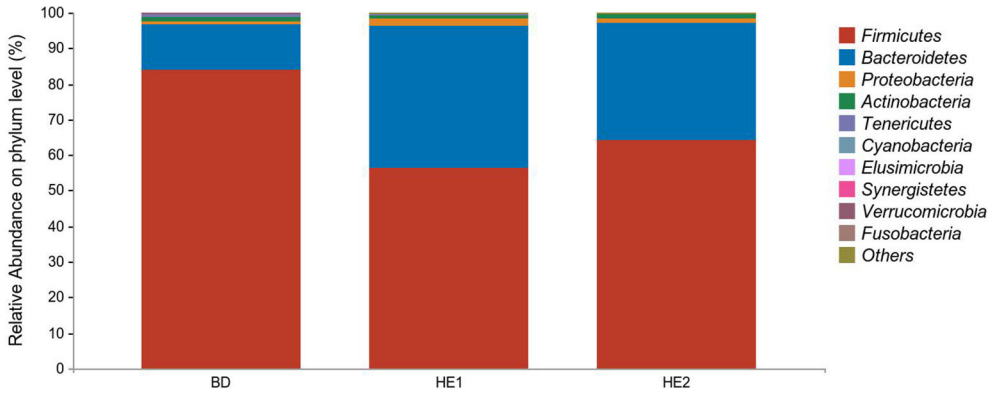

FIGURE 1
Relative abundance at the phylum level between groups.

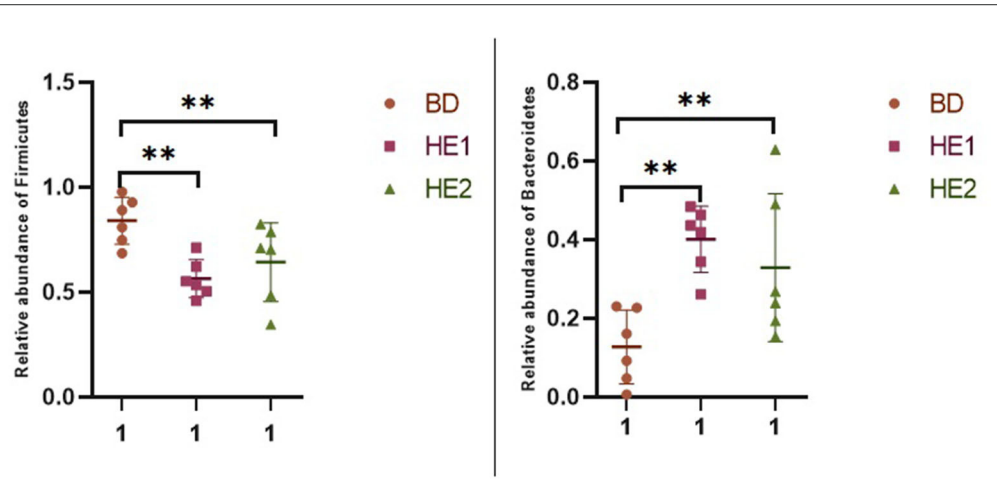

FIGURE 2
Scatter plot of the relative abundance of significantly different phylum in each group.

and there is no significant difference in groups. Figure 1 shows the average relative abundance at the phylum level. *Firmicutes*, *Bacteroidetes*, *Proteobacteria*, and *Actinobacteria* were the dominant species in the cecum. Figure 2 shows some phyla with significant differences between groups. Compared with the BD group, we found that the abundance of *Firmicutes* was significantly decreased ($P < 0.01$) while the abundance of *Bacteroidetes* was significantly increased ($P < 0.01$) in the HE1 and HE2 groups. Figure 3 shows the average relative abundance at the species level. *Bacteroides barnesiae, Subdoligranulum variabile, Bacteroides plebeius,* and *Faecalibacterium prausnitzii* were the dominant species in the cecum. Compared with the BD group, we found that the abundance of *Bacteroides plebeius* and *Faecalibacterium prausnitzii* were significantly increased ($P < 0.05$) in the HE1 group (Figure 4). Figure 5 shows the PCoA based on the Bray-Curtis distance. The HE1 and HE2 groups were gathered in a certain area and had a relatively obvious distance from the BD. The PCo1 value is 15.6%, and the PCo1 value is 9.4%. We used LEfSe to find out which genus levels were different in each group. As shown in Figure 6, BD enriched *g_Dorea* and *g_Dehalobacterium*; HE1 enriched *g_Faecalibacterium, g_Dialister, g_Prevotella, g_Megamonas, g_Phascolarctobacterium, g_Paraprevotella, g_Anaerostipes, g_Staphylococcus, g_Odoribacter, g_Succinivibrio,* and *g_Sutterella*; and HE2 enriched g_ *Parabacteroides*, *g_Olsenella, g_human,* and *g_Rikenella.*

Relationship between gut microbiota and main parameters

We used Spearman's correlation heatmaps to predict the relationship between the species and key parameters (Figure 7). *Bacteroides plebeius* was positively correlated with final BW, ADG, IgA, IgG, VH (ileum), and V/C (ileum) and was negatively correlated with F/G and CD (ileum); *Butyricicoccus*

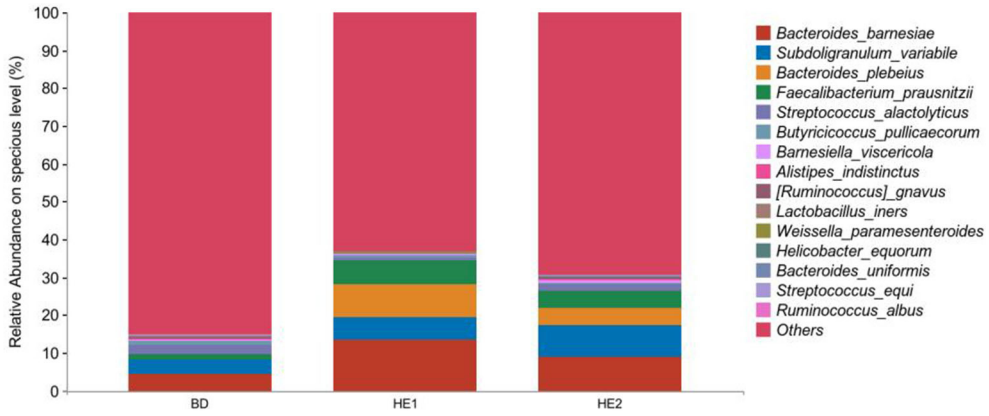

FIGURE 3
Relative abundance at the specious level between groups.

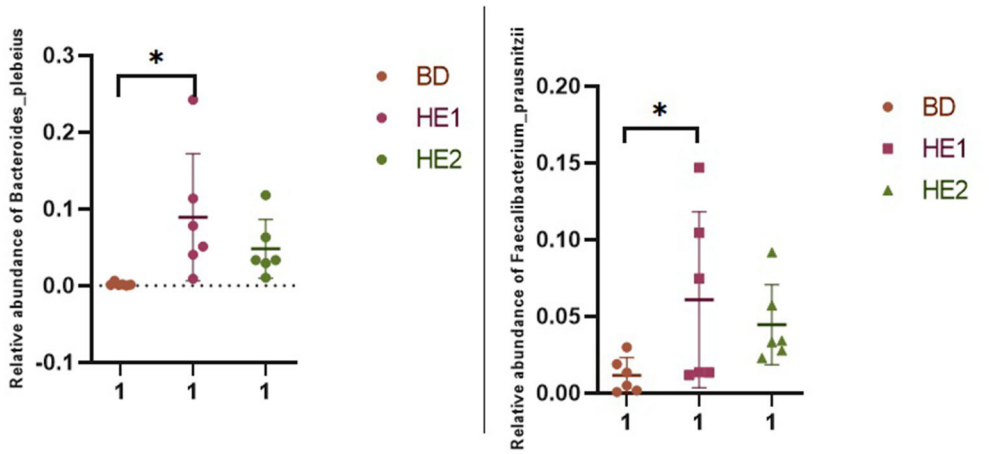

FIGURE 4
Scatter plot of the relative abundance of significantly different specious in each group.

pullicaecorum was negatively correlated with final BW and IgA; *Ruminococcus gnavus* was negatively correlated with final BW, ADG, IgA, and IgG; *Bacteroides eggtherii* was positively correlated with IgA and the bursa of the Fabricius index; *Clostridium ruminantium* was negatively correlated with the spleen index; *Desulfovibrio oxamicus* was positively correlated with final BW, ADG, the bursa of Fabricius index, and the thymus index and was negatively correlated with F/G; *Mucisoirillum schaedleri* was positively correlated with the thymus index and V/C (ileum) and was negatively correlated with IL-10; *Clostridium cocleatum was* negatively correlated with ADFI.

Discussion

Honeysuckle contains a variety of organic acids and polyphenols that can increase the secretion of enzymes in the stomach, improve the activity of digestive enzymes, and promote gastric motility and digestion of fatty foods, thereby enhancing the appetite and feed intake of young animals, promoting weight gain. Supplementing 400 g/day of honeysuckle to sheep diet can significantly increase final weight and average daily gain (14). Liu found that the supplementation of *Scutellaria baicalensis* and HE mixture (0.025 and 0.05%) improved growth performance, nutrient digestibility, and meat quality in finishing pigs (15). J.H. Park found that supplementing 0.2% HE improved broiler performance, blood cells, and meat quality (16). Chlorogenic acid (CGA) is a component of HE. Supplementation of 1 g/kg of CGA improved growth performance, immune function, antioxidant status, and intestinal barrier function in coccidial-infected broilers (17). Dietary CGA supplementation at 1 g/kg improved growth performance and quality and oxidative statuses of meat in pigs subjected or not to oxidative stress induced by dietary oxidized oil (18). A combination of CGA

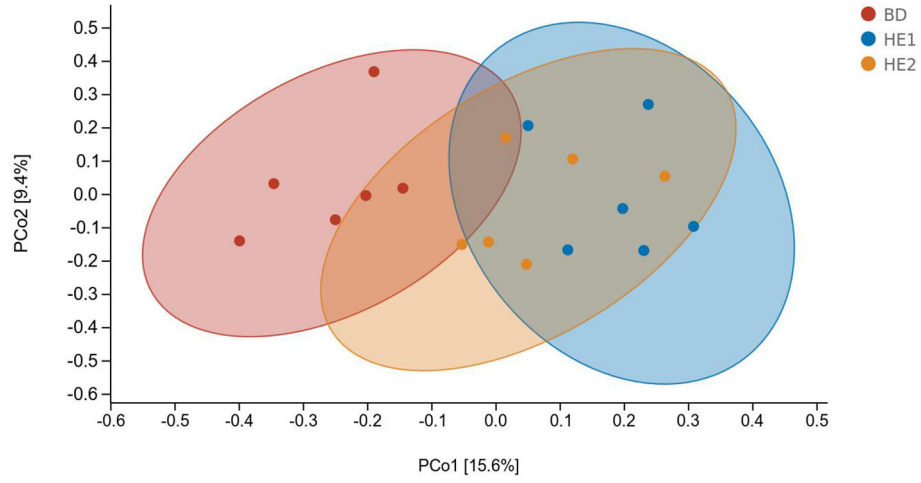

FIGURE 5
The PCoA based on the Bray-Curtis distance.

and bamboo charcoal particles can effectively improve the performance and husbandry environment of broilers (19). In this experiment, we found that the supplementation of 1 g/kg and 2 g/kg of HE to the goose diet can significantly improve the F/G ratio and the ADFI, and the ADG has a tendency to increase. At the same time, we found that the supplementation of 1 g/kg and 2 g/kg has a tendency to reduce the CD of the ileum and the jejunum and can significantly increase the V/C ratio of the ileum and the jejunum. This may be related to the fact that the supplementation of HE increased the V/C ratio, which in turn increased the daily gain and F/G ratio. Several studies found that there was a positive correlation between production performance and the V/C ratio (20–22).

Previous research showed that plant extracts can improve the immune organ index and the serum immune performance of animals (23, 24). The study found that HE improves cellular and innate immunity in immunosuppressed mice and promotes the secretion of immune-related cytokines through the iNOS-related signaling pathways, thereby exerting immunomodulatory activity (25). HE can modulate immunity by inhibiting the apoptosis of mouse lymphocytes (26). CGA at 215 μg/egg showed the significant potential of anti-infectious bursal disease virus (27). The above results are similar to the results of this experiment. We found that the supplementation of HE has a tendency to increase the bursa index and can significantly increase the serum IgA and IgG levels. It shows that HE can improve the immune performance of geese.

The normal microbial flora in the animal gastrointestinal tract plays an important role in maintaining animal health and normal physiological state, improving animal body resistance, and inhibiting the colonization of potentially pathogenic bacteria in the animal gastrointestinal tract (28). Bacteria in the microbiota produce a number of different compounds, including vitamins (vitamin K and B vitamins) (29, 30), volatile fatty acids (31), organic acids (lactic acid) (32), and antibacterial compounds (bacteriocins) (33). The microbiome provides protection and nutrition for animals. In the intestines of animals, the intestinal flora is mainly composed of *Firmicutes* and *Bacteroidetes*. *Bacteroidetes* are gram-negative and related to immune regulation. They are composed of lipopolysaccharides and flagellin, which interact with cellular receptors and enhance immune responses through cytokine synthesis (34). In this study, we found that the abundance of *Bacteroidetes* increased after supplementing HE. This may be positively correlated with improved immune properties such as the bursa index and serum IgA and IgG. The *Firmicutes*/*Bacteroidetes* ratio (F/B) is widely believed to play an important role in maintaining normal intestinal homeostasis (35). The high-fat diet causes an increase in the F/B ratio that can be recovered after treatment with plant extracts (36). This may be the reason for the decreased F/B ratio in this experiment. Therefore, we believe that supplementing HE changed the proportion of dominant flora at the cecal phylum level and promoted intestinal health.

Using LEfSe analysis, we found that *Faecalibacterium* was enriched in the HE1 group, and *Faecalibacterium prausnitzii* was significantly increased using a one-way ANOVA. *F. prausnitzii* is an acetate consumer that produces butyrate and bioactive anti-inflammatory molecules such as shikimic and salicylic acids. It improves intestinal inflammation (37, 38) and insulin resistance (39). Late weaning is associated with increased microbial diversity and the abundance of *F. prausnitzii* in the fecal microbiota of piglets (40). Supplementation of *F. prausnitzii* to lactating calves reduces the incidence of diarrhea, improves ADG, and increases weaning weight (41, 42). In this experiment, supplementing 1 g HE can significantly increase the abundance of *F. prausnitzii*.

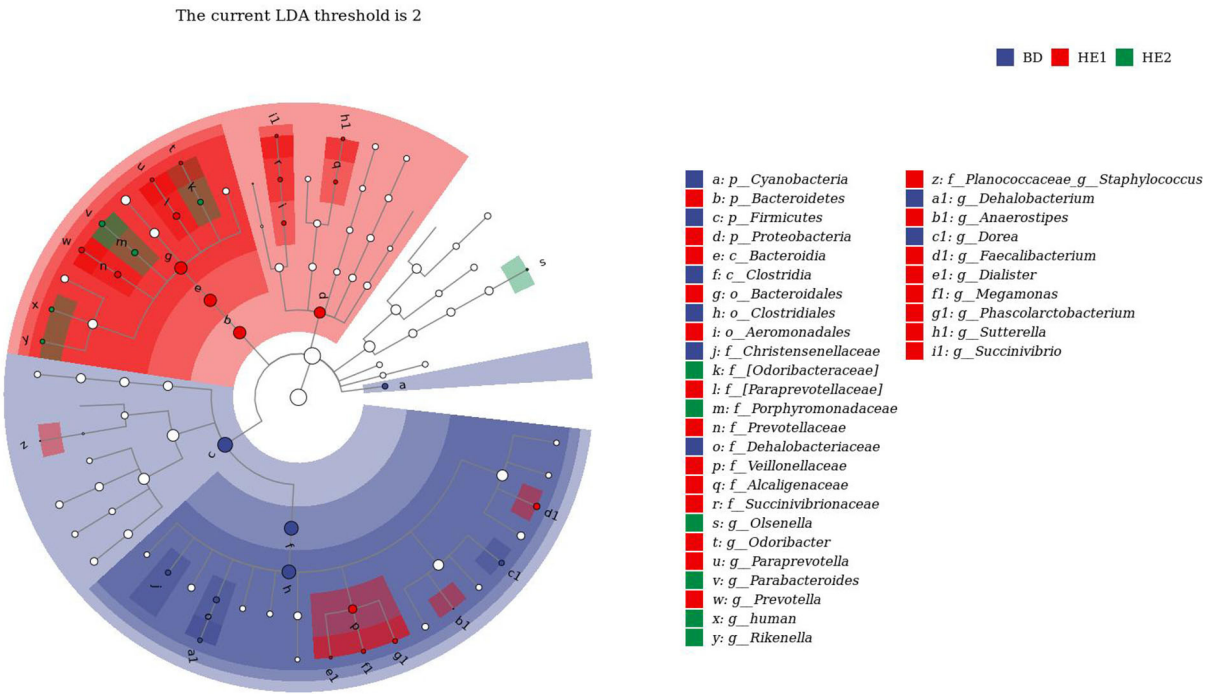

FIGURE 6
LEfSe analysis of differential microorganisms.

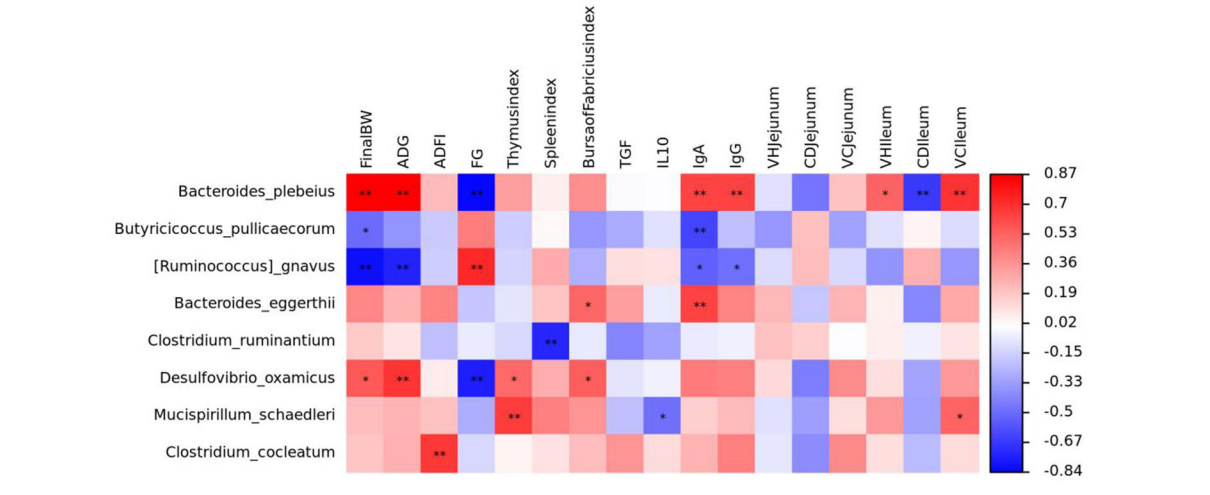

FIGURE 7
Relationship between gut microbiota and main parameters.

Through Spearman's analysis of the relationship between the main parameters and the gut microbiome, *Bacteroides plebeius* and *Bacteroides eggtherii* were positively correlated with many growth performance and immune indicators. *Bacteroides* are involved in many important metabolic activities in the human colon, including the fermentation of carbohydrates, the utilization of nitrogenous substances, and the biotransformation of bile acids and other steroids. The main by-products of anaerobic respiration are acetic acid, isovaleric acid, and succinic acid (43). *Bacteroides plebeius is* a key enzyme in initiating the depolymerization of agarose in the human gut (44). The abundance of *Ruminococcus gnavus* is linked to various diseases (45, 46). The adhesin produced by *R. gnavus* in the gut will preferentially bind IgA or IgG, which may be the reason

why *R. gnavus* was negatively correlated with IgA or IgG in this experiment.

Conclusions

Supplementation of honeysuckle extract in the diet can effectively improve growth performance, intestinal morphology, and immune performance by altering gut microbial composition and antioxidant capacity. The best supplementation level is 1 g/kg.

Data availability statement

The datasets presented in this study can be found in online repositories. The name of the repository and accession number can be found below: NCBI; PRJNA865537.

Ethics statement

The experimental procedure was approved by the Shanghai Academy of Agricultural Sciences (SAASPZ0522046) and the experimental methods and ethics complied with relevant regulations.

Author contributions

Writing-original draft: GL. Formal analysis: GL and XW. Investigation: GL, YY, and LZ. Conceptualization: SG, YL, and CW. Project administration: DH. Writing—review, editing, validation, and supervision: HW and DH. Funding acquisition: HW. All authors have read and agreed to the published version of the manuscript.

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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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Thermal manipulation modifies embryonic growth, hepatic free amino acid concentrations, and hatching performance in layer-type chicks

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Thermal manipulation (TM) of incubation temperature has been demonstrated to alter metabolism and post-hatch thermotolerance in broiler strains (meat-type chickens). Fewer reports were focused on layer-type chickens and there was no report on amino acid metabolism during TM in layer-type embryos. In this study, we investigated the effects of TM on embryonic development, hepatic amino acid metabolism, and hatching performance in layer-type chickens. Fertilized eggs were incubated under control thermoneutral temperature (CT, 37.6°C) and TM with high temperature (TMH, 39°C, 8 h/day) or low temperature (TML, 20°C, 1 h/day) from embryonic day (ED) 8 to ED 15. The embryonic weight and relative embryonic weight (yolk-free embryonic weight to the initial egg weight) significantly declined in the TML group at ED 13 ($P < 0.01$) and ED 16 ($P < 0.0001$), and were significantly increased ($P < 0.001$) in the TMH group at ED 16, in comparison with the embryos in the CT group. The concentrations of all hepatic free amino acids were significantly increased ($P < 0.01$) with embryonic development. Interestingly, TMH and TML caused similar effects on hepatic amino acid metabolism, in which most of the essential and non-essential amino acids were significantly declined ($P < 0.05$) under TM treatments at ED 13 but not affected at ED 16. Until hatching, TML, but not TMH, caused a significant ($P < 0.05$) delay (31–38 min/day from ED 8) in incubation duration. The hatchability in the TML group was lower than the other two groups, which indicated that 20°C as cold stimulation was not suitable for layer embryos. The body weight, yolk weight, yolk-free body mass, and chick quality were not affected by TM treatments. However, the relative weight of the liver, but not the heart, was significantly reduced ($P < 0.05$) at hatching by TML treatment. In conclusion, TML, but not TMH, caused to delay in embryogenesis and affected the internal organ of chicks at hatch. Similar

changes in amino acid metabolism under TMH and TML indicated that thermal stress induced by both high and low extreme ambient temperatures influences embryonic amino acid metabolism in a similar fashion in layer-type embryos.

KEYWORDS

amino acid, thermal manipulation, embryo, layer-type, hatching

Introduction

Incubation temperature is considered the most critical environmental factor for embryonic development and hatching efficiency (1), as the thermogenesis of embryos is limited and embryos are kept warm by incubators or hens before hatching (2). Over-heated incubation was reported to cause red hocks, unhealed navels, and lower yolk-free chick weight (3). An eggshell temperature of 40°C during the last 5 days of incubation caused hatchability to decline by 10–20% for broiler strains and meat-type chickens (4). Thermal manipulation (TM) means changing the standard incubation temperature during embryogenesis and results in the modification of the performance and fitness of chickens (3). Thermal manipulation (TM) of apposite high incubation temperature, such as 38.5–39.5°C for 6–8 h per day during embryonic days (EDs) 10–18, has been well studied and demonstrated to improve thermotolerance in post-hatch chicks and chickens of broiler strains (1, 5–7), as heat stress is a critical challenge for commercial broiler production in hot summer (8). Higher eggshell temperature (38.5°C) during incubation could improve bone morphology and ash contents of bones in layer strains (9). Improvement of cold resistance is a benefit to reducing ascites syndrome in broilers under cold conditions. A short-term cold exposure (15°C for 30 or 60 min) during incubation was considered to improve the adaptation of thermoregulatory and cardiovascular systems to cold conditions in post-hatch broilers (10). Few studies reported that TM of low temperature or cold exposure during embryogenesis supported birds to cope with low ambient temperature and caused positive effects on growth in post-hatch broilers (11, 12). Cyclic low incubation temperature (36.5°C) modified thyroid activity with an increased plasma thyroxine level under cold stress in post-hatch hens (13). However, few studies focused on the effects of TM on layer-type embryonic development.

Amino acids serve as building blocks of protein, and the free amino acid pool enlarges with embryonic growth during incubation (14). Amino acid administration during incubation was reported to increase body weight in post-hatched chicks and chickens (15). Stress-related research suggested that amino acids also play important roles in stress response and minimizing stress levels in poultry (16). Short- or long-term exposure to heat stress was reported to modify several free amino acids in blood and tissues in layer- and meat-type chicks or chickens (17–20). In our previous study, TM treatment during incubation caused to reduce of several free amino acids, including leucine (Leu) in the brain and the liver of broiler embryos (21), and *in ovo* administration of L-Leu was demonstrated to improve thermotolerance in broiler chicks and chickens (20, 22). However, the studies of amino acid profiles in layer-type embryos were less focused.

The initial body weight corresponded to the growth rate and later body weight in broiler chicks during the post-hatch first week (23). Thermal manipulation (TM) of high incubation temperature was reported to influence the incubation period and organ development in broiler strains (24, 25). Several hatching parameters of day-old chicks were considered predictors of growth potential in broilers (26). The average commercial life span of a layer hen is 72 weeks, which is longer than that of commercial broilers. However, the potential benefit of TM treatments in layer-type chicks was less focused. In the present study, the aims were to investigate the effects of cyclic heat or cold exposure to embryos on embryo growth, changes in hepatic amino acid concentrations, and chick quality in commercial layer strain. It was hypothesized that amino acid profiles as well as the growth of embryos will be modified by TM treatments.

Materials and methods

Incubation, thermal manipulation, and experimental design

The first experiment (Experiment 1) was conducted to investigate the effects of TM on embryo development and hepatic amino acid changes during embryogenesis. In Experiment 1, 150 fertilized layer eggs (Hy-Line Variety Brown strain; 41 weeks old of parent stock) were purchased from a local hatchery in Jiangsu, China. The eggs were weighted and delivered into three groups ($n = 50$/group): control thermoneutral temperature (CT) group, TM with high temperature (TMH) group, and TM with low temperature (TML) group. The average egg weights (means ± SEM) were 60.72 ± 0.60 g, 60.90 ± 0.67 g, and 60.57 ± 0.65 g for CT, TMH, and TML, respectively. All eggs were placed into an incubator (Hongde 2112 type incubator, Hongde Co., Shandong, China). According to our previous studies, the incubation temperature was 37.6°C with 60–70% relative humidity and auto-turning every 1.5 h (19, 20, 27). On ED 7, all eggs were candled and unfertilized eggs were discarded properly. The

number of unfertilized eggs was 9, 4, and 3 for CT, TMH, and TML, respectively. The hypothalamic-pituitary-thyroid (HPT) and hypothalamic-pituitary-adrenal (HPA) axes are responsible for the regulation of metabolism and thermoregulation, as well as response to stress. The HPT and HPA axes mainly develop and mature between ED 7 and 16 during embryogenesis in chicks (7, 28). The incubation temperature between 38.5 and 39.5°C was well tested to afford thermotolerance (1) and a short-term cold exposure at 15°C for 30 min or 60 min was reported to cause no effects on hatchability and body weight at hatching, as well as positive effects on growth in broiler chickens (10). Thus, eggs from the TMH group were exposed to 39°C (29) for 8 h every day and eggs from the TML group were exposed to 20°C for 1 h every day in other two small incubators (Hongde 440 type incubator, Hongde Co., Shandong, China) from ED 8 to 15. A cyclical treatment of 39 or 20°C caused no significant effects on the hatching rate in birds (10, 29). The relative humidity was set around 65% during TM treatments. After TM exposure, eggs from the TMH or TML group were returned to the previous incubator with the CT group (Hongde 2112 type incubator, Hongde Co., Shandong, China). The schedule of TM treatments was shown in Figure 1. To investigate the changes in embryo growth and amino acid concentrations during and after TM treatments, developing embryos (n = 15/group) were randomly selected from each group for sampling on ED 13 and 16. The sampling of the embryo was performed by the same person without knowing the grouping information. The yolk free body mass (YFBM) was determined after removing the yolk from the embryo. The relative embryo weight (%) was calculated as the ratio between YFBM (g) and the initial egg weight (g). The percent moisture loss from the egg during the incubation was calculated as follows: % moisture loss = [initial egg weight (g) – sampling egg weight (g)]/the initial egg weight (g) multiplied by 100. After the measurement of embryo weight, the liver was collected, snap frozen using liquid nitrogen, and then stored with plasma samples at −80°C until further analysis. After sampling, the remaining eggs were discarded properly.

The second experiment (Experiment 2) was conducted to investigate the effects of TM on hatching performance in layer chicks. In Experiment 2, 75 fertilized layer eggs (Hy-Line Variety Brown strain; 58 weeks old of parent stock) were purchased from a local hatchery as same as in Experiment 1, and 6 eggs were broken before incubation. On ED 7, 12 unfertilized eggs were detected by candling. The remaining eggs were divided into three groups: CT, TMH, and TML (n = 19/group) based on the initial egg weight. The average egg weights (means ± SEM) were 60.04 ± 0.70 g, 60.06 ± 0.83 g, and 60.07 ± 0.76 g for CT, TMH, and TML, respectively. The incubation and TM treatments were the same as described in Experiment 1. At the end of ED 18 (incubation time 432 h), all eggs (n = 19/group) were shifted to the hatching trays and the hatching process was recorded by a video system (HIKVISION, Hangzhou, China). For each egg, the incubation duration was defined as the time between setting and hatching (30). After 510 h of incubation, all the hatched chicks were assessed for chick quality of Tona score as described elsewhere (30), as chick quality was defined to encompass several qualitative characteristics and scored according to their importance, which could be a predictor of their later performance (30). Simply, the conditions of activity, down and appearance, retracted yolk, eyes, legs, navel area, remaining membrane, and remaining yolk were assessed and scored according to their importance within a total scale of 100 (30). The chicks were properly anesthetized with ethyl ether (Sinopharm Chemical Reagent Co., Ltd., Shanghai, China) after scoring before sampling. Then, the body weight (BW), yolk weight (YW), liver weight, and heart weight were measured by an electric balance (Shanghai Yingheng Electronic Scale Co., Ltd., Shanghai, China). The hatchability (%) was calculated as the ratio between the number of hatched chicks and the number of fertile eggs. The YFBM was calculated as the difference between BW and YW. The relative weight (%) of the heart and liver was calculated as the ratio between the liver or heart weight and the BW or YFBM.

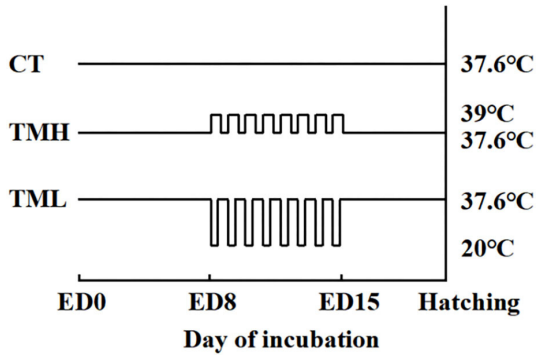

FIGURE 1
Incubation treatments. CT, control incubation temperature until hatching at 37.6°C; TMH, thermal manipulation with high temperature (39°C, 8 h/day) from embryonic day (ED) 8 to ED 15; TML, thermal manipulation with low temperature (20°C, 1 h/day) from ED 8 to 15. All eggs were transferred to the hatcher at ED 18.

This study was performed according to the Guidelines for the Care and Use of Laboratory Animals prepared by the Institutional Animal Care and Use Committee of Nanjing Agricultural University [Permit Number SYXK (Su) 2017-0007].

Free amino acid analysis in the liver

A fully automatic amino acid analyzer (L-8080 type, Hitachi, Japan) was used to measure the free amino acid

concentrations in the liver, according to the protocol described elsewhere (27). Six liver samples were randomly selected from each group for analysis. In brief, the liver samples were weighed and homogenized in a 5% sulfonic acid solution. After 30 min deproteinization on ice, the supernatant was filtered (0.22-μm filter, Biosharp, Guangzhou Saiguo Biotech Co., Ltd., Guangzhou, China) after 20 min of centrifugation (4°C, 20,000 *g*). The filtrate and standard solution were incorporated into the amino acid analyzer. The hepatic amino acid concentrations were expressed as pmol/mg on a wet tissue basis.

Statistical analysis

Since there were two factors (embryonic days and TM) in Experiment 1, data were statistically analyzed by two-way analysis of variance (ANOVA). Holm–Sidak's multiple comparisons test was applied as a *post-hoc* analysis when a significant interaction was detected. The data in Experiment 2 were statistically analyzed by one-way ANOVA and Turkey's multiple comparisons test was applied when a significant difference was detected. Statistical analyses were performed using a commercially available package—GraphPad Prism 8 (GraphPad Software Inc., San Diego, CA). Significant differences were denoted as $P < 0.05$. Data were expressed as mean ± SEM. All data in each group were first subjected to outlier identification ($P < 0.01$) by GraphPad Prism 8, and the remaining data were used for the analysis among groups. The number of chicks used for statistical analysis in each group is shown in the Figure legends and Table notes.

Results

Influence of TM on embryonic growth during incubation

The changes in embryonic weight, relative embryonic weight, and moisture loss are shown in Figure 2. The embryonic weight and relative embryonic weight were significantly ($P < 0.0001$) increased by the day of embryonic age and significantly ($P < 0.0001$) influenced by TM. A significant ($P < 0.0001$) interaction between embryonic age and TM suggested that the declined embryonic weight and relative embryonic weight by TML at ED 13 were fortified at ED 16. Moreover, the embryonic weight and relative embryonic weight were significantly improved by TMH at ED 16 but not at ED 13 (Figures 2A,B). The moisture loss in incubation was significantly increased with embryonic growth but was not affected by TM treatments (Figure 2C).

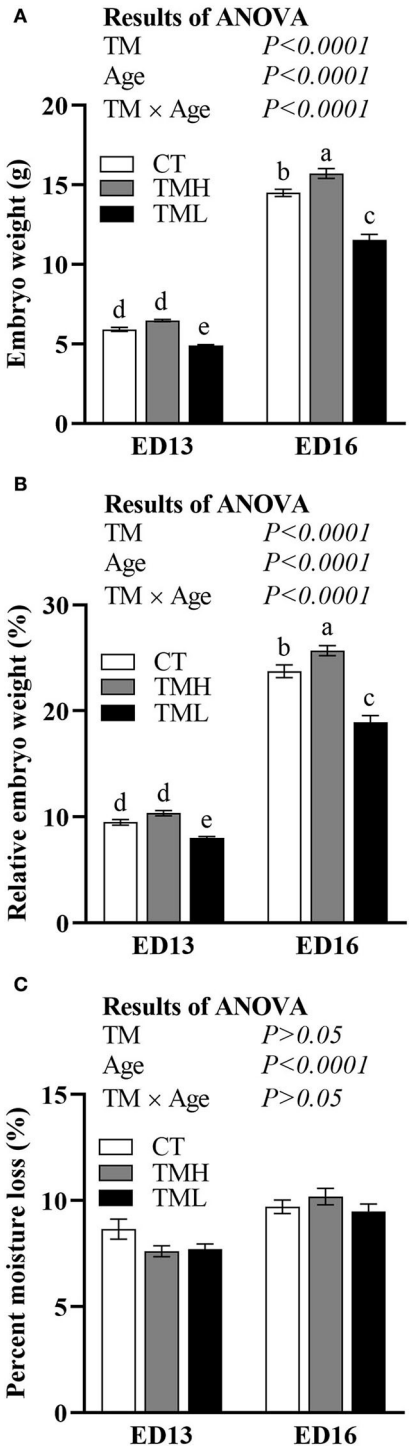

FIGURE 2
The changes in embryonic weight **(A)**, relative embryonic weight **(B)**, and percent moisture loss **(C)** exposed to control temperature (CT, 37.6°C) and TM with high temperature (TMH, 39°C, 8 h/day) or low temperature (TML, 20°C, 1 h/day) from embryonic day (ED) 8 to ED 15. The number of chicks in each group was $n = 12–15$. Values are mean ± SEM. Different superscripts indicate significant ($P < 0.05$) differences. TM, thermal manipulation.

TABLE 1 Free amino acid contents in the liver of embryos exposed to control or modified temperature during embryogenesis.

Amino acids	ED13			ED16			*P* value		
	CT	TMH	TML	CT	TMH	TML	Age	TM	Interaction
Essential amino acids									
Histidine	524 ± 138	167 ± 24	100 ± 21	822 ± 252	674 ± 218	735 ± 226	**	NS	NS
Threonine	5,788 ± 1,507^{b}	1,663 ± 271^{c}	1,066 ± 118^{c}	9,656 ± 452^{a}	10,151 ± 680^{a}	9,604 ± 438^{a}	****	**	**
Arginine	847 ± 172^{a}	178 ± 36^{b}	145 ± 18^{b}	956 ± 76^{a}	899 ± 86^{a}	1,270 ± 162^{a}	****	**	***
Valine	918 ± 238^{a}	297 ± 40^{b}	261 ± 30^{b}	1,257 ± 75^{a}	1,319 ± 6^{a}	1,123 ± 44^{a}	****	**	*
Isoleucine	425 ± 88^{b}	125 ± 17^{c}	118 ± 5^{c}	616 ± 31^{a}	605 ± 25^{a}	588 ± 37^{ab}	****	***	**
Methionine	260 ± 59^{b}	79 ± 11^{c}	64 ± 10^{c}	469 ± 35^{a}	477 ± 14^{a}	452 ± 23^{a}	****	**	**
Leucine	760 ± 173^{b}	223 ± 29^{c}	175 ± 22^{c}	1,484 ± 86^{a}	1,499 ± 43^{a}	1,431 ± 57^{a}	****	**	**
Lysine	1,246 ± 180^{b}	293 ± 44^{c}	268 ± 36^{c}	2,879 ± 166^{a}	3,056 ± 201^{a}	3,046 ± 139^{a}	****	*	***
Glycine	1,654 ± 366^{b}	558 ± 90^{c}	549 ± 64^{c}	2,743 ± 154^{a}	2,993 ± 142^{a}	3,129 ± 144^{a}	****	NS	**
Phenylalanine	494 ± 94^{b}	136 ± 17^{c}	99 ± 15^{c}	922 ± 52^{a}	892 ± 44^{a}	811 ± 33^{a}	****	****	**
Non-essential amino acids									
Aspartic acid	1,284 ± 322^{a}	456 ± 30^{b}	245 ± 35^{b}	1,700 ± 92^{a}	1,692 ± 13^{a}	1,934 ± 184^{a}	****	*	**
Tyrosine	807 ± 218	222 ± 30	235 ± 30	887 ± 159	952 ± 173	731 ± 147	***	*	NS
Cystathionine	104 ± 31^{b}	23 ± 4^{c}	20 ± 5^{c}	169 ± 11^{a}	180 ± 15^{a}	126 ± 10^{ab}	****	**	*
Glutamic acid	7,126 ± 1,667^{a}	2,005 ± 264^{b}	2,238 ± 258^{b}	7,318 ± 179^{a}	7,343 ± 547^{a}	6,920 ± 441^{a}	****	**	**
Serine	2,764 ± 638^{a}	777 ± 137^{b}	563 ± 57^{b}	3,303 ± 118^{a}	3,890 ± 218^{a}	3,728 ± 253^{a}	****	*	***
Ammonia	4,133 ± 1,085^{b}	1,286 ± 181^{c}	1,246 ± 92^{c}	7,684 ± 196^{a}	7,755 ± 218^{a}	8,262 ± 682^{a}	****	*	*
Alanine	1,559 ± 399^{b}	426 ± 62^{c}	302 ± 43^{c}	2,397 ± 142^{a}	2,497 ± 74^{a}	2,385 ± 112^{a}	****	**	**

The number of embryos used in each group was as n = 5–6. Different superscripts in the same row indicate significant differences (P < 0.05) between treatments. Values are means ± SEM in pmol/mg. CT, control treatment (37.6°C) during incubation; TMH, thermal manipulation with higher incubation temperature (39°C during ED 8–15 with daily 8-h treatment from 10:00 to 18:00) during incubation; TML, thermal manipulation with lower incubation temperature (20°C during ED 8–15 with daily 1-h treatment from 14:00 to 15:00) during incubation; ED, embryonic day.
*P < 0.05.
**P < 0.01.
***P < 0.001.
****P < 0.0001.
NS, not significant.

Influence of TM on free amino acid concentrations in the liver of embryos

The changes in free amino acid concentrations in the liver of layer-type embryos are shown in Table 1. The hepatic free amino acid pool was enlarged with the progress of embryonic age since the concentration for all the amino acids was significantly ($P < 0.01$) increased. Except for histidine and glycine, the concentration of other essential amino acids (leucine, valine, isoleucine, arginine, and so on) in the chicken as well as non-essential amino acids was significantly ($P < 0.05$) influenced by TM treatment. Significant interactions between age and TM suggested that most of the influenced amino acids were significantly ($P < 0.05$) declined by TM treatment at ED 13 but not at ED 16. Interestingly, the treatments of TMH and TML had a similar type of effects with declining hepatic amino acid concentrations at ED 13.

Influence of TM on hatching performance in layer-type chicks

The results of the hatching process, incubation duration, and hatching performance are shown in Figure 3 and Tables 2, 3, respectively. Each group has 19 fertile eggs for hatching, and the number of hatched chicks was 16, 17, and 14 for CT, TMH, and TML, respectively, and one bird in the TMH group was dead after hatching. The hatchability was 84.2, 89.5, and 73.7% for CT, TMH, and TML, respectively. The hatchability in the TML group was clearly lower than in the other two groups. The chick of the TMH group first hatched was 2.55 h earlier than that of the CT group (482.73 h for CT; 480.18 h for TMH); however, the THL treatment delayed 5.27 h for first hatching (488.00 h for TML) compared with the CT group (Figure 3). The incubation duration was significantly ($P < 0.05$) longer in the THL group than in CT and TMH groups, and the THL treatment

delayed incubation time 35–46 min/day for hatching after ED 8 in comparison with CT or TMH groups (Table 2). At hatching, TM treatments did not show significant effects on the BW, YW, YFBM, and chick quality (Tona score; Table 3). The weight of the heart and liver in chicks was also not affected by TMH or TML, even TML showed a trend of decline in liver weight ($P = 0.0596$ for CT vs. TML) at hatching. Importantly, the ratio of liver weight to BW or YFBM was significantly ($P < 0.05$) declined by the TML treatment at hatching.

Discussion

The objective of this study was to investigate embryonic growth, changes in hepatic amino acid metabolism, and hatching performance after TM with high or low incubation temperature in layer-type chickens. Temperature manipulation during the middle stages caused a significant modification in embryonic growth, incubation duration, hepatic amino acid concentrations, and several parameters of hatched chicks in comparison with the control group.

Temperature manipulation is considered a management tool to influence embryonic development. In the current study, the decreased incubation temperature induced significant retardation in embryo growth at ED 13 and 16. The threshold temperature for embryonic development is 27°C and embryonic development stops below at ~27°C (31). Shinder et al. (10) reported that the heart rate of embryos was reduced from 252 to 35 beats/min after 60 min cold exposure (15°C) and recovered under control incubation temperature. The cold exposure to 20°C for 60 min was thought to delay embryonic development for a short term, and 5 days (or less) accumulation of TML treatment resulted in lower embryonic weight. However, the hatchability, which is a critical parameter for hatchery managers, was clearly lower in the TML treatment than in

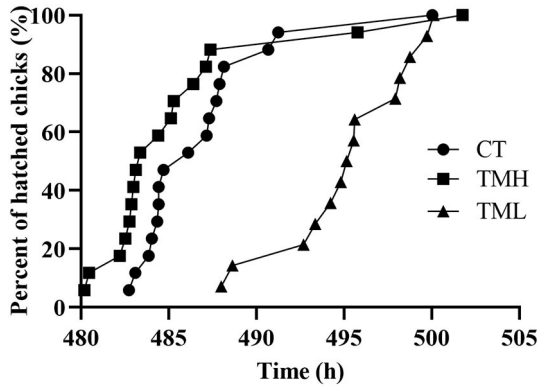

FIGURE 3
Distribution of hatch after exposure to control temperature (CT, 37.6°C) and thermal manipulation with high temperature (TMH, 39°C, 8 h/day) or low temperature (TML, 20°C, 1 h/day) from embryonic day (ED) 8 to ED 15. The Y axis is percent of hatched birds and the X axis is the incubation duration.

TABLE 3 Hatching performance after exposure to control or modified temperature during embryogenesis.

Items	CT	TMH	TML	*P*-value
BW (g)	40.64 ± 0.93	39.05 ± 1.10	42.99 ± 1.28	NS
YW (g)	4.53 ± 0.29	4.12 ± 0.27	5.15 ± 0.36	NS
YFBM (g)	36.11 ± 0.79	34.93 ± 0.87	37.65 ± 1.00	NS
Liver (g)	0.98 ± 0.03	0.95 ± 0.02	0.89 ± 0.03	NS
Heart (g)	0.29 ± 0.01	0.30 ± 0.01	0.31 ± 0.01	NS
Tona-score	90.6 ± 1.1	92.0 ± 1.5	91.4 ± 0.8	NS
Liver/BW (%)	2.39 ± 0.09^{a}	2.45 ± 0.05^{a}	2.06 ± 0.07^{b}	**
Heart/BW (%)	0.73 ± 0.03	0.77 ± 0.02	0.65 ± 0.05	NS
Liver/YFBM (%)	2.69 ± 0.11^{a}	2.73 ± 0.05^{a}	2.35 ± 0.07^{b}	**
Heart/YFBM (%)	0.82 ± 0.03	0.85 ± 0.26	0.75 ± 0.06	NS

The number of embryos used in each group was n = 8–10. Different superscripts in the same row indicate significant differences ($P < 0.05$) between treatments. Values are means ± SEM. CT, control treatment (37.6°C) during incubation; TMH, thermal manipulation with higher incubation temperature (39°C during ED 8–15 with daily 8-h treatment from 10:00 to 18:00) during incubation; TML, thermal manipulation with lower incubation temperature (20°C during ED 8–15 with daily 1-h treatment from 14:00 to 15:00) during incubation; ED, embryonic day; BW, body weight; YW, yolk weight; YFBM, yolk free body mass.
**$P < 0.01$.
NS, not significant.

TABLE 2 Incubation duration and calculated time delay (min/d) of hatching after exposure to control or modified temperature during embryogenesis.

Spread of hatch (%)	Incubation duration (h)			Delay from ED 8	
	CT	TMH	TML	THL-CT	THL-TMH
50	484.2 ± 0.3^{a}	482.3 ± 0.4^{a}	492.4 ± 1.1^{b}	35	43
75	485.2 ± 0.5^{a}	483.2 ± 0.5^{a}	494.0 ± 1.0^{b}	38	46
100	486.9 ± 1.0^{a}	485.5 ± 1.3^{a}	495.2 ± 1.0^{b}	36	42

Different superscripts in the same row indicate significant differences ($P < 0.05$) between treatments. Values are means ± SEM. CT, control treatment (37.6°C) during incubation; TMH, thermal manipulation with higher incubation temperature (39°C during ED 8–15 with daily 8-h treatment from 10:00 to 18:00) during incubation; TML, thermal manipulation with lower incubation temperature (20°C during ED 8–15 with daily 1-h treatment from 14:00 to 15:00) during incubation; ED, embryonic day.

the control group in this study. Our recent study agrees with these results that TML treatment of 20°C for 60 min reduced hatchability by 11% in layer chicks (unpublished data). It indicated that 20°C for 60 min might be too harsh for layer embryos, even 15°C for 60 min caused no negative effects on hatchability in broiler chicks (10). On the contrary, TMH treatment (39°C for 8 h/d during ED 8–15) was expected to accelerate embryonic development and increased the embryo's relative weight at ED 16, which agreed with the previous report that TM with 39.5°C for 12 h/d during ED 7–16 significantly increased embryonic relative weight at ED 11, 12, 14, and 16 in broiler strain (32). The threshold of heat injury is 40.5°C, and no embryos hatched under continuous temperatures above 40.5°C (31). However, the short periods of high temperature are not necessarily lethal for embryos. A continuous 40.6°C between ED 16 and 18.5 resulted in lower embryo weight and higher embryonic mortality (24). The hypothalamic-pituitary-adrenal and hypothalamic-pituitary-thyroid axes are the main efferent neuro-hormonal axis for stress responses and metabolic regulation (1, 33), which are developed from ED7 to ED16 in chick embryos (7, 28). Elevated incubation temperature during the middle stages (ED 7–ED 16) promoted myoblast proliferation and muscle growth in broiler embryos, as well as caused a long-term effect on post-hatch broiler chicken (34). As mentioned above, TM treatments during ED 8–15 significantly affected the embryo growth in layer-type chicks. However, embryo growth and physiological development are different between layer and broiler strains (14, 35), as different purposes of intensive selection. Then, the most appreciated period or "critical" developmental period by TM treatments, as well as the suitable temperature for cold stimulation needs to be further investigated in layer strains.

The effects of TM, cold, or heat, in embryonic growth, are controversial due to the differences in temperature, duration, and embryonic stages of TM treatments. However, it is unanimous that TM of low or high temperature could affect the physiology and anti-stress ability of post-hatch chicks or chickens in a long term (12, 36, 37). Thermal manipulation with high temperature (TMH) has been demonstrated to afford thermotolerance to later heat exposure in broiler chickens (1, 38). Cyclic cold exposure during incubation caused to increase cold resistance in broilers (36). Moreover, embryonic TM was also reported to afford cross-adaptation to stress in broilers (39). In the present study, TMH and TML induced opposite effects on embryonic growth, thus, it is possible to apply both TMH and TML to regulate embryonic growth and to improve the acquisition of cold, heat, or cross resistance with avoiding harmful consequences of TM treatments in post-hatch chickens. Therefore, the collective effects of TMH and TML during incubation on physiology, growth, and stress responses in layer-type chicks and hens are needed to be investigated in future studies.

The hatching process and day-old chick quality are affected by the incubation environment and egg characteristics (30, 40). Although both TMH and TML significantly affected embryonic growth, only TML significantly delayed the incubation duration. However, there was no significant effect of TM treatments on the speed of hatching and Tona score. The YFBM and BW of day-old chick are important parameters of day-old chick quality and closely related to slaughter weight in broilers (41), but the relationship between YFBM (or BW) and laying performance is still unclear in layer hens. The higher eggshell temperature during incubation significantly decreased YFBM with 0.7 g at hatching in layer-type chicks, which was contributed by improved percent moisture loss under high incubation temperature (42). Although significant differences were not detected, the average YFBM in the TMH group declined to 1.18 g compared with the control group. Interestingly, both TMH and TML caused significant effects on embryo growth on ED 16, but no differences were found at hatching. The body mass is correlated with egg weight, and percent moisture loss was not affected by TM treatments in the current study. Similar results were reported that *in ovo* feeding of L-Leu caused growth retardation of embryos but no effects on body weight at hatching in broilers (21, 27). However, the laying performance and eggshell quality are more concerned than slaughter weight in layer-type strains. Thus, TM-mediated effects on the laying performance of hens should be clarified in the future.

During incubation, the free amino acids play an important role in layer- and meat-type embryonic growth (14). The free amino acid concentrations were increased during incubation, which agrees with previous reports that the albumin uptake into the embryo was increased and the free amino acid pool was enlarged to match the amino acid requirements of embryogenesis (21, 43). Our previous study demonstrated that TMH decreased some essential amino acid concentrations in the embryos and the altered amino acid was demonstrated to afford thermotolerance in broiler chicks and chickens (20–22). Similarly, most amino acids declined under TMH in the embryonic liver. The declined hepatic amino acids are speculated to use to cope with the increased incubation temperature, which has been demonstrated in broiler strains, as L-Leu *in ovo* feeding affords thermotolerance (22). Interestingly, the TML showed similar effects with declining hepatic amino acid concentrations. One possibility could be that the decreased free amino acids played the same roles in coping with cold exposure in developing embryos, or the nutritional mechanisms of heat- or cold-resistance were similar in poultry. Both heat and cold exposures are identified as thermal stress. During stressful conditions, some amino acids are used to attenuate stress response in the chicken (44). Promoted protein synthesis or gluconeogenic phenomenon under high incubation temperature might contribute to the decline of free amino acid in the liver

(45). Saleh et al. (38) reported that TMH caused to increase in body weight and declined body temperature after both cold and heat stress in broiler chickens. Epigenetic modification or improved oxidative function might be the reasons for TM-mediated cross adaptation to later stress in post-hatch broilers (38, 39). In addition, TML caused retardation in embryonic growth, which might be another contributor to declined amino acid concentrations in the TML group, as the free amino acid pool is correlated with stages of embryonic development. However, the TM-mediated difference in the concentration of free amino acid disappeared after TM treatment at ED 16, even though the growth was significantly slowed down in the TML group and improved in the TMH group. This is consistent with our previous report that the TMH treatment (38.6°C for 6 h/d during ED 10–18) caused a decline in some hepatic amino acid levels at ED 14 but not at ED 19, in broiler embryos (21). The liver plays an important role in amino acid metabolism; however, the brain was considered the center of thermoregulation (33). The effects of TM treatments on amino acid metabolism in the blood, brain, and other tissues of layer embryos need to be investigated in the future, which is expected to provide important clues for the potential agent (s) in affording stress-resistance in layer chicks and hens.

Conclusion

In summary, TM of high or low incubation temperature during the middle stages of embryogenesis caused different modifications in embryonic growth, incubation duration, and hatching process in layer strain. Interestingly, the changes in hepatic amino acid concentrations were similar after both cold and heat exposure in layer embryos. Future studies will investigate the effects of TM treatments on post-hatch performance in layer-type chicks and hens.

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

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

Author contributions

GH and CL designed this research. GH, SL, and YL conducted animal experiments. GH, SL, PT, and TB performed the sample analysis and statistical analysis. GH and VC wrote the manuscript. MF, ZB, and CL reviewed and edited the manuscript. All authors read and approved the final manuscript.

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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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Effects of *Lactobacillus plantarum* and *Pediococcus acidilactici* co-fermented feed on growth performance and gut microbiota of nursery pigs

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The fermented feed has been used extensively as a growth promoter in agricultural animal production. However, the effects of fermented feed on swine gut microbiota are still largely unknown. The work presented here aimed to investigate the growth performance and gut microbiota of nursery pigs receiving the LPF diet (10% *Lactobacillus plantarum* and *Pediococcus acidilactici* co-fermented feed + basal diet) compared with pigs receiving the NC diet (basal diet). The data showed LPF diet numerically improved average daily gain and significantly increased fecal acetate, butyrate, and total short-chain fatty acid (SCFA) concentrations. Furthermore, gut microbiota structure and membership significantly changed in response to the addition of fermented feed in the diet. Gut microbiota results indicated that LPF treatment significantly enriched SCFA-producing bacteria such as *Megasphaera*, *Roseburia*, *Faecalibacterium*, *Blautia*, *Selenomonas*, *Dialister*, *Acidaminococcus*, *Ruminococcus*, and *Bifidobacterium*. Some of these bacteria also had anti-inflammatory and other beneficial functions. Overall, these findings suggested that *Lactobacillus plantarum* and *Pediococcus acidilactici* co-fermented feed benefited growth performance and established potential health impacts on the gut microbiota of nursery pigs.

KEYWORDS

fermented feed, *Lactobacillus plantarum*, *Pediococcus acidilactici*, nursery pig, growth performance, gut microbiota

Introduction

Nowadays the beneficial effects of fermented feed on animal growth performance and health have received increasing attention. Fermentation is a metabolic process that produces biochemical changes in the primary food matrix through the action of microorganism enzymes (1). The importance of fermented feed is to improve the nutrient digestibility of raw material (e.g., corn and soybean meal) and increase the availability of vitamins and minerals by reducing the level of anti-nutritional factors (2). Apart from improved nutritional quality, fermented feed could benefit animal growth performance by boosting immune function and improving intestinal morphology (3, 4). Zhou et al. reported that fermented feed not only increased serum immunoglobulin levels but also improved lymphocyte proliferation and transformation (5). Another study revealed that supplementing piglet diets with fermented soybean meal significantly increased both villus height and villus: crypt ratio, which enlarged intestinal surface area and thereby improved nutrition uptake ability (6). Furthermore, Kiers et al. determined that fermented soybeans promoted feed intake and weight gain, as well as reduced diarrhea incidence of weaned piglets challenged with *Escherichia coli* (7).

Evidence is accumulating to demonstrate that incorporating fermented feed into swine diets has many beneficial effects on intestinal microbiota. Lactic acid-producing bacteria such as *Lactobacillus plantarum* and *Pediococcus acidilactici* are commonly used for fermentation, which generates a large amount of lactic acid and lowers the pH of the feed. Thus, the fermented feed can inhibit pathogenic bacterial growth, deliver probiotics, and prevent pathogenic bacteria from attaching to the intestinal walls (8). Studies have shown that fermented products reduced the proliferation of certain enteropathogens like *E. coli* and *Salmonella* in both swine and broiler (4, 8).

Although previous studies have remarkably expanded our knowledge regarding the impacts of fermented feed on gut microorganisms, they delivered information only on a limited set of microbial taxa. A gap in the understanding of how fermented feeds modulate the entire complex gut microbial ecosystem still exists. In this study, we tested *Lactobacillus plantarum* and *Pediococcus acidilactici* co-fermented feed and applied next-generation sequencing technology to achieve a depth insight into how the gut microbiota of nursery pigs evolves under the influence of fermented feed.

Materials and methods

Animal management and care followed the Institute of Animal Husbandry and Veterinary Medicine of Hebei Province Animal Care and Use Committee guidelines (IAHVM20190910-1, Hebei, China).

Animals and experimental design

This study was carried out at a commercial swine farm (Zhangjiakou, China). On the weaning day, a total of 32 piglets (body weight 14.65 ± 0.46 kg; 25 ± 1 d of age) were transferred to a nursery facility and were randomly assigned to the negative control (NC, $n = 16$) or fermented feed (LPF, $n = 16$) groups. The ambient temperature was set at 30°C upon pig arrival and was reduced 2°C per week until a 24°C setting was achieved. Each pen was fully slatted (1.8 × 2.0 m^2) and was equipped with a nipple drinker and a feeder for *ad libitum* access to diets and water. Each pen housed eight pigs. After a 14-day adaption period, a 31-day feeding trial was conducted. All pigs were supplied with a common diet during the adaption period and then switched to the experimental diets at 39-day old. NC diet: basal diet; LPF diet: NC + 10% *Lactobacillus plantarum* and *Pediococcus acidilactici* co-fermented feed (M108, Dacheng (Wanda) Tianjin Co., Ltd, China). Diets meet or exceed the NRC (9) nutrient requirements and the Supplementary Tables S1, S2 detailed the basal diet and LPF diet formulations.

Data recording and sample collection

Individual pig body weight (BW) was measured at the beginning and the end of the study. Each dietary treatment data was used to calculate average daily gain (ADG).

Blood samples ($n = 5$) were collected into tubes *via* jugular vena cava from randomly selected pigs (69-day-old) at the end of the study and were assayed for total protein (TP), blood urea nitrogen (BUN), glutamate oxaloacetate transaminase (GOT), glutamate pyruvate transaminase (GPT), and superoxide dismutase (SOD). At the same time, ~1 g ($n = 4$) and 2 g ($n = 10$) rectal swab samples for individual animals were randomly collected for short-chain fatty acid analysis and gut microbiota analysis, respectively. Samples were temporally kept on dry ice and then stored in an ultra-low temperature freezer until further analysis.

Growth performance data analysis

Independent Samples *T*-Test program in IBM SPSS 22.0 statistical software was used for data statistical analysis and each animal served as the experimental unit. The probability value of $p < 0.05$ was considered significant.

16S rRNA sequencing and data analysis

Microbial genome DNA was extracted from the fecal samples using the DNeasy PowerLyzer PowerSoil Kit (Qiagen, Germantown, MD, USA), following the manufacturer's

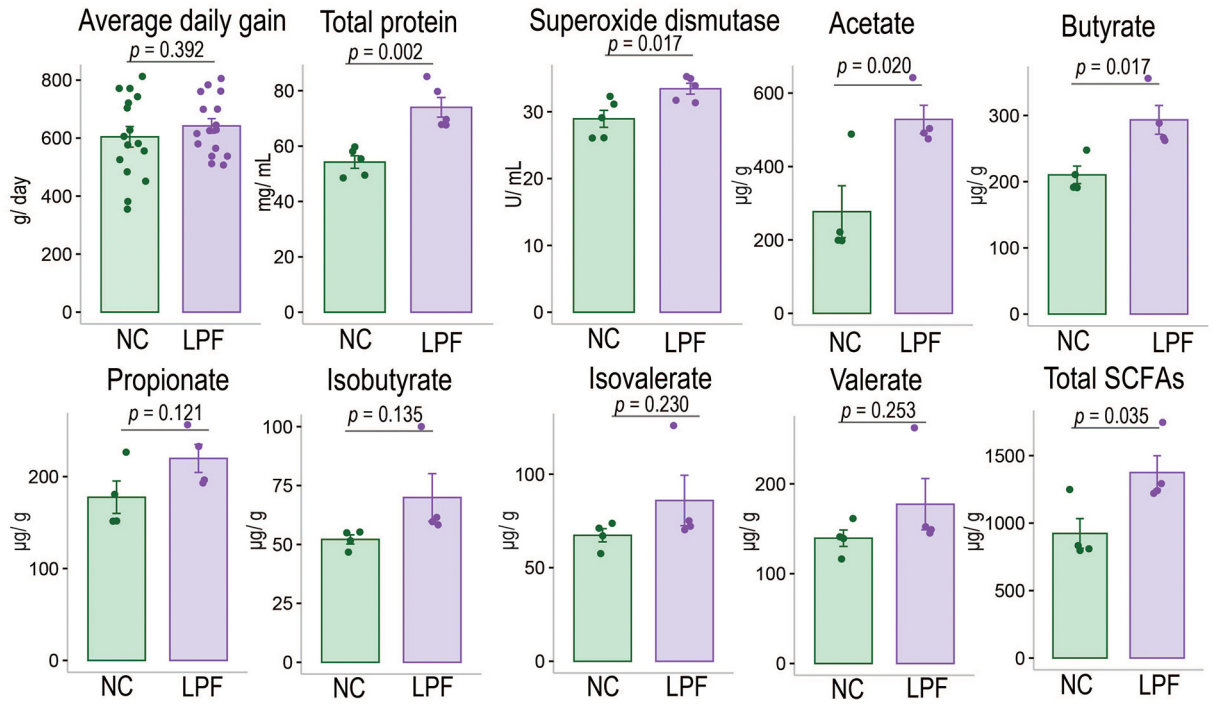

FIGURE 1
Effects of LPF diet on average daily gain, serum indexes, and fecal SCFAs of nursery pigs compared with NC diet. NC diet: basal diet, LPF diet: NC + 10% *Lactobacillus plantarum* and *Pediococcus acidilactici* co-fermented feed.

instructions. DNA concentration and purity were decided by NanoDrop One (Thermo Fisher Scientific, Madison, WI, USA) and then diluted to 20 ng/μL for downstream application.

The 16S rRNA gene hypervariable regions V3–V4 were used to identify bacteria and were amplified using primers 341F (5′-ACTCCTACGGGAGGCAGCA-3′) and 806R (5′-GGACTACHVGGGTWTCTAAT-3′). PCR reaction conditions are initial denaturation at 98°C for 2 min, followed by 30 cycles, including denaturation at 98°C for 15 s, annealing at 55°C for 30 s, and extension at 72°C for 30 s, with a final extension at 72°C for 5 min and 4°C hold. Agarose gel (1.2%) electrophoresis was applied to assess the success of PCR reactions. In addition, VAHTS DNA Clean Beads (Vazyme Biotech, Nanjing, Jiangsu, China) and Quant-iT PicoGreen dsDNA Assay Kit (Invitrogen, Carlsbad, CA, USA) were used to purify and quantify the amplicons, respectively. Purified PCR amplicons were then pooled together to generate a sequencing library. Agilent 2100 Bioanalyzer (Agilent, Santa Clara, CA, USA) and the Quant-iT PicoGreen dsDNA Assay Kit were applied to detect the quality and the concentration of the library, respectively. To detect potential bias introduced during PCR amplification and the MiSeq run, a mock community [ZymoBIOMICS^{TM}Microbial Community Standard (Zymo, Irvine, CA, USA)] was included in the sequencing library as a standard. Finally, the library was sequenced on the Illumina MiSeq sequencer with MiSeq Reagent Kit V3 (600 cycles) to generate paired-end reads.

The fastq files downloaded from the Illumina sequencer were analyzed using the QIIME2 (2019.4 release) microbiome bioinformatics platform (10). QIIME 2 plugin DADA2 processed sequencing data including quality control, denoising, merging, and removing chimera as well as singleton, and generated a feature table for the downstream analysis (11). Greengenes reference database (V13_8) trained Naive Bayes classifier was used to annotate sequences (12, 13).

Alpha diversity and beta diversity were estimated in QIIME2 at a sub-sampling depth of 62380 sequences for each sample. The analysis of similarity (ANOSIM) was performed to compare the dissimilarity between the treatments. In addition, the most differentially abundant bacteria between treatments were identified by LEfSe (Linear discriminant analysis Effect Size, LDA score > 2) at each taxonomic rank. Random forest R package with default setting was used to identify microbial signatures that best differentiate treatments at the feature level (14).

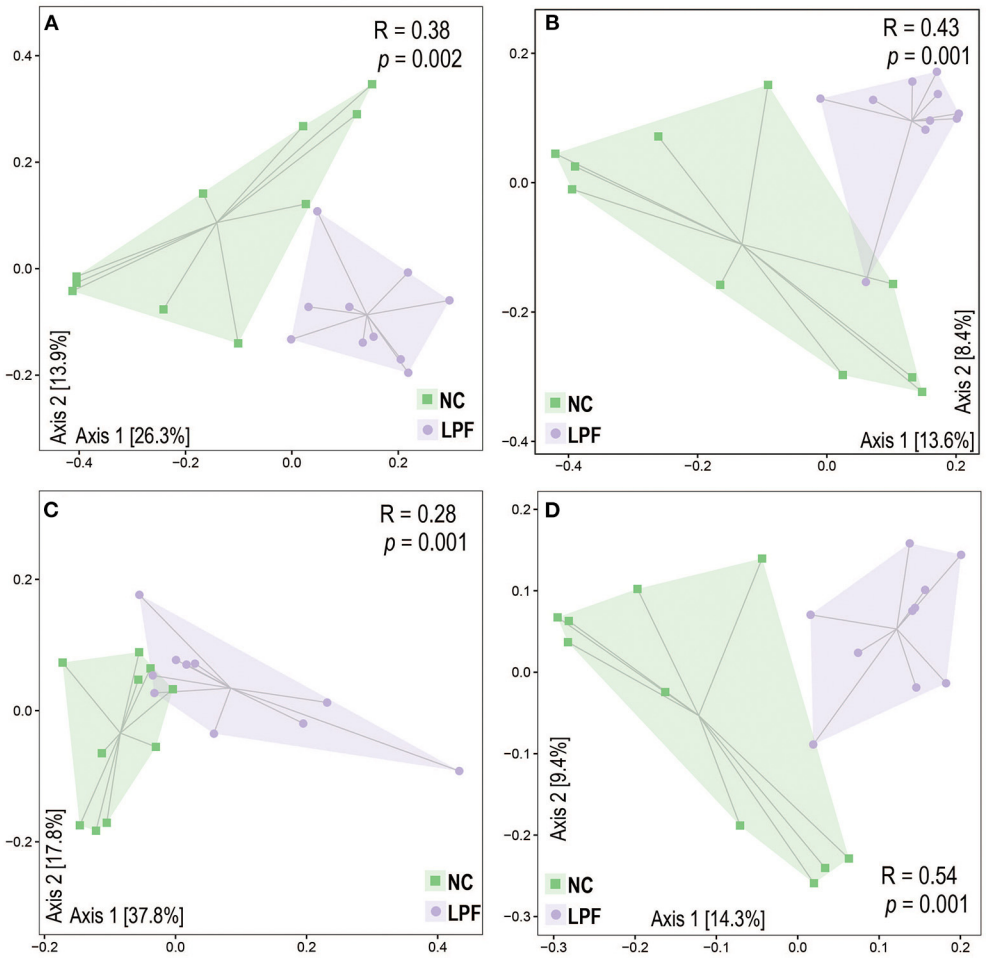

FIGURE 2
LPF diet modulated gut microbiota beta diversity based on **(A)** Bray-Curtis, **(B)** Jaccard, **(C)** Weighted UniFrac, and **(D)** Unweighted UniFrac distances. The analysis of similarity (ANOSIM) was applied to estimate the dissimilarity between NC and LPF treatments. NC diet: basal diet, LPF diet: NC + 10% *Lactobacillus plantarum* and *Pediococcus acidilactici* co-fermented feed.

Results

The effects of co-fermented feed on swine growth performance

Experimental data such as BW was provided in Supplementary Table S3. The results indicated that the LPF diet numerically improved average daily gain ($p = 0.392$) and significantly improved the TP concentration ($p = 0.002$) as well as SOD ($p = 0.017$) compared with the NC diet (Figure 1). We also found that the LPF diet had no significant impacts on BUN, GPT, and GOT (Supplementary Table S4).

As shown in Figure 1, the LPF diet numerically improved the concentrations of propionate, isobutyrate, isovalerate, as well as valerate ($p > 0.05$) and significantly improved acetate ($p = 0.020$), butyrate ($p = 0.017$), and total SCFAs ($p = 0.035$) compared with NC diet (more detailed information are provided in Supplementary Table S5).

The influence of co-fermented feed on gut microbial diversity

The gut microbiota alpha diversity was measured by the Shannon index, Observed_species, and Chao1, however, there were no significant differences between the treatments (Supplementary Figure S1). Beta diversity, including Bray-Curtis, Jaccard, Weighted Unifrac, and Unweighted Unifrac distances, was used to determine gut microbiota structural changes in response to co-fermented feed (Figure 2). The gut microbiota profiles of the NC and LPF groups were distinctly different. Principal coordinate analysis (PCoA) based on Bray-Curtis dissimilarity and Jaccard distance showed remarkable

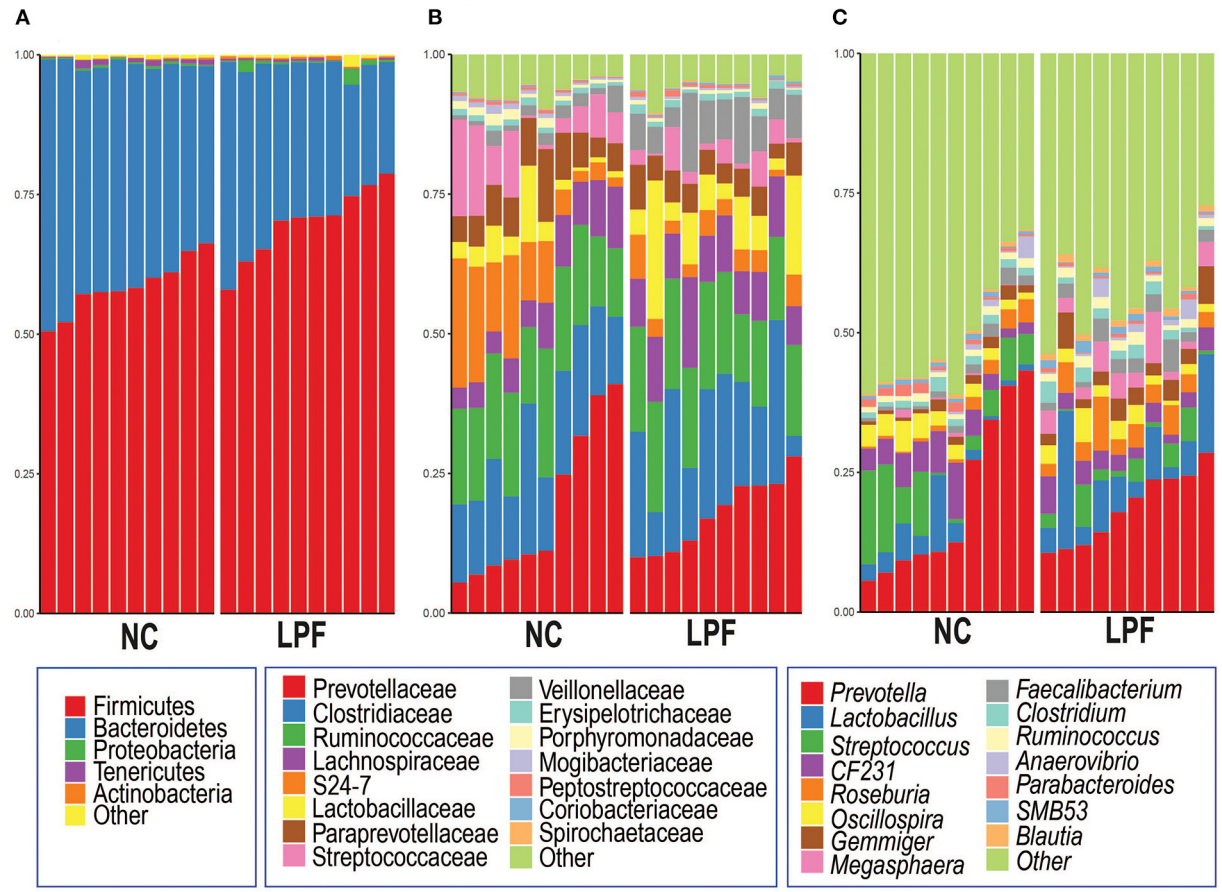

FIGURE 3
Gut microbiota compositions at **(A)** phylum, **(B)** family, and **(C)** genus levels for each treatment. NC diet: basal diet, LPF diet: NC + 10% *Lactobacillus plantarum* and *Pediococcus acidilactici* co-fermented feed.

clusters for each experimental group. PCoA based on the Weighted and Unweighted Unifrac distances also revealed the distinct changes induced by co-fermented feed. The analysis of similarities (ANOSIM) confirmed the pattern that the swine gut microbiome was significantly different between NC and LPF treatments (Bray-Curtis: $R = 0.38$, $P = 0.002$; Jaccard: $R = 0.43$, $P = 0.001$; Weighted Unifrac: $R = 0.28$, $P = 0.001$; and Unweighted Unifrac: $R = 0.54$, $P = 0.001$).

Gut microbiota composition changes induced by co-fermented feed

At the phylum level, the two treatment groups had a similar pattern that the dominant phyla Firmicutes and Bacteroidetes accounted for more than 95% of total sequences (Figure 3A). The top 15 bacteria at the family level are shown in Figure 3B, the most represented bacteria are Prevotellaceae, Clostridiaceae, and Ruminococcaceae in both groups. But the subdominant gut microbiota component varied at different treatments. For example, S24-7 and Streptococcaceae, the subdominant gut microbiota component in NC (10.7 and 7.4%, respectively) group, strikingly decreased in the LPF (3.8 and 3.1%, respectively) treatment. In addition, the relative abundance of Veillonellaceae was dramatically promoted by the LPF diet compared to that in the NC diet (7.5 vs. 1.9%). At the genus level (Figure 3C), *Prevotella* is the predominant bacteria in both groups (NC: 20.1% and LPF: 18.7%) among the top 15 genera. The relative abundance of *Lactobacillus* (8.6 vs. 3.8%), *Roseburia* (4.2 vs. 1.6%), *Gemmiger* (3.5 vs. 1.4%), *Megasphaera* (3.8 vs. 0.3%), and *Faecalibacterium* (2.7 vs. 1.3%) were largely enriched by the LPF diet compared to the NC diet. However, *Streptococcus* (7.2 vs. 2.9%) dramatically decreased in the LPF treatment compared to the NC group.

A heatmap with cluster analysis showed the abundance of the top 50 bacterial taxa at the genus level, revealing visible compositional differences between piglets that received LPF feed and those fed the NC diet (Figure 4). Complete linkage hierarchical clustering based on Euclidean distance generated a separation of LPF and NC treatments, except for one sample

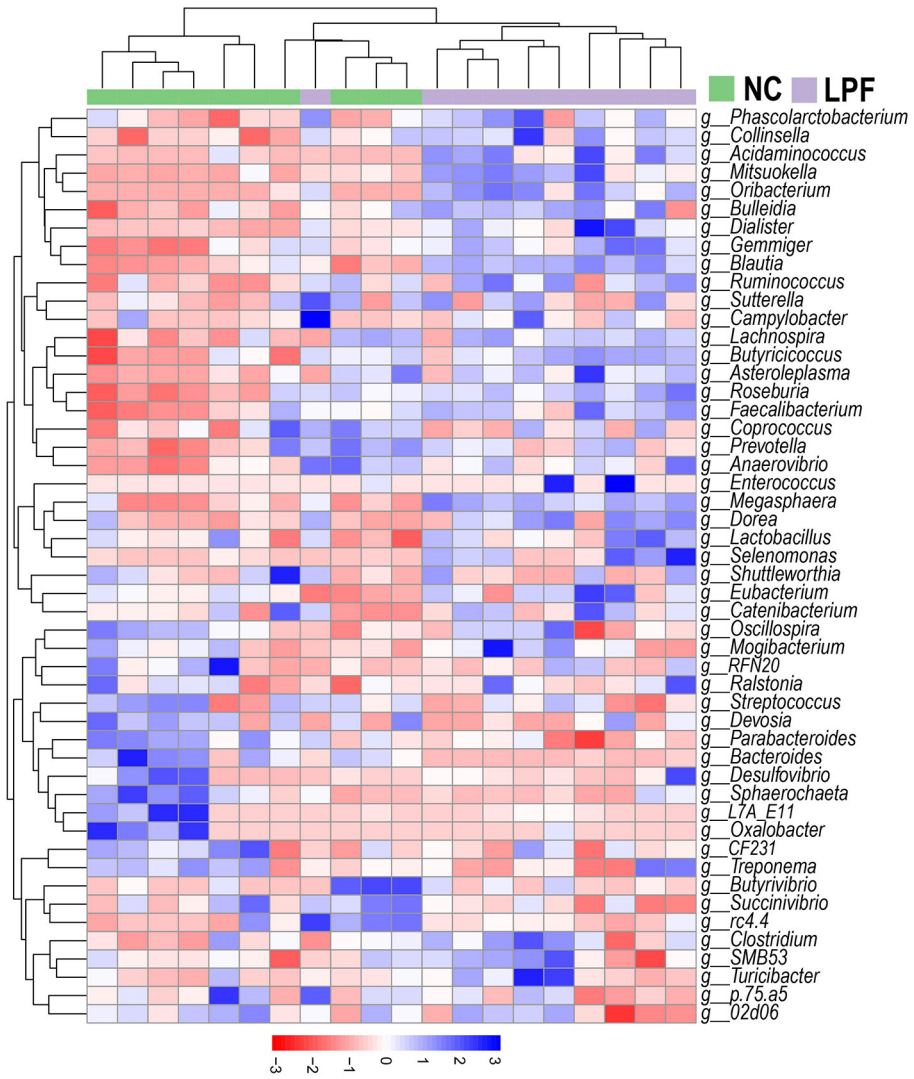

FIGURE 4
Heatmap of the relative abundance of top 50 genera. Clusters based on Euclidean distance using complete linkage clustering. NC diet: basal diet, LPF diet: NC + 10% *Lactobacillus plantarum* and *Pediococcus acidilactici* co-fermented feed.

from the LPF group joined the cluster containing samples from the NC group.

Linear discriminant analysis of the gut microbiota

We next performed LEfSe analysis to detect the most differentially abundant bacterial taxa between the NC and the LPF groups. A total of 44 represented bacterial taxa were identified (Figure 5A). Many biomarker genera like *Megasphaera*, *Roseburia*, *Faecalibacterium*, *Blautia*, *Selenomonas*, *Dialister*, *Acidaminococcus*, *Ruminococcus*, and *Bifidobacterium* were significantly more abundant in the LPF group compared with the NC group, whereas the relative abundance of *Bacteroides* was distinctly decreased by the LPF diet (Figure 5B).

For the other bacteria such as *Gemmiger*, *Phascolarctobacterium*, *Peptococcus*, *Collinsella*, *Butyricicoccus*, *Oribacterium*, *Dorea*, *Bulleidia*, *Mitsuokella*, Veillonellaceae, OPB56, Tremblayales, Actinobacteria, Coriobacteriia, Coriobacteriales, Coriobacteriaceae, Actinobacteria, Campylobacterales, Epsilonproteobacteria, Bifidobacteriales, and Bifidobacteriaceae were also higher in the LFP group, whereas the NC group had significantly more abundant *Epulopiscium*, *Devosia*, *Anaerovorax*, *Parabacteroides*, Bacteroidia, Ruminococcaceae, Lachnospiraceae,

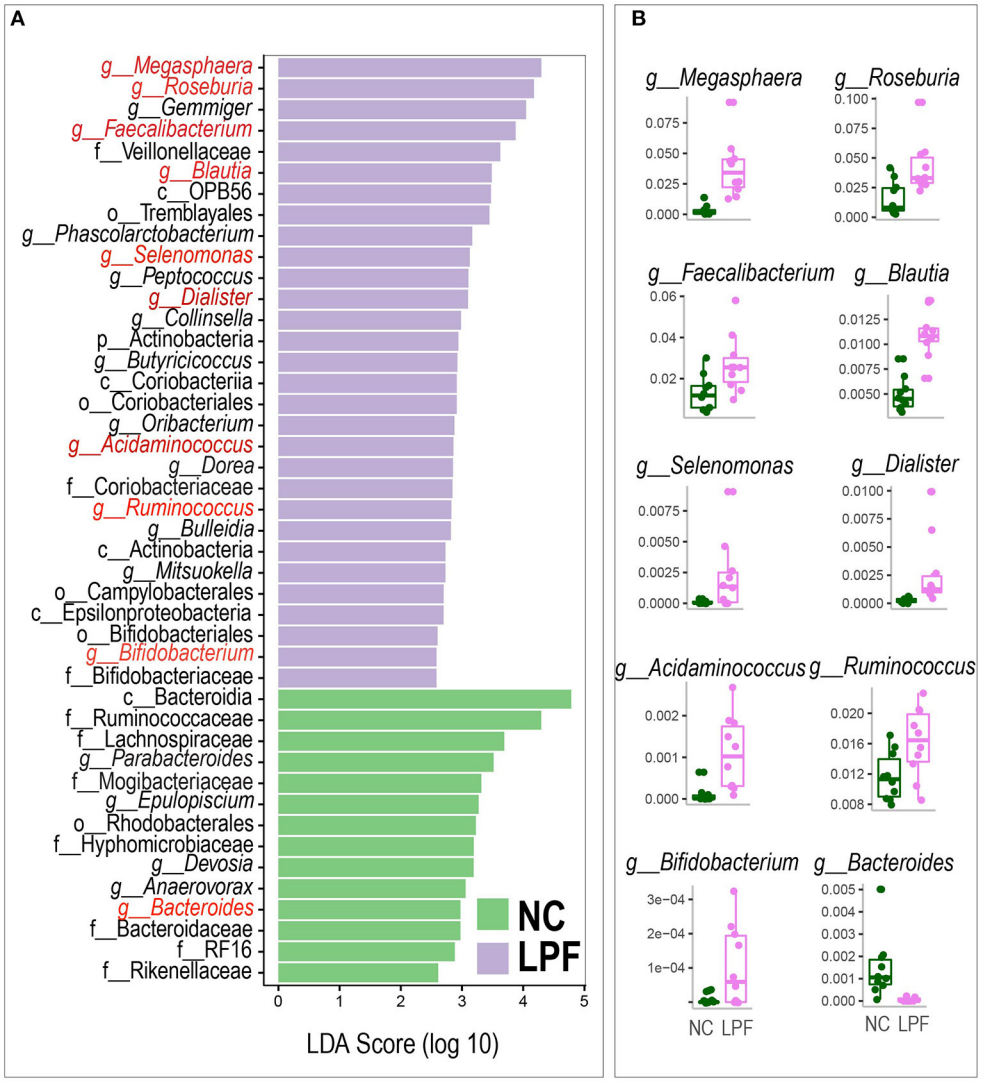

FIGURE 5
(A) Linear discriminant analysis effect size (LefSe) of significantly different relative abundant bacterial taxa between groups. **(B)** Relative abundance of the important bacteria selected by LEfSe. NC diet: basal diet, LPF diet: NC + 10% *Lactobacillus plantarum* and *Pediococcus acidilactici* co-fermented feed.

Mogibacteriaceae, Rhodobacterales, Hyphomicrobiaceae, Bacteroidaceae, RF16, and Rikenellaceae.

Gut microbiota signature of pigs fed with co-fermented diet

Microbial signatures that best differentiate the NC and LPF treatments were identified by random forest at the species level. The relative abundances of the top 500 bacterial features were included in the random forest model and the top 20 bacterial features that best predicted treatment are listed in Figure 6A. The relative abundances of these features for individual piglets are visualized on a heatmap (Figure 6B). Two members of *Lactobacillus* (Features #50 and #124) and *Blautia* (Features #224 and #440) were more abundant in the LPF group.

Discussion

In the current study, the supplementation of 10% *Lactobacillus plantarum* and *Pediococcus acidilactici* co-fermented feed numerically increased the ADG, which is in accordance with previous reports that dietary fermented feed exhibited beneficial effects on swine production (15–18). However, several studies showed inconsistent results on the effects of fermented feed on growth performance. For example,

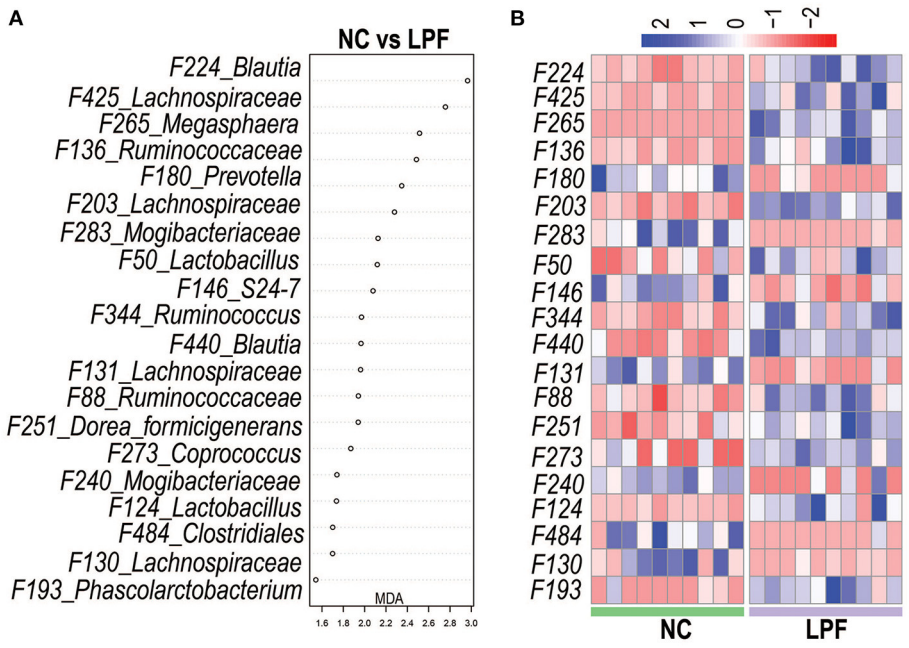

FIGURE 6
Gut microbiota signature of the pigs fed with LPF diet was determined by Random Forest. **(A)** Top 20 most predictive features that differentiate LPF-fed pigs from those fed with NC diet. **(B)** Heatmap shows the relative abundance of Features (log 10 transformed) selected by Random Forest. NC diet: basal diet, LPF diet: NC + 10% *Lactobacillus plantarum* and *Pediococcus acidilactici* co-fermented feed.

Le et al. reported that *Lactobacillus reuteri* fermented wheat failed to improve the growth performance of weaned piglets (19). Liu et al. also observed that *Bacillus subtilis* fermented corn bran had no effects on ADFI and ADG of finishing pigs (20). The contradictory results could, at least in part, be explained by the different experimental animal ages and the different composition of fermented substrates, probiotic strains, as well as addition amount applied to the diet.

Weaning is the most stressful event in pig life, which could cause adverse impacts on gut health, such as villous atrophy, crypt hyperplasia, increased gut permeability, and intestinal inflammation (21). However, the SCFAs are considered beneficial to the gut and thus could help reduce weaning stress. A study conducted by Diao et al. demonstrated that SCFAs improved intestinal barrier function and reduced *E. coli* count in the ileal digesta in weaned piglets. Other studies confirmed that SCFAs help maintains gut barrier integrity (22, 23), which prohibits pathogens, toxins, or food proteins to pass into the blood. In addition, SCFAs have beneficial immune system effects in the intestinal mucosa (24, 25). Butyrate, a four-carbon short-chain fatty acid, is a primary energy source for intestinal epithelial cells and is anti-inflammatory (26). A study showed that butyrate improved the growth performance of weaning pigs fed diets containing 0.5% benzoic acid (27). Researchers also found that a mixture of SCFAs (propionic and formic) and capric acid significantly improved the growth performance of piglets (28). Overall, these findings suggested that SCFAs could reduce the detrimental effects of weaning stress and improve the growth performance of piglets. In the current study, the significantly increased acetate, butyrate, and total SCFAs may contribute to improved growth performance.

Consistent with previous studies (27, 29), our data showed that the Firmicutes and Bacteroidetes were the two dominant phyla and *Prevotella* was the most abundant genus in the gut microbiota of nursery piglets. The abundance of *Prevotella* might be linked with the diet style. Studies have shown that the relative abundance of *Prevotella* strikingly increased after the dietary transition from sow milk to corn/soybean meal-based diets (27). A human study also found that the relative abundance of *Prevotella* was associated with dietary habits and a high percentage of *Prevotella* could be a consequence of high fiber intake, improving metabolic energy absorption from consumed plant polysaccharides (30). These indicated that the gut microbiota coevolved with the diet.

LEfSe analysis was applied to identify the microbiota that was significantly changed by the LPF diet. Compared with the NC diet, LPF treatment significantly enriched SCFA-producing bacteria such as *Megasphaera*, *Roseburia*, *Faecalibacterium*, *Blautia*, *Selenomonas*, *Dialister*, *Acidaminococcus*, *Ruminococcus*, and *Bifidobacterium* (31–41). Some of those bacteria also have other beneficial functions, for example, *Megasphaera* could enhance large intestine functions and effectively prevent hyper-lactate accumulation-related diarrhea (42), *Roseburia* has benefits from immune modulation to

inflammatory regulation (43), *Blautia* and *Faecalibacterium* had anti-inflammatory functions to help support gut health (44–46). In addition, this study indicated that the LPF diet selectively decreased the relative abundance of *Bacteroides*, an acetate producer (47). It may be that co-fermented feed additive prohibited the growth of *Bacteroides* directly or assisted the growth of antagonistic species against *Bacteroides* within the gastrointestinal tract. Taken together, our data indicated that the LPF diet improved the relative abundance of bacteria with potential probiotic properties.

In conclusion, this study demonstrated that the *Lactobacillus plantarum* and *Pediococcus acidilactici* co-fermented feed additive improved weaning pigs' growth performance, modulated gut microbiota diversity and composition, leading to enrichments of SCFA-producing bacteria.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found at: https://www.ncbi.nlm.nih.gov/, https://www.ncbi.nlm.nih.gov/bioproject/PRJNA891080.

Ethics statement

The animal study was reviewed and approved by Institute of Animal Husbandry and Veterinary Medicine of Hebei Province Animal Care and Use Committee.

Author contributions

YY, XWe, and QZ conceived and designed this experiment. YY, GY, XM, XWa, GL, ZZ, and SZ collected rectal samples and growth performance data. XWe and YY analyzed the data. XWe, YY, and QZ drafted and revised the manuscript with input from GY, XM, ZZ, SZ, GL, and XWa. All authors read and approved the final manuscript.

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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.2022.1076906/full#supplementary-material

SUPPLEMENTARY FIGURE S1
Alpha diversity for different dietary supplements was measured by **(A)** Shannon index, **(B)** Observed species, and **(C)** Chao1.

SUPPLEMENTARY TABLE S1
Composition and nutrient levels of basal diets (air-dry basis).

SUPPLEMENTARY TABLE S2
Composition of *Lactobacillus plantarum* and *Pediococcus acidilactici* co-fermented feed.

SUPPLEMENTARY TABLE S3
Effects of fermented feed additive on growth performances of nursery pigs.

SUPPLEMENTARY TABLE S4
Effects of fermented feed additive on serum chemistry of nursery pigs.

SUPPLEMENTARY TABLE S5
Effects of fermented feed additive on short chain fatty acid concentration in fecal samples.

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Effects of dietary supplementation with quercetagetin on nutrient digestibility, intestinal morphology, immunity, and antioxidant capacity of broilers

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Quercetagetin (QG) is gaining increased attention as a potential alternative to in-feed antioxidants due to its antioxidant activity. This experiment was conducted to investigate the effects of dietary supplementation with QG on nutrient digestibility, intestinal morphology, immunity, and antioxidant capacity of broilers. Four hundred 1-day-old Ross 308 broilers were randomly assigned into 4 groups with 10 replicates in each group and 10 broilers in each replicate. The four dietary treatments included the basal diet supplemented with 0, 3.2, 4.8, or 6.4 mg/kg QG. The results showed that dietary supplementation with QG significantly promoted the broilers' apparent digestibility of phosphorus ($P < 0.05$), increased the villus height in jejunum and ileum, and reduced the crypt depth in jejunum and ileum, which significantly increased the ratio of villus height to crypt depth in the jejunum and ileum ($P < 0.05$). The dietary supplementation with QG also significantly enhanced the immunoglobulin G (IgG) and complement 4 (C4) levels in the blood ($P < 0.05$), the activity of total antioxidant capacity (T-AOC) in serum, jejunum mucosa, and ileum mucosa, the activity of superoxide dismutase (SOD) in the serum and liver ($P < 0.05$), and significantly up-regulated the kelch-like ECH-associated protein 1 (*Keap1*), nuclear factor E2 related factor 2 (*Nrf2*), heme oxygenase-1 (*HO-1*), NAD(P)H: quinone oxidoreductase 1 (*NQO-1*), glutathione peroxidase (*GSH-Px*) and superoxide dismutase 1 (*SOD1*) mRNA expression levels in the jejunum mucosa, ileum mucosa, and liver tissues of broilers. Therefore, supplementing broilers' diets with QG can enhance the apparent digestibility of phosphorus, improve the structure and morphology of jejunum and ileum, promote immunity, and increase the activity of antioxidant enzymes and the antioxidantive capacity through the Nrf2/antioxidant response element (ARE) signaling pathway mediated by Keap1.

KEYWORDS

quercetagetin, broiler, nutrient digestibility, intestinal morphology, immunity, antioxidant capacity

1. Introduction

Normally, fewer free radicals are produced of poultry, which can be timely eliminated by the antioxidant system to prevent oxidative stress and injury (1). However, with the increase in poultry production scale and intensity, various factors, such as excessive feeding, lower feeding management, unstable feed quality (excessive metal element content, mycotoxin pollution, and lipid oxidation), and pathogen infestation increase the free radical production in poultry through the respiratory burst mechanism, which exceeds the scavenging capacity of the antioxidant system, triggering a free radical chain reaction, which leads to oxidative stress, and damage (2–7). In chicks, oxidative stress also reduces disease resistance, survival rate, feed return, and production performance and causes maldevelopment. In adult chickens, oxidative stress reduces immunity, lowers product quality, and decreases production performance (8, 9).

Enhancing the anti-stress potential and reducing the negative effects of oxidative stress on poultry production is the key to transforming, upgrading, and developing a green and high-quality poultry breeding industry. Quercetagetin ($C_{15}H_{10}O_8$, QG) is a flavonol compound chemically identified as 3,3,4,5,6,7-hexahydroxyflavone extracted from marigold (*Tagetes erecta* L.) (10). QG is a safe, efficient, and economical natural antioxidant (11), with strong scavenging activities against hydroxyl radical (OH·), 2,2 -biphenyl-1-picrylhydrazino (DPPH), and 2,2′- diazabibis (3-ethylbenzothiazolin-6-sulfonic acid) diammonium salt (ABTS) (12).

QG has greater development potential and research value in alleviating oxidative stress and improving the antioxidant capacity of poultry. However, there are few reports on the influence of QG on broiler production. Therefore, this study evaluated the effects of different QG dosages as dietary supplements on nutrient digestibility, intestinal morphology, immunity, and antioxidant capacity of broilers. The findings in this study will provide a reference for the application of QG in broiler production.

2. Materials and methods

2.1. Experimental diet

Quercetagetin (>80% purity) was purchased from Chenguang Biotech Group Co., Ltd. (Handan, China). The basal diet was formulated according to National Research Council (NRC) (13). The composition and nutritional levels of the basal diet is shown in Table 1.

TABLE 1 Composition and nutrient levels of the basal diet (air-dry basis, %).

Item	Trial period	
	1–21 d	22–42 d
Ingredients		
Corn	60.00	64.37
Soybean meal	28.37	25.20
Fishmeal	5.00	2.00
Vegetable oil	3.00	5.00
$CaHPO_4$	1.40	1.20
Limestone	1.20	1.30
$C_5H_{14}CINO$	0.10	0.10
Premix[a]	0.18	0.18
Lysine	0.35	0.25
Methionine	0.15	0.10
NaCl	0.25	0.30
Total	100.00	100.00
Nutrient levels[b]		
Metabolic energy (MJ/kg)	13.25	13.74
Crude protein	21.35	20.02
Calcium	1.00	0.95
Available phosphorus	0.48	0.44
Total phosphorus	0.68	0.65
Lysine	1.16	1.03
Methionine	0.47	0.40

[a]The premix provided the following per kg of diets: vitamin A, 10,000 IU; vitamin D, 34,000 IU; vitamin E, 28 mg; vitamin K3, 3 mg; vitamin B1, 4 mg; vitamin B2, 10 mg; vitamin B6, 6 mg; vitamin B12, 0.08 mg; niacin, 1,000 mg; pantothenic acid, 18 mg; pyridoxine, 8 mg; folic acid, 1 mg; biotin, 0.3 mg; $C_5H_{14}ClNO$, 600 mg; Fe, 60 mg; Cu, 10 mg; Zn, 80 mg; Mn, 90 mg; I, 1 mg; Se, 0.3 mg.
[b]Metabolic energy, Crude protein, Calcium and Total phosphorus were calculated value, while the others were measured values.

2.2. Experimental design, animal, and management

Four hundred 1-day-old Ross 308 broilers were randomly assigned into 4 groups (three experimental groups and control) with 10 replicates in each group and 10 broilers in each replicate. The control group was fed with the basal diet, while the experimental groups were fed on the basal diet supplemented with 3.2, 4.8, and 6.4 mg/kg QG (calculated based on a 100% purity). The broilers were reared for 42 d.

The experimental protocols were approved by the Animal Care and Use Committee of Hebei Agriculture University (Baoding, China).

All animal experiments complied with the ARRIVE guidelines were carried out in accordance with the U.K. Animals

(Scientific Procedures) Act, 1986 and associated guidelines, EU Directive 2010/63/EU for animal experiments.

2.3. Determination indexes and methods

2.3.1. Nutrient digestibility

The nutrient digestibility of broilers in each group was measured using the endogenous indicator method (acid-insoluble ash). Fresh feces were collected from each group five days to the end of the experimental period. The feces were weighed and assigned to two fecal samples. One sample was mixed with 10% hydrochloric acid to determine the level of nitrogen fixation and crude protein content. The second sample was used to detect the contents of other nutrients. All fecal samples were dried, crushed, and placed into a sample bottle for subsequent testing. The chemical composition of the ingredients was determined as proposed by AOAC (14). The basal diet nutrient composition in each group was also determined. The apparent digestibility of nutrients in each group was determined as follows:

$$\text{Nutrient digestibility } \% = 100 - 100(b \times c) \div (a \times d) \times 100.$$

Where a is the content of X nutrient in feed; b is the content of X nutrient in fecal samples; c is the content of acid-insoluble ash in feed; d is the content of acid-insoluble ash in fecal samples.

2.3.2. Indicators in serum and tissue

At the end of the experimental period, the broilers were fasted for 12 h; then, one chicken was randomly selected from each replicate for blood collection (10 mL) from the wing vein. The blood samples were collected in vacutainers and centrifuged at 3,000 ×g at 4°C for 10 min to separate the serum. Serum samples were stored at −20°C until further analysis. In addition, one chicken/replicate was randomly selected and euthanized by cervical dislocation. The jejunum, ileum, and liver samples were collected using surgical instruments that had been rigorously sterilized, then cryopreserved at −80°C for testing. The levels/activities of immunoglobulin A (IgA), immunoglobulin G (IgG), immunoglobulin M (IgM), complement 3 (C3), complement 4 (C4), total antioxidant capacity (T-AOC), glutathione peroxidase (GSH-Px), superoxide dismutase (SOD), and malondialdehyde (MDA) in the blood and tissues were determined to use ELISA kits [Nanjing Jiancheng Bioengineering Institute (Nanjing, China)] according to the manufacturer's instructions.

2.3.3. The jejunum and ileum tissues morphology

The jejunum and ileum tissues were picked under aseptic conditions and fixed using a 10% neutral formaldehyde fixing solution. The fixed tissues were dehydrated using a full-automatic dehydrator (TSJ-II, Zhongshan, Changzhou, China). After embedding and slicing, the following operations were performed: the fixed tissues were subjected to slice dewaxing. After that, it was put in hematoxylin stain for 15 min and rinsed with tap water for 2 min. Thereafter, hydrochloric acid alcohol differentiation was done for 10 s, rinsed with tap water for 2 min, and put in warm water at 50°C until the solution turned blue. It was then rinsed with tap water for 2 min, and put in 85% alcohol for 4 min. The tissues were then stained with eosin for 4 min, washed with distilled water for 5 s. The tissues were then dehydrated using gradient alcohol, made transparent using xylene, and sealed with neutral gum. The trinocular biological microscope camera system (BA200 digital, motic, Xiamen, China) was used for slice observation and image acquisition. All tissues of each section were observed using a microscopic camera system at ×40, and the tissue images were taken at ×100 and ×400.

2.3.4. Relative gene expression in the tissues

The relative content of mRNA of tissue samples were detected by quantitative real-time PCR (qRT-PCR). The specific primers were designed using primer 6.0 software based on the gene sequences of kelch-like ECH-associated protein 1 (*Keap1*), nuclear factor E2 related factor 2 (*Nrf2*), heme oxygenase-1 (*HO-1*), NAD(P)H: quinone oxidoreductase 1(*NQO-1*), *GSH-pX*, superoxide dismutase 1 (*SOD1*), glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) in the GenBank and were synthesized by Bioengineering Co., Ltd. (Shanghai, China) (Table 2).

The total RNA was extracted using 50–100 mg of the jejunal, ileal and hepatic samples, respectively, following the Trizol reagent according to the manufacture's instruction (Invitrogen, Carlsbad, USA). The extracted RNA concentration was detected using an RNA concentration meter (Nanodrop Lite, Thermo Fisher Scientific, Massachusetts, USA). A total of 1 μg RNA were used for cDNA synthesis with the kit of HiScript III RT SuperMix for qPCR (+gDNA wiper) (Number: R323-01, Vazyme, Nanjing, China) based on the manufacture's instruction. The qRT-PCR was performed with CFX96 touch real-time PCR detection system (Bio-Rad, Hercules, CA, USA) using the primers displayed in Table 2. GAPDH was selected as internal control to compare the amplification efficacy. The ChamQ Universal SYBR qPCR Master Mix (Number: Q711-02, Vazyme, Nanjing, China) was used in qRT-PCR. A total of 20 μL qRT-PCR mixture consisted of 10 μL SYBR Green Master Mix, 0.8 μL of forward and reverse primers mix (stock concentration of 10 μmol/L), 2 μL (200 ng) template cDNA and 7.2 μL DNase/RNase Free H_2O. The heat-cycling conditions of qRT-PCR: 95°C for 5 min, followed by 40 cycles at 95°C for 10 s, 60°C for 30 s of melt curve analysis. The experiment was repeated three times and the target gene expression was calculated by the $2^{-\Delta\Delta Ct}$ method.

TABLE 2 Sequence of primers for real-time PCR.

Target gene	Primer sequence (5′ to 3′)	Product size (bp)	GenBank accession no.
Keap1	Forward: CCAACTTCGCCGAGCAGA	120	XM_010728179.2
	Reverse: GCTGGCAGTGGGACAGGTT		
Nrf2	Forward: CACCAAAGAAAGACCCTCCT	197	XM_015289381.3
	Reverse: GAACTGCTCCTTCGACATCA		
HO-1	Forward: CCGCTATTTGGGAGACCT	166	NM_205344.1
	Reverse: CTCAAGGGCATTCATTCG		
NQO-1	Forward: TCTCTGACCTCTACGCCAT	93	NM_001277621.1
	Reverse: TCTCGTAGACAAAGCACTCGG		
GSH-Px	Forward: GATGAGATCCTGAGAGTGGTGGAC	116	NM_000581.4
	Reverse: TCATCAGGTAAGGTGGGCACAA		
SOD1	Forward: AGGGAGGAGTGGCAGAAGT	163	NM_205064.1
	Reverse: GCTAAACGAGGTCCAGCAT		
GAPDH	Forward: GGCTGCTAAGGCTGTGGG	136	NM_204305.1
	Reverse: ATCATCATACTTGGCTGGTTTC		

Keap1, kelch-like ECH-associated protein 1; Nrf2, nuclear factor E2 related factor 2; HO-1, heme oxygenase-1; NQO-1, NAD(P)H: quinone oxidoreductase 1; GSH-Px, glutathione peroxidase; SOD1, superoxide dismutase 1.

TABLE 3 Effect of quercetagetin on apparent metabolic rate of nutrients in broilers (%).

Items	Control group	The amount of quercetagetin			SEM	*P*-value
		3.2 mg/kg	4.8 mg/kg	6.4 mg/kg		
Ether extract	83.14 ± 1.57	82.65 ± 1.18	85.55 ± 2.32	85.57 ± 2.13	0.510	0.093
Crude protein	62.92 ± 3.53^{ab}	62.30 ± 3.23^{b}	65.43 ± 2.33^{ab}	66.42 ± 2.17^{a}	0.680	0.044
Calcium	54.70 ± 2.04	56.89 ± 1.67	56.11 ± 2.78	55.82 ± 2.00	0.466	0.481
Total phosphorus	52.62 ± 2.94^{b}	53.57 ± 3.34^{ab}	56.56 ± 2.86^{a}	57.05 ± 2.30^{a}	0.700	0.040

^{a,b}Within a row, means with different superscripts differ significantly ($P < 0.05$). Values are means ± standard deviation ($n = 10$).
SEM, the standard error of the means.

2.4. Statistical analysis

The statistical data analysis was done using Excel 2016 and SPSS 20.0 software. One-way analysis of variance (ANOVA) was used to test the significant differences between each group data, while the Duncan method was used for multiple comparisons. A p-value <0.05 ($P < 0.05$) showed a significant difference between the groups.

3. Results

3.1. Nutrient digestibility

Compared to the control group, the apparent digestibility of total phosphorus in the basal diet was significantly increased ($P < 0.05$) in groups supplemented with 4.8 and 6.4 mg/kg QG (Table 3). In addition, the apparent digestibility of crude protein in 6.4 mg/kg QG supplement group was higher than that in 3.2 mg/kg QG supplement group ($P < 0.05$). However, there were no significant differences in apparent digestibility of crude fat, crude protein, and calcium in the experimental groups compared to the control group ($P > 0.05$).

3.2. Intestinal morphology

Compared to the control group, the villus height in the jejunum and ileum of broilers fed on a basal diet supplemented with 4.8 mg/kg QG was significantly increased, while the crypt depth in the jejunum and ileum in the three experimental groups was significantly decreased ($P < 0.05$, Table 4). However, the ratios of villus height to crypt depth in the jejunum and ileum of broilers in the experimental groups were significantly increased ($P < 0.05$) compared to the control group.

TABLE 4 Effect of quercetagetin on intestinal morphology of broilers.

Items	Control group	The amount of quercetagetin			SEM	*P*-value
		3.2 mg/kg	4.8 mg/kg	6.4 mg/kg		
Jejunum						
Villus height, μm	1296.04 ± 123.11[b]	1317.50 ± 54.89[b]	1430.49 ± 201.22[a]	1385.10 ± 102.00[ab]	17.207	0.009
Crypt depth, μm	190.44 ± 52.75[a]	152.52 ± 26.56[b]	161.52 ± 42.85[b]	163.98 ± 27.17[b]	4.688	0.031
Villus height/crypt depth	7.30 ± 2.09[b]	8.87 ± 1.46[a]	9.34 ± 2.48[a]	8.64 ± 1.41[a]	0.240	0.009
Ileum						
Villus height, μm	1114.26 ± 140.30[b]	1172.57 ± 52.42[ab]	1200.79 ± 100.62[a]	1131.71 ± 115.84[ab]	11.871	0.046
Crypt depth, μm	203.22 ± 41.21[a]	150.59 ± 29.51[b]	153.20 ± 43.45[b]	155.09 ± 31.35[b]	4.703	<0.001
Villus height/Crypt depth	5.76 ± 1.63[b]	8.08 ± 1.66[a]	8.36 ± 2.20[a]	7.56 ± 1.70[a]	0.222	<0.001

[a,b]Within a row, means with different superscripts differ significantly ($P < 0.05$). Values are means ± standard deviation ($n = 10$).
SEM, the standard error of the means.

TABLE 5 Effect of quercetagetin on organ indices of broilers (g/kg).

Items	Control group	The amount of quercetagetin			SEM	*P*-value
		3.2 mg/kg	4.8 mg/kg	6.4 mg/kg		
Spleen	1.03 ± 0.14	1.06 ± 0.16	1.13 ± 0.03	1.24 ± 0.33	0.021	0.359
Thymus	3.35 ± 0.83	3.84 ± 0.37	3.83 ± 0.63	4.27 ± 0.64	0.178	0.307
Bursa	2.26 ± 0.66	2.50 ± 0.54	2.25 ± 0.37	2.48 ± 0.46	0.126	0.872

Values are means ± standard deviation ($n = 10$).
SEM, the standard error of the means.

3.3. Immune-organ index

There were no significant differences in spleen, thymus, and bursa of fabricius indexes in the experimental groups compared to the control group ($P > 0.05$, Table 5).

3.4. Blood index

The T-AOC activity in broilers supplemented with 3.2 mg/kg QG in feed was significantly increased relative to the control. In addition, the C4 content in the blood of chickens supplemented with 3.2 and 4.8 mg/kg QG was significantly increased ($P < 0.05$, Table 6). At the same time, IgG level, GSH-Px, and SOD activities in the blood of all experimental groups were significantly increased ($P < 0.05$) compared to the control.

3.5. Antioxidant indexes of the jejunum and ileum mucosa

The T-AOC activity in the jejunum mucosa and the T-AOC and GSH-Px activities in the ileum mucosa of broilers supplemented with 4.8 mg/kg QG were significantly increased relative to the control ($P < 0.05$). In addition, the T-AOC activity in the ileum mucosa of chickens supplemented with 6.4 mg/kg QG was significantly increased compared to the control ($P < 0.05$, Table 7).

3.6. Antioxidant indexes of liver

The SOD activity in the liver of broilers supplemented with 6.4 mg/kg QG in feed was significantly increased relative to the control group ($P < 0.05$). Meanwhile, the MDA level in the liver of groups supplemented with 3.2 and 6.4 mg/kg QG was significantly decreased ($P < 0.05$, Table 8).

3.7. Expression of antioxidation-related genes in the intestinal mucosa

3.7.1. Expression of antioxidation-related genes in jejunum mucosa

Compared to the control group, the relative expression levels of *Keap1* and *GSH-Px* mRNA in the jejunum mucosa of broilers supplemented with 3.2 mg/kg QG and the *Keap1*, *Nrf2*, and *SOD1* mRNA levels in the group supplemented with 4.8 mg/kg QG was significantly increased ($P < 0.05$, Figure 1).

TABLE 6 Effect of quercetagetin on serum biochemical indices of broilers.

Items	Control group	The amount of quercetagetin			SEM	*P*-value
		3.2 mg/kg	4.8 mg/kg	6.4 mg/kg		
IgA, μg/mL	515.01 ± 62.18	531.10 ± 10.58	573.23 ± 55.56	580.04 ± 11.39	14.250	0.342
IgG, μg/mL	817.01 ± 95.47^{b}	1043.73 ± 140.43^{a}	1138.92 ± 156.74^{a}	1106.14 ± 42.25^{a}	42.547	0.010
IgM, μg/mL	8.54 ± 0.41	10.48 ± 0.65	10.59 ± 1.11	10.36 ± 0.96	0.305	0.054
C3, g/L	2.59 ± 0.46	2.87 ± 0.45	2.87 ± 0.28	2.89 ± 0.31	0.101	0.552
C4, g/L	1.84 ± 0.20^{b}	2.19 ± 0.15^{a}	2.16 ± 0.18^{a}	2.03 ± 0.09^{ab}	0.057	0.001
T-AOC, U/mL	0.24 ± 0.03^{b}	0.29 ± 0.03^{a}	0.27 ± 0.01^{ab}	0.25 ± 0.06^{ab}	0.012	<0.001
GSH-Px, U/mL	514.68 ± 39.31^{b}	612.19 ± 67.24^{a}	630.72 ± 71.09^{a}	594.32 ± 54.98^{a}	14.600	0.015
SOD, U/mL	149.17 ± 43.83^{b}	218.31 ± 20.21^{a}	214.87 ± 28.31^{a}	221.44 ± 10.85^{a}	9.990	0.009
MDA, nmol/mL	3.01 ± 0.22	2.76 ± 0.09	2.84 ± 0.42	2.82 ± 0.23	0.068	0.717

^{a,b}Within a row, means with different superscripts differ significantly ($P < 0.05$). Values are means ± standard deviation ($n = 10$).
IgA, immunoglobulin A; IgG, immunoglobulin G; IgM, immunoglobulin M; C3, complement 3; C4, complement 4; T-AOC, total antioxidant capacity; GSH-Px, glutathione peroxidase; SOD, superoxide dismutase; MDA, malondialdehyde; SEM, the standard error of the means.

TABLE 7 Effect of quercetagetin on antioxidant performance of intestinal mucosa of broilers.

Items	Control group	The amount of quercetagetin			SEM	*P*-value
		3.2 mg/kg	4.8 mg/kg	6.4 mg/kg		
Jejunal mucosa						
T-AOC, U/mg prot	0.61 ± 0.06^{b}	0.62 ± 0.08^{b}	0.70 ± 0.07^{a}	0.66 ± 0.06^{ab}	0.015	0.129
GSH-Px, U/mg prot	42.99 ± 8.97	49.4 ± 15.48	48.03 ± 10.57	42.25 ± 6.7	2.176	0.596
SOD, U/mg prot	570.43 ± 13.18	558.21 ± 14.55	566.75 ± 33.11	555.07 ± 14.14	4.948	0.326
MDA, nmol/mg prot	0.17 ± 0.03	0.22 ± 0.08	0.21 ± 0.03	0.20 ± 0.09	0.038	0.179
Ileum mucosa						
T-AOC, U/mg prot	0.60 ± 0.06^{b}	0.62 ± 0.02^{ab}	0.71 ± 0.08^{a}	0.72 ± 0.10^{a}	0.020	0.038
GSH-Px, U/mg prot	61.65 ± 8.78^{b}	59.30 ± 13.73^{b}	74.71 ± 7.11^{a}	64.37 ± 10.75^{ab}	2.327	0.044
SOD, U/mg prot	534.49 ± 12.99	538.47 ± 12.12	532.58 ± 22.38	538.51 ± 23.82	4.212	0.957
MDA, nmol/mg prot	0.12 ± 0.04	0.14 ± 0.04	0.15 ± 0.02	0.15 ± 0.05	0.010	0.741

^{a,b}Within a row, means with different superscripts differ significantly ($P < 0.05$). Values are means ± standard deviation ($n = 10$).
T-AOC, total antioxidant capacity; GSH-Px, glutathione peroxidase; SOD, superoxide dismutase; MDA, malondialdehyde; SEM, the standard error of the means.

TABLE 8 Effect of quercetagetin on liver antioxidant performance of broilers.

Items	Control group	The amount of quercetagetin			SEM	*P*-value
		3.2 mg/kg	4.8 mg/kg	6.4 mg/kg		
T-AOC, U/mg prot	0.83 ± 0.16	0.90 ± 0.12	0.91 ± 0.13	0.94 ± 0.12	0.027	0.648
GSH-Px, U/mg prot	120.16 ± 23.47	142.99 ± 35.9	153.73 ± 31.36	129.11 ± 18.53	5.989	0.206
SOD, U/mg prot	663.46 ± 46.78^{b}	703.13 ± 51.06^{ab}	727.59 ± 69.86^{ab}	748.00 ± 73.93^{a}	14.205	0.034
MDA, nmol/mg prot	1.31 ± 0.02^{a}	1.19 ± 0.07^{b}	1.36 ± 0.05^{a}	1.17 ± 0.10^{b}	0.026	0.012

^{a,b}Within a row, means with different superscripts differ significantly ($P < 0.05$). Values are means ± standard deviation ($n = 10$).
T-AOC, total antioxidant capacity; GSH-Px, glutathione peroxidase; SOD, superoxide dismutase; MDA, malondialdehyde; SEM, the standard error of the means.

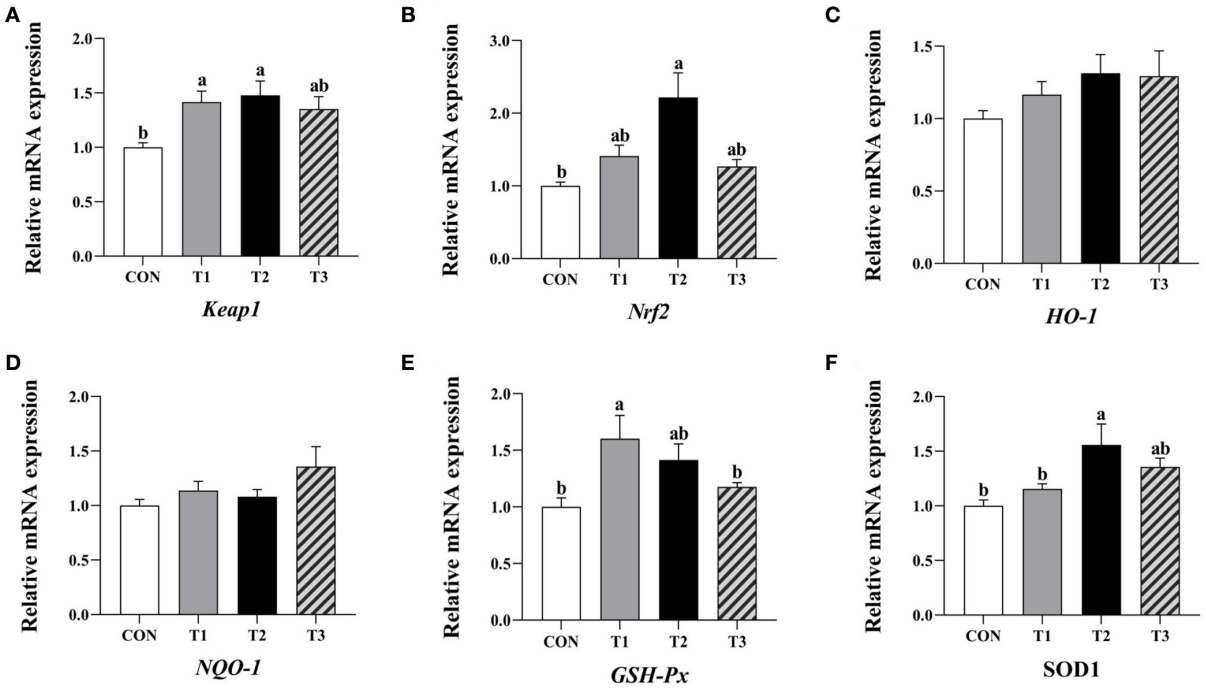

FIGURE 1
Effect of quercetagetin on expression level of antioxidant related genes in jejunal mucosa of broilers. Values are means ± standard deviation (n = 10). Above the bar no letter or the same letter mean no significant ($P > 0.05$), while with different letter mean significant difference ($P < 0.05$). Kelch-like ECH-associated protein 1 (Keap1) **(A)**, Nuclear factor E2 related factor 2 (Nrf2) **(B)**, Heme oxygenase-1 (HO-1) **(C)**, NAD(P)H: quinone oxidoreductase 1 (NQO-1) **(D)**, Glutathione peroxidase (GSH-Px) **(E)**, Superoxide dismutase 1 (SOD1) **(F)**.

3.7.2. Expression of antioxidation-related genes in the ileum

Compared to the control group, the relative expression levels of *HO-1*, *GSH-Px*, and *SOD1* mRNA in the ileum mucosa of broilers supplemented with 3.2 mg/kg QG, *Nrf2*, and *SOD1* mRNA in the group supplemented with 4.8 mg/kg QG, and *GSH-Px* and *SOD1* mRNA in the group supplemented with 6.4 mg/kg QG were significantly increased ($P < 0.05$, Figure 2).

3.8. Expression of antioxidation-related genes in the liver

The relative expression levels of *Nrf2* and *GSH-Px* mRNA in the liver of broilers in the three experimental groups were significantly increased compared to the control ($P < 0.05$, Figure 3). In addition, the relative expression levels of *NQO-1* and *SOD1* mRNA in the liver of broilers supplemented with 4.8 mg/kg QG in feed, and *SOD1* mRNA in the group supplemented with 6.4 mg/kg QG were significantly increased compared to the control ($P < 0.05$).

4. Discussion

Flavonols have numerous biological functions. For instance, quercetin enhances the antioxidant capacity of broilers' intestines, alleviates intestinal inflammatory reactions, regulates the internal intestinal environment, maintains the integrity of the intestinal barrier, and improves the structure and morphology of the intestinal tract (15–17). Rutin exhibits antioxidant and anti-inflammatory effects by inhibiting the production of reactive oxygen species and cell apoptosis (18). In the present study, the dose range of QG was selected based on the results of free radical scavenging experiments of QG *in vitro* and the pre-experiments of QG in broilers. And the results implied that the dietary supplementation with QG improved the morphology of broilers' jejunum and ileum, enhanced the functions. The positive effect of QG on the intestinal development of broilers is similar to that induced by quercetin, probably due to its antioxidant, anti-inflammatory, and antiviral activities, which strengthen intestinal resistance and homeostasis. Specifically, QG has a strong scavenging capacity against OH·, DPPH, and ABTS (12), significantly inhibits the intestinal inflammatory response induced by silver nanoparticles (AgNP) (19), and has a strong inhibitory effect

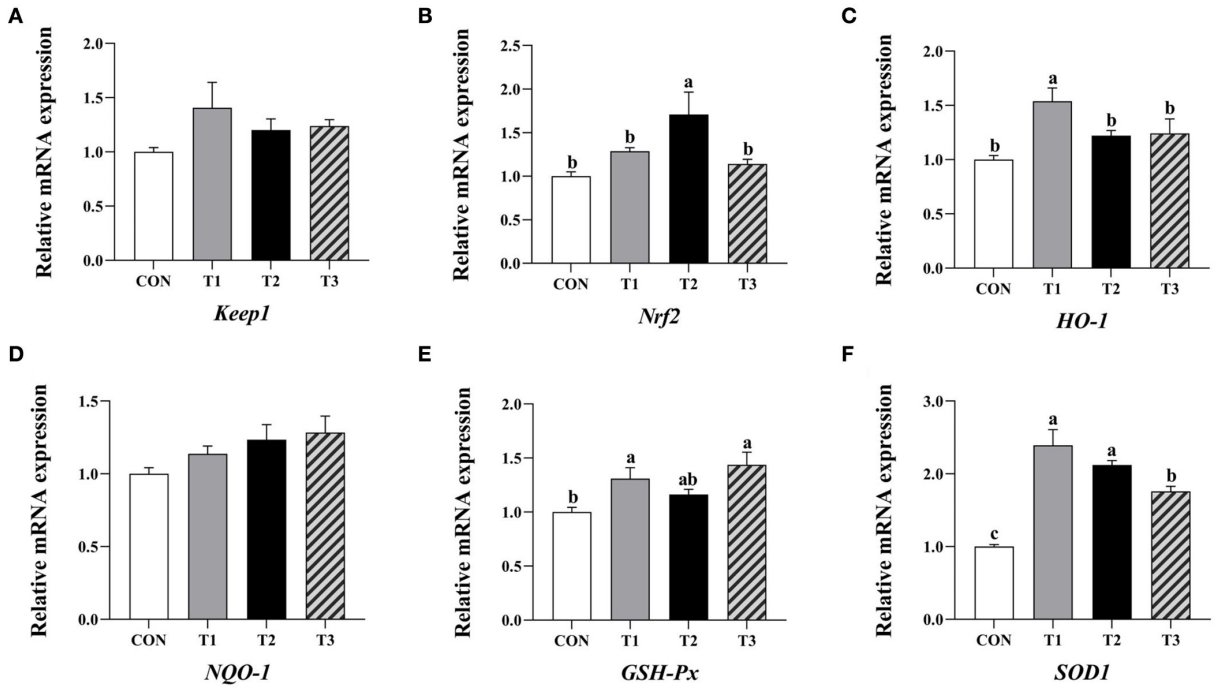

FIGURE 2
Effect of quercetagetin on expression level of antioxidant related genes in inileal mucosa of broilers. Values are means ± standard deviation (n = 10). Above the bar no letter or the same letter mean no significant ($P > 0.05$), while with different letter mean significant difference ($P < 0.05$). Kelch-like ECH-associated protein 1 (Keap1) **(A)**, Nuclear factor E2 related factor 2 (Nrf2) **(B)**, Heme oxygenase-1 (HO-1) **(C)**, NAD(P)H: quinone oxidoreductase 1 (NQO-1) **(D)**, Glutathione peroxidase (GSH-Px) **(E)**, Superoxide dismutase 1 (SOD1) **(F)**.

against Chikungunya virus infection with IC_{50} of 13.85 μg/ml (43.52 μM) (20).

Flavonol compounds affect the utilization efficiency of some nutrients in broilers. For example, the relative expression levels of glucose transporter 2, peptide transporter 1, and fatty acid synthase mRNA in 1-day-old Ross 308 broilers fed with 200, 400, and 800 mg/kg quercetin for 35 d were significantly increased (17). Quercetin prevents the inhibitory effect of menadione and other glutathione-consuming substances on calcium absorption in broilers' intestines by alleviating oxidative stress and inhibiting the activation of the FasL/Fas/caspase-3 pathway (21). In addition, the results in this study revealed that dietary supplementation with QG has no significant effect on the apparent digestibility of crude protein, crude fat, and calcium in broilers but promotes the digestion and absorption of phosphorus (4.8 and 6.4 mg/kg QG supplement groups), which is key to reducing the feed cost. This is because dietary supplementation with QG improves the intestinal morphology of jejunum and ileum, promoting phosphorus absorption. In addition, QG supplements alleviate lipid and protein oxidation in the diet, improving the diet stability, which benefits nutrient absorption. Antioxidation induced by QG provides beneficial conditions for the absorption and utilization of nutrients. However, there were no significant differences in the apparent digestibility of crude protein, crude lipid, and calcium in this study, which may be related to the quantity of QG supplements fed to the broilers.

Flavonol compounds have immunomodulatory effects, with sufficient supplementation improving the immunity of broilers. For example, dietary supplementing Tianfu broilers with 0.4 g/kg quercetin for 14 weeks significantly increases the secretory immunoglobulin A concentrations in the duodenum, jejunum, and ileum (16). Besides, supplementing 1-day-old broilers with 0.5 mg/kg quercetin exerts immunomodulatory, antioxidant, and anti-apoptotic effects and alleviates ochratoxin A-induced immunotoxicity by regulating the PI3K/AKT pathway (22). At the same time, supplementing 1-day-old AA broilers with 0.02% quercetin for 6 weeks significantly increases the blood C3 content, while 0.06% quercetin significantly increases the thymus and spleen index, IgA, IgM, C4, interleukin 4, and tumor necrosis factor alpha contents in the blood (23). In the present study, dietary supplementation with QG improved the immunity of broilers by increasing IgG and C4 (3.2 and 4.8 mg/kg QG supplement groups) levels in the blood, given the antioxidant effect of QG, which alleviates oxidative stress on immune cells. On human lymphoblasts (Jurkat T), QG reduces the effects of oxidative stress by scavenging free radicals and enhancing antioxidant enzyme activity (24). Besides,

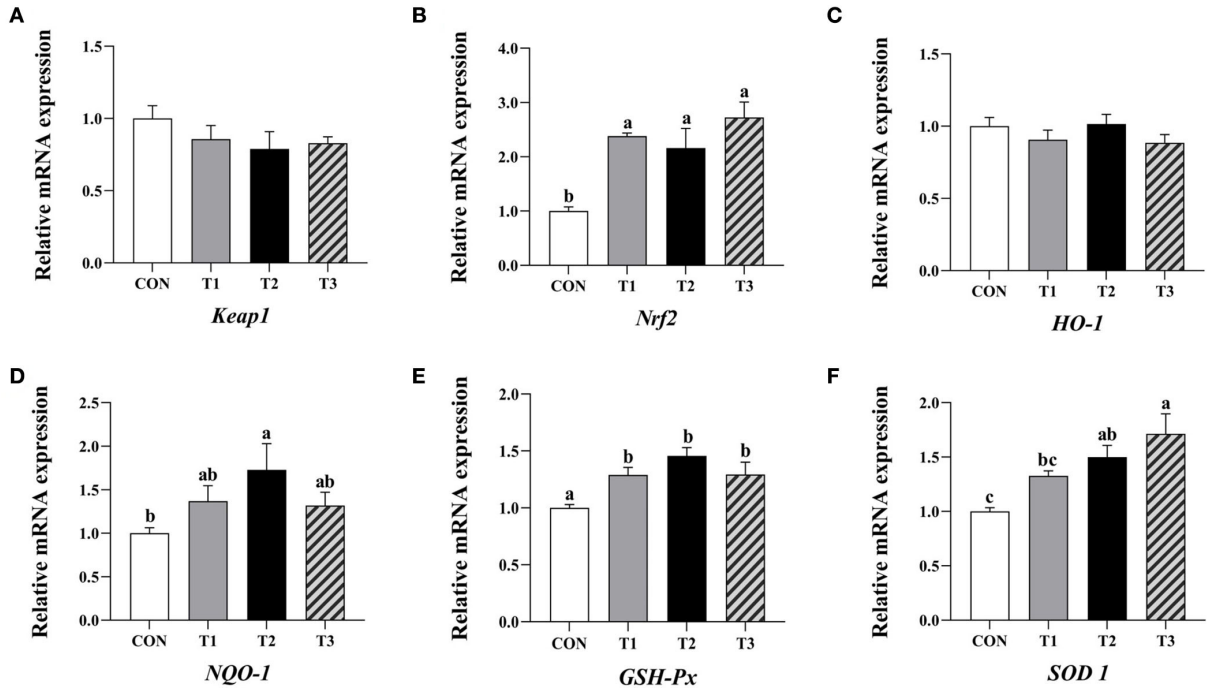

FIGURE 3
Effect of quercetagetin on expression level of antioxidant related genes in liver of broilers. Values are means ± standard deviation ($n = 10$). Above the bar no letter or the same letter mean no significant ($P > 0.05$), while with different letter mean significant difference ($P < 0.05$). Kelch-like ECH-associated protein 1 (Keap1) **(A)**, Nuclear factor E2 related factor 2 (Nrf2) **(B)**, Heme oxygenase-1 (HO-1) **(C)**, NAD(P)H: quinone oxidoreductase 1 (NQO-1) **(D)**, Glutathione peroxidase (GSH-Px) **(E)**, Superoxide dismutase 1 (SOD1) **(F)**.

the immunomodulatory and anti-inflammatory effects of QG influence the immune response of the body. Kang et al. (25) revealed that QG inhibits the formation of macrophage-derived chemokine (MDC/CCL22) in the human keratinocytes (HaCaT) by mediating signal transducer and activator of transcription 1 (STAT1), suppressor of cytokine signaling 1 (SOCS1) and transforming the growth factor-β1 (TGF-β1); hence its potential as an immunotherapeutic agent against inflammatory diseases such as atopic dermatitis (AD). QG also alleviates the effects of oxidative stress induced by silver nanoparticles on human neutrophils (19).

Flavonol compounds exhibit antioxidant activity; thus, sufficient supplements could improve the antioxidant capacity of broilers and alleviate the effects of oxidative stress and injury on broilers. Supplementation of a 1-day-old AA broilers diet with 400 and 800 mg/kg quercetin for 11 d significantly reduced MDA increase in the blood and intestinal mucosa induced by oxidized soybean oil. At the same time, the mRNA expression levels of *Nrf2*, *CAT*, *SOD1*, *GSH-Px2*, and *HO-1* in the AA broilers ileum mucosa were significantly up-regulated, the antioxidant capacity was improved, and the effect of oxidized soybean oil was alleviated (26). In addition, with supplementation of 1-day-old Ross 308 broilers diet with 1 g/kg rutin for 42 d, the SOD, CAT, and GSH-Px activities in the serum were significantly increased, but the MDA level was significantly decreased, which enhanced the antioxidant capacity of the broilers (27). In this study, the results revealed that QG enhanced the antioxidant capacity of the broilers through the Keap1-mediated Nrf2/ARE signaling pathway. Since Nrf2/ARE signaling pathway is a critical antioxidant and defense signaling pathway, the free radical scavenging capacity and antioxidant activity are promoted by regulating the expression of antioxidases, such as HO-1, NQO-1, GSH-Px, and SOD1. The antioxidant activity of quercetin correlates to the hydroxyl groups on three positions of the A and C rings and the catechol group on the B ring (28). Compared to quercetin, QG has an extra phenolic hydroxyl at six positions of the A ring. Furthermore, the oxygen atom on the 4-carbonyl group in the parent nucleus of QG has a stronger coordination ability, and the spatial structure of its polyhydroxy groups is conducive to the formation of metal complexes with various structures, which is an important source of antioxidant and other biological activities of QG (10). *In vitro*, QG has similar ABTS and DPPH scavenging activities to quercetin, with an IC_{50} of 12.16 ± 0.56 and 12.38 ± 0.50 μmol/L, and 27.12 ± 1.31 and 27.85 ± 1.13 μmol/L, respectively (29). Besides, major antioxidant components such as gallic acid, epigallocatechin, quercetin, and QG have been detected in an alcohol extract of defatted marigold residue, among which QG had the strongest

antioxidant activity (30), which may be one of the reasons for the difference in the dosage of QG and quercetin used in broiler production. QG is extracted from marigold, which is rich in resources. The application of QG as feed additive in broiler production is economical and easily available.

5. Conclusions

Dietary supplementation with QG improves broilers' apparent digestibility of phosphorus in feed, improves the jejunum and ileum morphology, and enhances their immunity. In addition, QG increases the activity of antioxidant enzymes and strengthens the antioxidant capacity of broilers through the Keap1-mediated Nrf2/ARE signaling pathway.

Data availability statement

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

Ethics statement

The animal study was reviewed and approved by the Animal Care and Use Committee of Hebei Agriculture University (Baoding, China). All animal experiments complied with the ARRIVE guidelines and were carried out in accordance with the UK. Animals (Scientific Procedures) Act, 1986 and associated guide-lines, EU Directive 2010/63/EU for animal experiments.

Author contributions

FW and HW are the primary investigators in this study. SL participated in the animal experiments. ZW participated in sample analysis and statistical data analysis. SH revised the manuscript. BC designed this study and wrote the manuscript as corresponding author. All authors read and approved the final manuscript.

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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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Effects of dietary supplementation with dandelion tannins or soybean isoflavones on growth performance, antioxidant function, intestinal morphology, and microbiota composition in Wenchang chickens

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Many benefits have been found in supplementing tannins or soybean isoflavones to poultry, including increased body weight gain, antioxidant activity, and better intestinal morphology. However, few studies tested the influence of dandelion tannins or soybean isoflavones supplementation on Wenchang chickens. This study investigates the effects of dietary supplementation with dandelion tannins or soybean isoflavones on the growth performance, antioxidant function, and intestinal health of female Wenchang chickens. A total of 300 chickens were randomly divided into five groups, with six replicates per group and 10 broilers per replicate. The chickens in the control group (Con) were fed a basal diet; the four experimental groups were fed a basal diet with different supplements: 300 mg/kg of dandelion tannin (DT1), 500 mg/kg of dandelion tannin (DT2), 300 mg/kg of soybean isoflavone (SI1), or 500 mg/kg of soybean isoflavone (SI2). The experiment lasted 40 days. The results showed that the final body weight (BW) and average daily gain (ADG) were higher in the DT2 and SI1 groups than in the Con group ($P < 0.05$). In addition, dietary supplementation with dandelion tannin or soybean isoflavone increased the level of serum albumin ($P < 0.05$); the concentrations of serum aspartate aminotransferase and glucose were significantly higher in the SI1 group ($P < 0.05$) than in the Con group and the concentration of triglycerides in the DT1 group ($P < 0.05$). The serum catalase (CAT) level was higher in the DT1 and SI1 groups than in the Con group ($P < 0.05$). The ileum pH value was lower in the DT2 or SI1 group than in the Con group ($P < 0.05$). The jejunum villus height

and mucosal muscularis thickness were increased in the DT2 and SI1 groups ($P < 0.05$), whereas the jejunum crypt depth was decreased in the DT1 or DT2 group compared to the Con group ($P < 0.05$). In addition, the messenger RNA (mRNA) expression level of zonula occludens 1 (*ZO-1*) in the duodenum of the SI1 group and those of *occludin*, *ZO-1*, and *claudin-1* in the ileum of the DT2 and SI1 groups were upregulated ($P < 0.05$) compared to the Con group. Moreover, the DT2 and SI1 groups exhibited reduced intestinal microbiota diversity relative to the Con group, as evidenced by decreased Simpson and Shannon indexes. Compared to the Con group, the relative abundance of Proteobacteria was lower and that of *Barnesiella* was higher in the DT2 group ($P < 0.05$). Overall, dietary supplementation with 500 mg/kg of dandelion tannin or 300 mg/kg of soybean isoflavone improved the growth performance, serum biochemical indexes, antioxidant function, and intestinal morphology and modulated the cecal microbiota composition of Wenchang chickens.

KEYWORDS

growth performance, biochemical blood indexes, antioxidant function, intestinal health, dandelion tannin, soybean isoflavones, Wenchang chicken

1. Introduction

Wenchang chicken is a Chinese local breed with delicious meat and unique flavor (1). Antibiotics are used as feed additives, mixed with chicken feed, and given to this breed of Wenchang chicken to ward off diseases and to promote their growth (2).However, the abuse of antibiotics by way of overdose administered to chickens has led to the emergence of an entirely new crop of antibiotic-resistant bacteria that threaten and endanger human health (3). China has banned the use of adding antibiotics to chicken feed for the very same reason. However, noninclusion of antibiotics in the feed brings with it numerous issues that need to be sorted out to boost chicken production. First, the noninclusion of antibiotics in the feed renders the chickens vulnerable to bacterial attack; second, the prevention of disease in chickens is rendered inefficient; and third, the noninclusion of antibiotics in feed makes it expensive as the chickens become weak and fall sick. These ailing chickens have to be nursed back to health, which is costly, compared to the inclusion of antibiotics in feed which does not entail these extra costs and so is less expensive (4, 5). Therefore, it is extremely urgent to find alternative compounds to replace antibiotics in chicken production.

Plant extracts are natural bioactive compounds with green, safe, and efficient characteristics, and are widely used in animal production. It was reported that plant extracts can improve growth performance and intestinal histology and regulate the immune system (6, 7). There are many kinds of plant extracts, including flavonoids, essential oils, alkaloids, polyphenols, and polysaccharides (8). Specifically, soybean isoflavones are naturally occurring nonsteroidal phenolic plant compounds (9). Numerous studies confirmed their biological functions in promoting the growth of livestock, improving intestinal morphology, and enhancing antioxidant activity and immunity (10–13). Tannin is widely distributed in plant tissues and belongs to the secondary metabolite type and is considered to be an antinutritional factor. Tannin plays an important role in anti-inflammatory (14, 15), antioxidant (16), and antibacterial properties (17) and is reported to promote the growth and improve the health of broilers (18, 19). Therefore, tannins or soybean isoflavones have the potential to serve as an alternative to antibiotics in chicken production (20–22).

Tannin and soybean isoflavones have many similar biological functions, as both of them can promote the growth of poultry. However, there is less information on the application of tannins or soybean isoflavones and the comparison of the methods of application between these two plant extracts in Wenchang chickens. Therefore, this study aimed to evaluate the effects of dietary supplementation with dandelion tannins or soybean isoflavones on the growth performance, biochemical blood indexes, antioxidant function, and intestinal morphology and the immunity of Wenchang chickens.

2. Materials and methods

2.1. Animal ethics

All the experimental procedures applied in this study were reviewed and approved by the Experimental Animal Ethics Committee of Animal Husbandry and Veterinary Research Institute, Hainan Academy of Agricultural Sciences.

TABLE 1 Feed and nutrient composition of the experimental chicken diet (as-fed basis, %).

Feed ingredient	Content	Nutrients[a]	Content
Corn	56.20	Crude protein (%)	20.00
Soybean meal	31.80	Metabolizable energy (MJ/kg)	12.18
Wheat	4.00	Methionine	0.32
Fish meal	1.00	Lysine	1.05
Soy oil	3.00	Calcium	0.85
Premix[b]	4.00	Non-phytate	0.40
Total	100		

[a]Nutrients are analyzed value.

[b]Premix provided the following per kilogram diet: 15,000 IU (international units) of vitamin A, 3,300 IU of vitamin D_3, 20 mg of vitamin E, 6 mg of vitamin K_3, 3 mg of vitamin B_1, 8 mg of vitamin B_2, 6 mg of vitamin B_6, 0.03 mg of vitamin B_{12}, 60 mg of niacin, 18 mg of calcium pantothenate, 1.5 mg of folic acid, 0.36 mg of biotin, 600 mg of choline chloride, 80 mg of Fe, 12 mg of Cu, 75 mg of Zn, 100 mg of Mn, 0.35 mg of I, and 0.15 mg of Se. The nutrition level indicators are calculated values.

2.2. Experimental design and diets

The feeding experiments were conducted at the Institute of Animal Husbandry and Veterinary Medicine, Hainan Academy of Agricultural Sciences. Dandelion tannins (95% purity) and soybean isoflavones (26% purity) were purchased from Zhaoqing Baishike Biotechnology Co., Ltd. A total of 300 1-day-old female Wenchang chickens (average initial weight = 28.17 g) were randomly assigned into five groups, with six replicates per group and 10 broilers per replicate each. The chickens in the control group (Con) were fed a basal diet; the four experimental groups were fed a basal diet with different supplements: 300 mg/kg of dandelion tannin (DT1), 500 mg/kg of dandelion tannin (DT2), 300 mg/kg of soybean isoflavone (SI1), or 500 mg/kg of soybean isoflavone (SI2). During the experiment, the temperature inside the house was kept at 32°C and the humidity was set at 55%−65%. The experiment lasted 40 days. All experimental chickens were raised in three-layer cages, with two different types of lighting systems (artificial light and natural light) and access to food and water *ad libitum*. Body weight (BW) and feed intake were recorded at days 1 and 41 of the experiment. The average daily gain (ADG), average daily feed intake (ADFI), and feed weight ratio (F:G) were calculated based on the final weight and feed intake. The basic diet (Table 1) met the nutrition requirements of yellow feather broilers (NY/T 3645-2020).

2.3. Sample collection

At the end of the experiment, two chickens close to the average weight were selected for sample collection in each replicate. After weighing both the chickens, the blood sample was collected from the Pterygoid venous plexus using a coagulation tube and then centrifuged at 3,000 r/min (revolutions per minute) for 10 min to recover the serum.

After the blood was collected, the chickens were euthanized for duodenum, jejunum, and ileum sample collection. Each intestine was divided into two parts, one part of the intestinal segment (about 2 cm) was fixed in 10% paraformaldehyde fixative and the other part of the intestinal segment (about 2 g) was collected into 1.5 ml freezing tubes and stored at −80°C for RNA extraction. Cecal contents of samples were collected into 1.5 ml freezing tubes and stored at −80°C until analysis.

2.4. Serum biochemical indexes and antioxidant indexes

The concentrations of total protein, albumin, globulin, aspartate aminotransferase, uric acid, total cholesterol, triglycerides, and glucose in serum were determined using an automatic biochemical analyzer (Hitachi LABOSPECT 008 AS). The levels of malondialdehyde (MDA), superoxide dismutase (SOD), total antioxidant capacity (T-AOC), and catalase (CAT) in serum were determined using the commercial kits (A003-1-2, A001-3-2, A015-2-1, and A007-1-1; Nanjing Jiancheng Bioengineering Institute, Nanjing, China) according to the manufacturer's instructions.

2.5. Intestinal pH value

The intestinal pH value was determined using a pH meter (OHAUS, Beijing, China) by inserting the electrode into the anterior, middle, and posterior parts of the jejunum and the ileum. The average value of these three points was calculated.

2.6. Intestinal morphology

The fixed jejunum and ileum tissues were cut into sections with a thickness of 3–4 μm and stained with hematoxylin–eosin. Five photographs were selected randomly for each slide using a bright-field microscope (Eclipse Ci-L) at 40× magnification. The villus height (VH), the crypt depth (CD), and mucosal muscularis thickness (MMT) were analyzed using Image-Pro Plus 6.0. The villus height:crypt depth ratio (VH/CD) was calculated based on the VH and CD.

2.7. Quantitative real-time PCR

The total RNA was extracted from duodenum and ileum tissues using an RNA Extraction Kit (Tiangen, China) following

TABLE 2 Primers used for quantitative real-time polymerase chain reaction (PCR) in this study.

Gene	Primer sequence (5′-3′)
β-ACTIN	F: ACCTGAGCGCAAGTACTCTGTCT R: CATCGTACTCCTGCTTGCTGAT
TNF-α	F: AGTGCTGTTCTATGACCGCC R: CGCTCCTGACTCATAGCAGA
TGF-β4	F: AGGATCTGCAGTGGAGTGGAT R: CCCCGGGTTGTGTTGGT
ZO-1	F: GCCAGCCATCATTCTGACTCCAC R: GTACTGAAGGAGCAGGAGGAGGAG
Claudin-1	F: GATCCAGTGCAAGGTGTACGA R: AAAGACAGCCATCCGCATCT
Occludin	F: ACGGCAGCACCTACCTCAA R: GGGCGAAGAAGCAGATGAG

TNF-α, tumor necrosis factor alpha; TGF-β, transforming growth factor beta; ZO, tight junction protein; F, forward primer; R, reverse primer.

TABLE 3 Effects of dietary supplementation with dandelion tannins or soybean isoflavones on the growth performance of Wenchang chickens.

Items	Control	DT1	DT2	SI1	SI2	*P*-value
Initial BW, g	28.17 ± 0.26	28.17 ± 0.26	28.17 ± 0.26	28.17 ± 0.26	28.17 ± 0.26	1.00
Final BW, g	495.22 ± 35.24^{b}	531.19 ± 35.94^{ab}	548.24 ± 23.19^{a}	548.30 ± 22.76^{a}	525.81 ± 32.34^{ab}	0.04
ADG, g/d	11.68 ± 0.88^{b}	12.58 ± 0.90^{ab}	13.00 ± 0.58^{a}	13.00 ± 0.57^{a}	12.44 ± 0.80^{ab}	0.03
ADFI, g/d	25.59 ± 1.46	26.41 ± 1.98	27.73 ± 1.21	26.99 ± 1.41	25.71 ± 1.79	0.14
F/G, g/g	2.21 ± 0.30	2.10 ± 0.09	2.13 ± 0.03	2.08 ± 0.07	2.07 ± 0.13	0.79
Mortality rate, %	20.00	6.67	6.67	10.00	10.00	0.33

BW, body weight; ADG, average daily gain; ADFI, average daily feed intake; F/G, feed intake/body weight gain. DT1, basal diet +300 mg/kg dandelion tannins; DT2, basal diet +500 mg/kg dandelion tannins; SI1, basal diet +300 mg/kg soybean isoflavones; SI2, basal diet +500 mg/kg soybean isoflavones.
The data were expressed as the means ± standard deviation.
^{ab}Marks indicate statistically significant differences ($P < 0.05$).

TABLE 4 Effects of dietary supplementation with dandelion tannins or soybean isoflavones on serum biochemical indexes of Wenchang chickens.

Items	Control	DT1	DT2	SI1	SI2	*P*-value
Total protein, g/L	42.24 ± 6.85	40.30 ± 6.19	40.85 ± 3.83	39.72 ± 4.64	40.78 ± 4.96	0.82
Albumin, g/L	12.72 ± 1.28^{b}	13.67 ± 1.55^{ab}	14.05 ± 1.00^{a}	14.07 ± 1.00^{a}	14.13 ± 0.84^{a}	0.04
Globulin, g/L	29.52 ± 6.56	26.63 ± 6.26	26.79 ± 3.28	25.65 ± 3.99	26.65 ± 4.73	0.66
Aspartate aminotransferase, U/L	229.30 ± 23.99^{b}	251.75 ± 26.36^{b}	245.55 ± 28.85^{b}	289.09 ± 32.59^{a}	254.08 ± 30.75^{b}	<0.01
Uric acid, μmol/L	210.30 ± 96.15	232.67 ± 67.15	205.09 ± 54.40	192.45 ± 49.46	165.08 ± 36.69	0.13
Total cholesterol, mmol/L	3.04 ± 0.66	2.79 ± 0.52	2.93 ± 0.46	3.02 ± 0.34	2.89 ± 0.50	0.77
Triglycerides, mmol/L	0.65 ± 0.13^{ab}	0.77 ± 0.21^{a}	0.65 ± 0.16^{ab}	0.54 ± 0.13^{b}	0.54 ± 0.17^{b}	0.03
Blood glucose, mmol/L	6.86 ± 3.48^{b}	6.93 ± 3.13^{b}	8.92 ± 2.07^{ab}	10.18 ± 1.70^{a}	9.47 ± 1.25^{ab}	0.02

DT1, basal diet +300 mg/kg dandelion tannins; DT2, basal diet +500 mg/kg dandelion tannins; SI1, basal diet +300 mg/kg soybean isoflavones; SI2, basal diet +500 mg/kg soybean isoflavones.
The data were expressed as the means ± standard deviation.
^{a,b}Marks indicate statistically significant differences ($P < 0.05$).

the manufacturer's instructions. The complementary DNA (cDNA) was synthesized from 2 μg of total RNA by reverse transcription in a 20 μl reaction mixture using a reverse transcriptase kit (Tiangen, China). The reverse transcription procedure was employed as follows: 42°C for 15 min and 95°C for 3 min. The cDNA samples were stored at −20°C. The quantitative reverse transcription-polymerase chain reaction (qRT-PCR) was carried out on a Bio-Rad CFX96 Touch Real-Time PCR Detection System using the SYBR Green Real-time PCR Master Mix. Target gene expression was quantified using the $2^{-\Delta\Delta Ct}$ method. The real-time PCR procedure was employed as follows: denaturation at 95°C for 2 min, followed by 39 cycles at 95°C for 0.5 s and 60°C for 10 s. The primers are listed in Table 2.

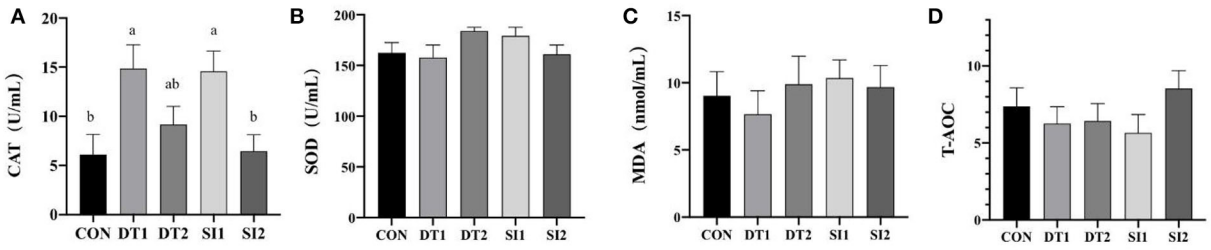


FIGURE 1
(A–D) Effects of dietary dandelion tannins or soybean isoflavones on serum antioxidant indexes. ^{a,b,c}Marks indicate statistically significant differences ($P < 0.05$).

2.8. Cecal microbiota

The cecal content was collected into a sterile Eppendorf (EP) tube, immediately frozen in liquid nitrogen, and stored at −80°C until analysis. The DNA of cecal content was extracted by cetyltrimethylammonium bromide (CTAB) method, and the purity and concentration of DNA were detected by agarose gel electrophoresis. The V3-V4 variable region was amplified using the primer (Forward: CCTAYGGGRBGCASCAG; Reverse: GGACTACNNGGGTATCTAAT). The PCR system (30 μl) was comprised of 15 μl of Phusion High-Fidelity PCR Master Mix (2×), 1 μl of each primer (1 μM), 10 μl of genomic DNA (gDNA) (1 ng/μl), and 3 μl of water. The PCR procedure was employed as follows: 98°C predenaturation for 1 min, followed by 30 cycles of 98°C for 10 s, 50°C for 30 s, 72°C for 30 s, and finally 72°C for 5 min. The PCR products were diluted to the same concentration. Then, 2% agarose gel electrophoresis was used to purify the PCR products. A library building kit (Illumina, San Diego, USA) was used for library construction. After the library was qualified, a NovaSeq 6000 was employed for online sequencing (Entrust Nuohe Zhiyuan Biological Information Technology Co., Ltd).

2.9. Statistical analysis

All data were analyzed using SPSS version 26.0 (IBM Corp., USA). Data were expressed as the means ± standard deviation. Between-group differences were analyzed using one-way analysis of variance (ANOVA), with a *P*-value of <0.05 as the significance level, and Duncan's multiple range test was used for multiple comparisons.

3. Results

3.1. Growth performance

The final body weight and average daily gain were higher in the DT2 and SI1 groups ($P < 0.05$) than in the Con group. Additionally, there were no differences in the ADFI or feed weight ratio in the experimental groups as in the Con group ($P > 0.05$). However, the feed weight ratio in the experimental groups exhibited a downward trend ($0.05 < P < 0.1$) compared to the controls (Table 3).

3.2. Serum biochemical indexes

As shown in Table 4, there was no difference in the level of serum total protein, globulin, uric acid, or total cholesterol among the groups ($P > 0.05$). However, the level of serum albumin was increased in the DT2, SI1, and SI2 groups compared to the Con group ($P < 0.05$). In addition, the SI1 group was higher than the Con group in the levels of serum aspartate aminotransferase and glucose ($P < 0.05$). Similarly, the level of triglycerides was higher in the DT1 group ($P < 0.05$) than in the Con group. As shown in Figure 1, the serum CAT level in the DT1 and SI1 groups was higher than that in the Con group ($P < 0.05$). There was no significant difference in the serum MDA, SOD, or T-AOC levels among the groups ($P > 0.05$).

3.3. Intestinal pH and histomorphology

As shown in Table 5, the ileum pH value was the lowest ($P < 0.05$) in the DT2 and SI1 groups. As shown in Table 6, an increasing trend ($P < 0.05$) in jejunum VH and MMT was found in the DT2 and SI1 groups compared to the Con group. The jejunum CD was decreased ($P < 0.05$) in the DT1 and DT2 groups compared to the Con group. In addition, the jejunum V:C was increased significantly in the DT2 group compared to the Con group ($P < 0.05$). However, no significant difference was observed in the jejunum V:C between the SI1 group and the SI2 group ($P > 0.05$). Similarly, no significant differences were observed in the ileum VH, CD, MMT, and V:C among the groups ($P > 0.05$).

TABLE 5 Effects of dietary supplementation with dandelion tannins or soybean isoflavones on the intestinal pH of Wenchang chickens.

Item	Control	DT1	DT2	SI1	SI2	*P*-value
Ileum pH	6.76 ± 0.11^{ab}	6.87 ± 0.29^{a}	6.62 ± 0.12^{b}	6.61 ± 0.26^{b}	6.82 ± 0.33^{ab}	0.04
Jejunum pH	6.44 ± 0.10	6.49 ± 0.25	6.36 ± 0.17	6.31 ± 0.36	6.44 ± 0.11	0.32

DT1, basal diet +300 mg/kg dandelion tannins; DT2, basal diet +500 mg/kg dandelion tannins; SI1, basal diet +300 mg/kg soybean isoflavones; SI2, basal diet +500 mg/kg soybean isoflavones.
The data were expressed as the means ± standard deviation.
^{a,b}Marks indicate statistically significant differences ($P < 0.05$).

TABLE 6 Effects of dietary supplementation with dandelion tannins or soybean isoflavones on jejunum and ileum histomorphology of Wenchang chickens.

Item	Control	DT1	DT2	SI1	SI2	*P*-value
Jejunum						
VH, mm	1.23 ± 0.26^{a}	1.03 ± 0.20^{b}	1.31 ± 0.22^{a}	1.32 ± 0.18^{a}	1.18 ± 0.25^{ab}	0.02
CD, mm	0.22 ± 0.04^{a}	0.16 ± 0.03^{b}	0.18 ± 0.04^{b}	0.21 ± 0.02^{a}	0.19 ± 0.03^{ab}	<0.01
MMT, mm	1.48 ± 0.28^{a}	1.26 ± 0.24^{b}	1.54 ± 0.21^{a}	1.58 ± 0.13^{a}	1.41 ± 0.27^{ab}	0.01
V:C	5.77 ± 1.25^{b}	6.41 ± 1.52^{ab}	7.51 ± 1.38^{a}	6.22 ± 0.91^{b}	6.38 ± 1.55^{ab}	0.05
Ileum						
VH, mm	0.91 ± 0.15	0.84 ± 0.16	0.93 ± 0.18	0.97 ± 0.14	0.95 ± 0.14	0.19
CD, mm	0.21 ± 0.03	0.18 ± 0.04	0.19 ± 0.03	0.21 ± 0.02	0.21 ± 0.03	0.07
MMT, mm	1.20 ± 0.20	1.07 ± 0.18	1.17 ± 0.18	1.24 ± 0.16	1.24 ± 0.16	0.06
V:C	4.29 ± 0.68	4.79 ± 1.53	4.93 ± 1.37	4.62 ± 0.48	4.50 ± 0.55	0.64

DT1, basal diet +300 mg/kg dandelion tannins; DT2, basal diet +500 mg/kg dandelion tannins; SI1, basal diet +300 mg/kg soybean isoflavones; SI2, basal diet +500 mg/kg soybean isoflavones; VH, villus height; CD, crypt depth; MMT, mucosal muscularis thickness; V:C, villus height:crypt depth ratio.
The data were expressed as the means ± standard deviation.
^{a,b}Marks indicate statistically significant differences ($P < 0.05$).

3.4. The mRNA expression level of genes

As shown in Figure 2, the mRNA expression level of *occludin* in the duodenum was the highest in the DT2 groups. The mRNA level of *ZO-1* in the SI1 group was significantly upregulated ($P < 0.05$) compared to the Con group. In the ileum, the mRNA expression level of *occludin* was higher in the DT2 and SI1 groups than in the Con group. The mRNA expression level of *ZO-1* was significantly higher in the DT2 and SI1 groups than in the Con group ($P < 0.01$). Relative to the Con group, the mRNA expression level of *claudin-1* was significantly increased in the DT2 group ($P < 0.01$).

Dietary supplementation with dandelion tannins or soybean isoflavones has no significant effect on the mRNA levels of tumor necrosis factor-alpha (*TNF-α*) and transforming growth factor beta (*TGF-β*) in the duodenum and ileum (Figure 3).

3.5. Cecal microbiota

The Chao1 and abundance-based coverage estimator (ACE) indexes were higher in the SI2 group than in the Con group. By contrast, the Shannon and Simpson indexes were significantly decreased in the DT2 and SI1 groups ($P < 0.01$; Table 7). At the phylum level, the top 10 most abundant taxa of cecal microbiota were Bacteroidota, Firmicutes, Proteobacteria, Euryarchaeota, Campylobacterota, Cyanobacteria, Actinobacteria, unidentified-bacteria, Desulfobacterota, and Verrucomicrobiota. The abundance of Bacteroidota and Firmicutes accounted for more than 90% of the total bacteria found in the cecal microbiota (Figure 4). The relative abundance of Actinobacteria was the highest, while the relative abundance of Bacteroidota was decreased significantly in the SI2 group compared to the Con group ($P < 0.01$). The relative abundance of Proteobacteria was decreased in the DT2 group compared to the Con group ($P < 0.01$; Figure 5).

At the genus level (Figure 6), the most dominant microbiota (>5%) were *Barnesiella* (22.88%), *Bacteroides* (8.58%), *Alistipes* (16.87%), and *Faecalibacterium* (15.39%). The relative abundance of *Barnesiella* was significantly increased in the DT2 group compared to the Con group (Figure 7). The linear discriminant analysis (LDA) effect size (LEfSe) analysis indicated that DT1 increased the relative abundance of *Lactobacillaceae*, while SI2 increased the relative abundance of *Bifidobacteriaceae*, *Unidentified-chloroplast*, *Streptococcaceae*,

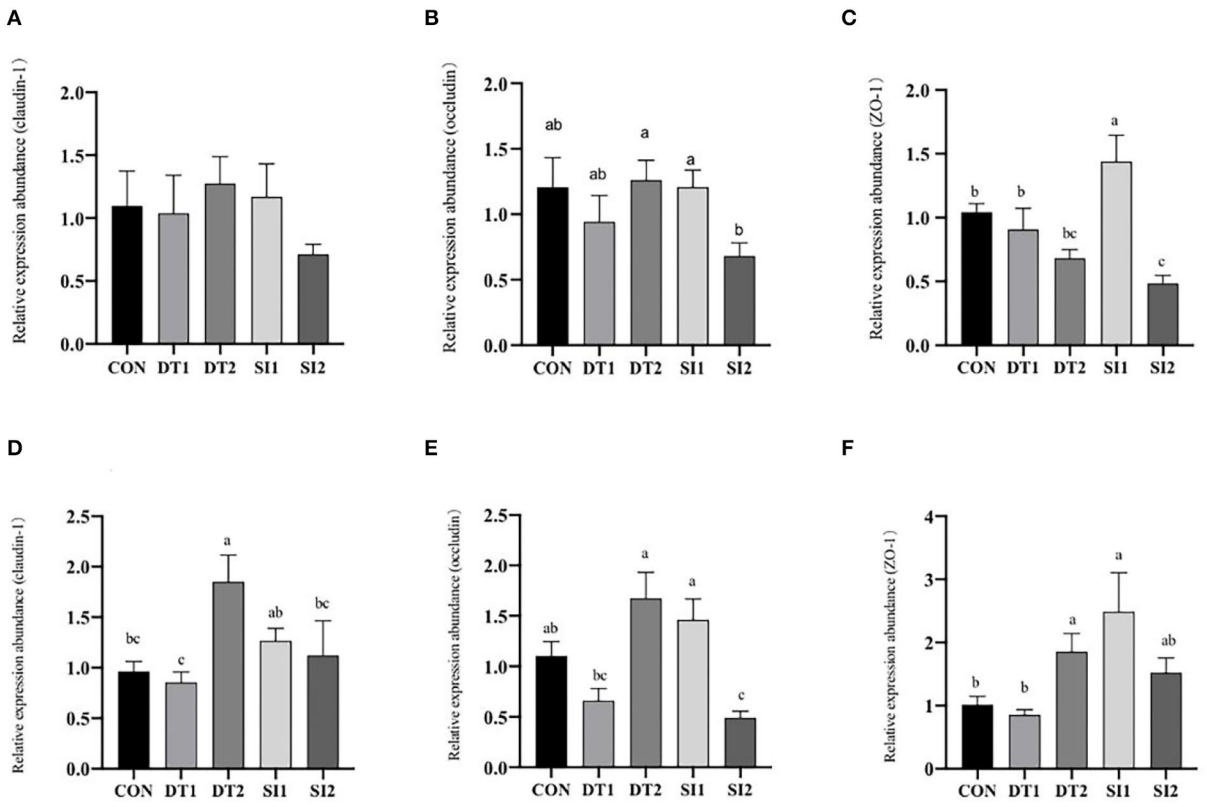


FIGURE 2
Effects of dietary dandelion tannins or soybean isoflavones on the messenger RNA (mRNA) expression of tight junction-related genes in duodenum **(A–C)** and ileum **(D–F)**. ^{a,b,c}Marks indicate statistically significant differences ($P < 0.05$).

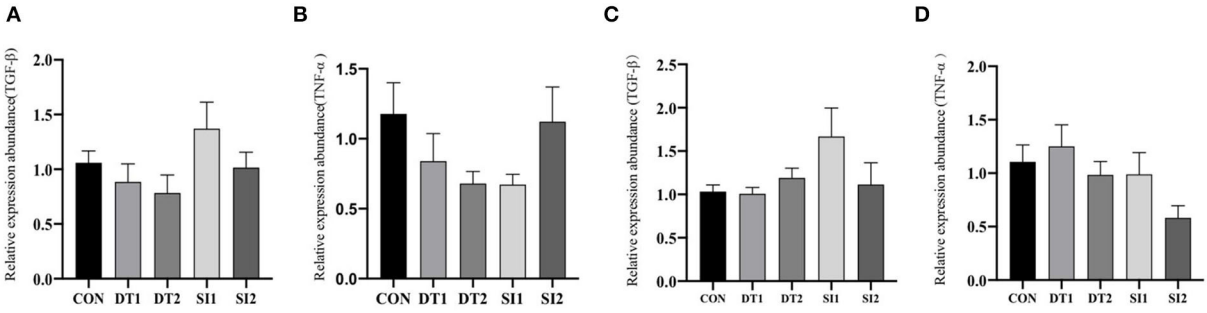


FIGURE 3
Effects of dietary dandelion or soybean isoflavones on the messenger RNA (mRNA) expression of inflammation-related genes in duodenum **(A, B)** and ileum **(C, D)**.

Staphylococcaceae, *Burkholderiaceae*, *and Akkermansiaceae* (Figure 8).

4. Discussion

The use of antibiotics as growth promoters in chicken production proved to be harmful to human health (23). Therefore, alternative innovative methods are urgently needed to be implemented to increase chicken production. The beneficial effects of tannins or soybean isoflavones have been well recognized for improving growth performance (18, 21, 24). In the present study, 1-day-old chickens were selected as models to evaluate the effects of dietary supplementation with dandelion tannins or soybean isoflavones on Wenchang chickens. Consistent with published studies (25, 26), an increase

TABLE 7 The Effects of dietary supplementation with dandelion tannins or soybean isoflavones on the α-diversity indexes of cecal microbiota.

Item	Control	DT1	DT2	SI1	SI2	*P*-value
Shannon	5.25 ± 0.37^{a}	5.22 ± 0.34^{a}	4.68 ± 0.34^{b}	4.76 ± 0.40^{b}	5.31 ± 0.43^{a}	<0.01
Simpson	0.91 ± 0.03^{a}	0.91 ± 0.03^{ab}	0.87 ± 0.03^{c}	0.88 ± 0.02^{bc}	0.90 ± 0.03^{ab}	<0.01
Chao1	854.34 ± 115.52^{b}	863.12 ± 132.77^{b}	781.54 ± 92.46^{b}	812.25 ± 186.09^{b}	977.90 ± 125.53^{a}	<0.01
ACE	718.36 ± 86.56^{b}	710.86 ± 97.51^{b}	644.77 ± 64.83^{b}	657.22 ± 146.01^{b}	816.91 ± 99.40^{a}	<0.01

DT1, basal diet +300 mg/kg dandelion tannins; DT2, basal diet +500 mg/kg dandelion tannins; SI1, basal diet +300 mg/kg soybean isoflavones; SI2, basal diet +500 mg/kg soybean isoflavones.
^{a,b}Marks indicate statistically significant differences ($P < 0.05$).

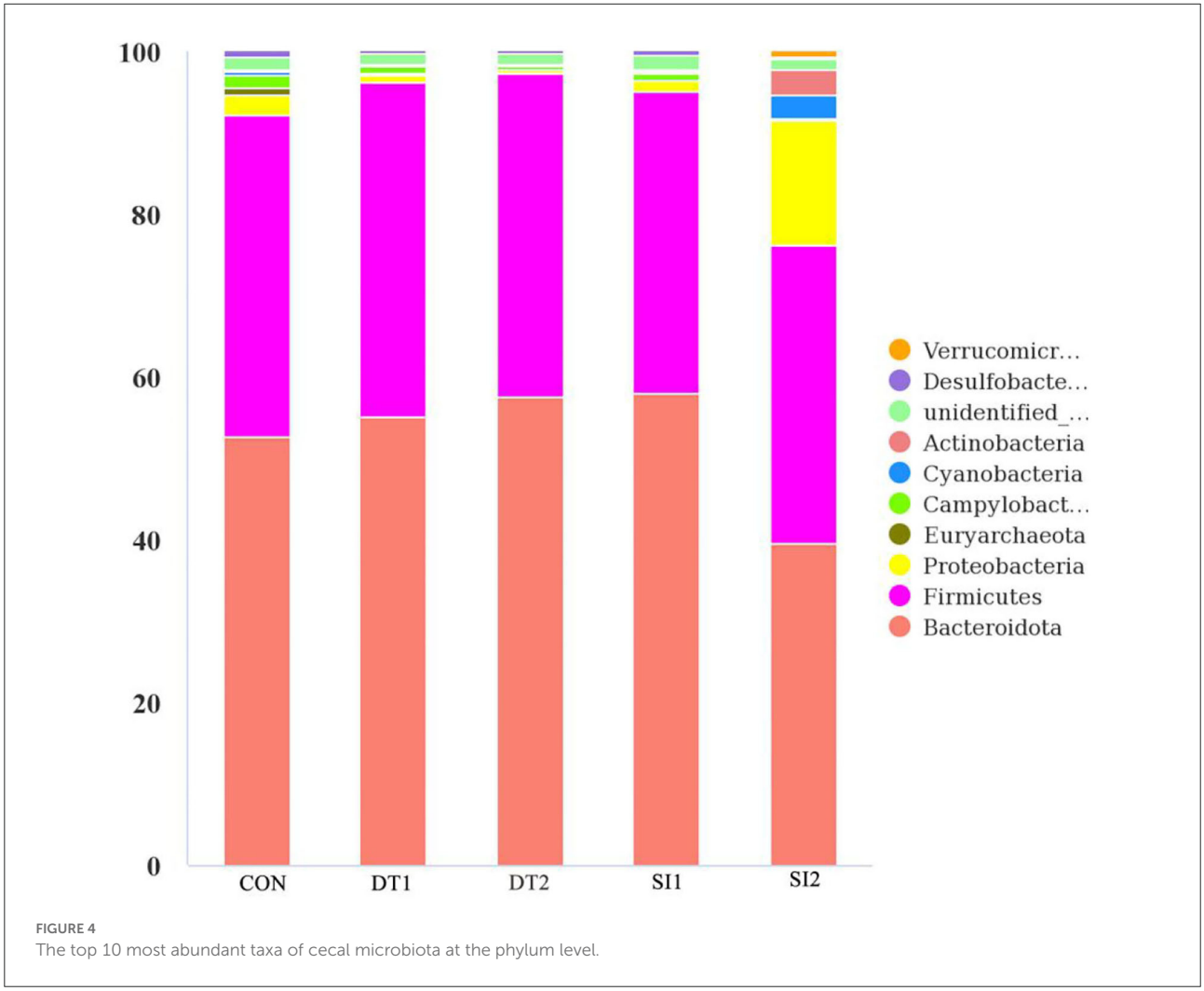


FIGURE 4
The top 10 most abundant taxa of cecal microbiota at the phylum level.

in BW and ADG was observed in the DT2 and SI1 groups. These results indicate that the growth performance improved with dandelion tannins' or soybean isoflavones' supplementation. The optimal growth performance is directly linked to the health of the intestine (27). Evidence showed that dandelion tannins could improve intestinal morphology and regulate the composition of intestinal microbiota (28, 29). These results indicate that dandelion tannins could maintain the health status of the intestine, thereby indirectly promoting the growth of the broiler (30). Evidence showed that soybean isoflavones could improve growth performance by regulating the hormone level related to growth and improving the metabolism and utilization of substances (9, 31). However, in the present study, a diet supplemented with 300 mg/kg of dandelion tannin or 500 mg/kg of soybean isoflavone did not lead to a significant improvement in growth performance, indicating that the effects of tannins or flavonoids on growth performance are dosage-dependent.

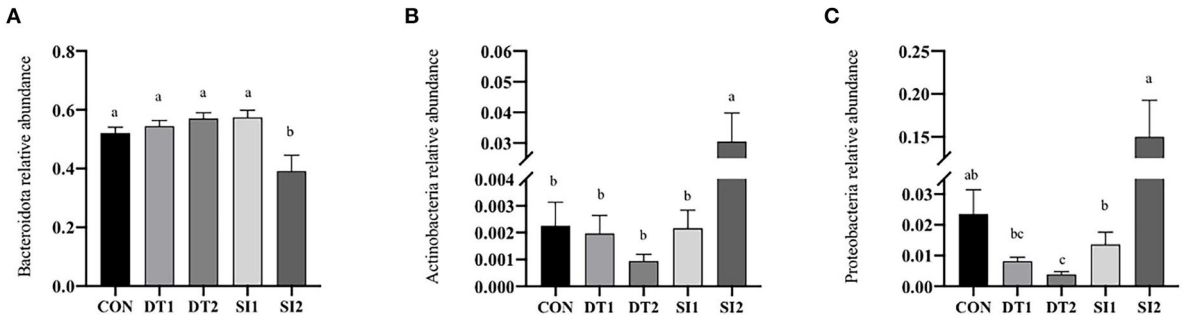


FIGURE 5
Effects of dietary dandelion tannins and soybean isoflavones of cecal microbiota at the phylum level. The relative abundance of Bacteroidota **(A)**, Actinobacteria **(B)**, and Proteobacteria **(C)**. ^{a,b,c}Marks indicate statistically significant differences ($P < 0.05$).

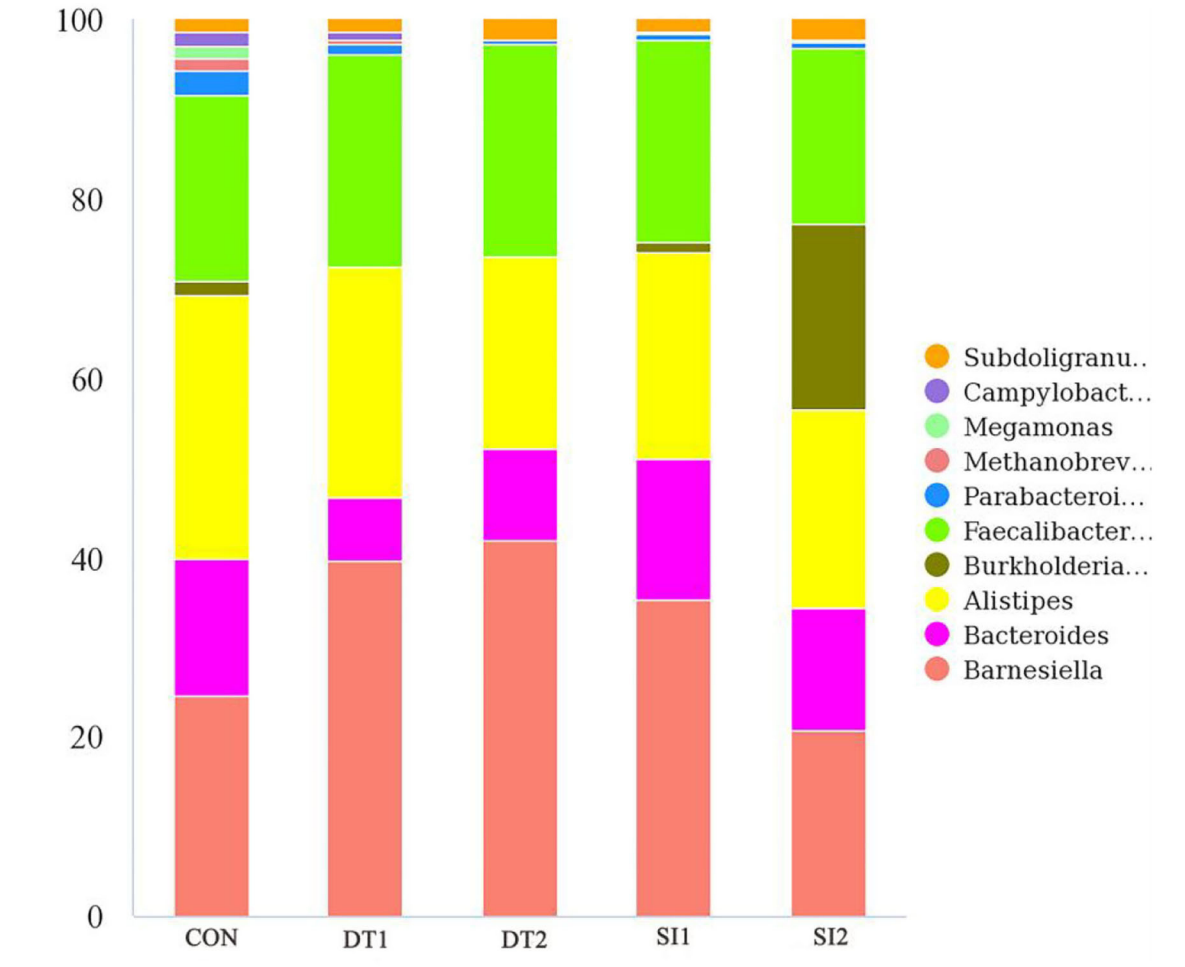


FIGURE 6
The top 10 most abundant taxa of cecal microbiota at the genus level.

Blood biochemical indicators reflect the health status of the animal. Albumin is mainly produced by the liver, which indicates an improvement in protein synthesis at a higher level, whereas it causes liver damage at a lower level (32, 33). Blood glucose reflects energy metabolism (34) and is the main source of energy (35). Maintaining a normal blood glucose level is

important for the physiological function of animals (36). In the present study, the serum albumin and blood glucose levels in experimental groups were increased, which was consistent with the changes in growth performance. Triglycerides are synthesized in the liver and adipose tissues. Most of the tissues in the human body can use the energy provided by the fatty acids and glycerol released by triglyceride decomposition (37). However, studies reported that the intake of meat with a high level of triglycerides is not beneficial to human health (38). In the present study, soybean isoflavones could decrease the serum triglyceride levels. Consequently, the results suggested that an appropriate level of dandelion tannins or soybean isoflavones in diets can promote growth by improving protein synthesis and lipid metabolism.

Poultry growth can be hampered by oxidative stress induced by nutrition, environment, disease, and other factors (39). Antioxidation is one of the most important biological functions of dandelion tannins or soybean isoflavones (40, 41). The antioxidant enzymes, including SOD, CAT, and glutathione peroxidase (GSH-Px), can effectively clean up free radicals and restore the balance between oxidants and antioxidants. As the product of lipid peroxidation, MDA indirectly reflects the extent of oxidative damage. Previous studies showed that the addition of dandelion tannins or plant flavonoids to a broiler diet could improve the serum antioxidant capacity (42, 43). Our study also found that the serum CAT levels became significantly higher with dandelion tannins' or soybean flavonoids' supplementation, suggesting that the dietary supplementation with low-dose dandelion tannins or soybean isoflavones could improve the serum antioxidant status of broilers.

The intestinal pH value is an important index reflecting the digestive environment *in vivo*. When the pH is close to 7 or slightly higher, it is conducive to the growth of pathogens (44). In contrast, a lower intestinal pH promotes the growth of beneficial microorganisms and nutrient absorption (45, 46). In the present study, the pH of the ileum in the DT2 and SI1 groups was significantly decreased. These results were in agreement with those of the previous study, where it was reported that adding a plant-based supplement (Galibiotic^{TM}) to broiler feed significantly decreased intestinal pH (44). Similar results were also found with the addition of turmeric meal individually or in combination with a wheat-soybean meal (47). Furthermore, 500 mg/kg of dandelion tannins and 300 mg/kg of soybean isoflavones had brought about the effect of reducing the intestinal pH to a certain degree, potentially improving nutrient absorption and promoting the growth of chickens.

The intestinal histomorphology can reflect the health status of the intestine, as well as the digestion and absorption of nutrients (48). To understand the underlying mechanism of the growth-promoting effect of dandelion tannins and soybean isoflavones, the intestinal villus length and crypt depth were measured (49). In this study, the jejunal villus height to crypt depth ratio significantly increased in the DT2 group than in

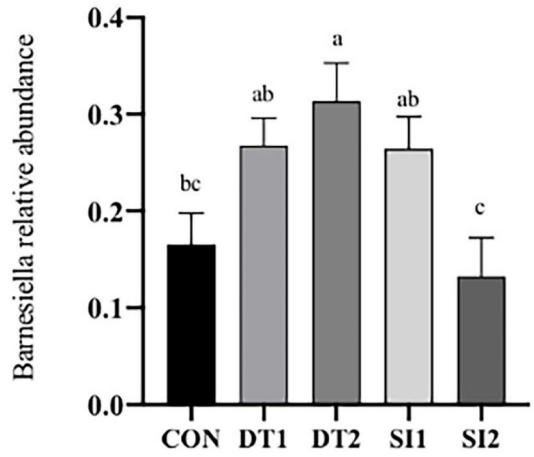
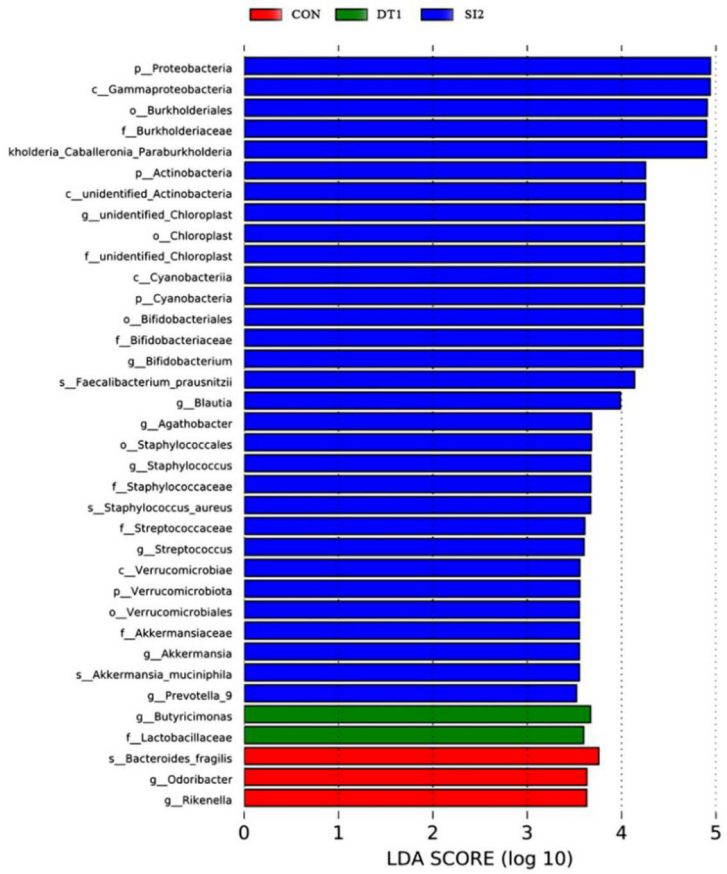


FIGURE 7
Effects of dietary dandelion tannins or soybean isoflavones on cecal microbiota at the genus level. ^{a,b,c}Marks indicate statistically significant differences ($P < 0.05$).

the Con group, and the jejunal villus length was higher in the DT2 and SI1 groups, which indicates that dandelion tannins or soybean isoflavones could enhance the function of the small intestine (50). In line with our study, evidence showed that the addition of tannins to the diet significantly increased the jejunal villus length and alleviated the negative effects of heat stress on intestinal histomorphology (42). In addition, soybean isoflavones were shown to increase the villus length and protect intestinal health (13). The intestine is not only an absorptive organ but also an important immune organ (51). The mucosal muscularis is the first barrier to preventing the destruction of intestines by pathogenic microorganisms (52). *Occludin* and *claudin*, two transmembrane proteins, are crucial components of tight junctions (53, 54) and play an important role in the maintenance of the epithelial barrier (55). In the present study, the jejunal muscularis thickness was higher in the DT2 and SI1 groups. The mRNA expression of *ZO-1* in the duodenum was higher in the SI1 group and those of *occludin*, *ZO-1*, and *claudin-1* were higher in the DT2 and SI1 groups. Collectively, these results indicate that dandelion tannins or soybean isoflavones contribute to the growth of chickens which might be attributed to the improved intestinal health.

Microbiota is a highly complex microbial community that directly affects the health, immunity, and productivity of animals (56). Previous studies found that intestinal microbes could affect the feed conversion ratio of chickens, whereas chickens with greater feed conversion ratios had lower α-diversity of gut microbiota (57, 58). In this study, there was lower community richness and diversity in cecum in the DT2 and SI1 groups. This may be one of the reasons for the improved growth performance of Wenchang chickens. It was reported that Firmicutes and Bacteroidetes are the



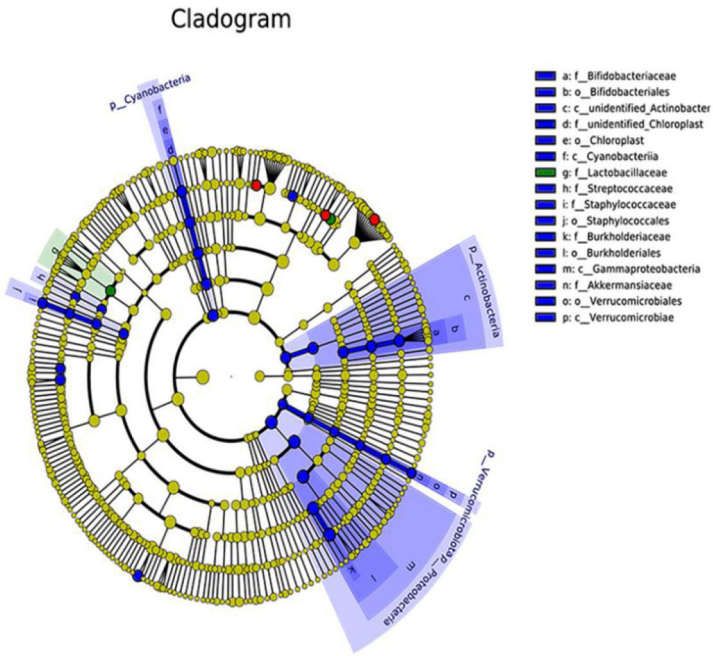


FIGURE 8
Linear effect size (LDA) effect size (LEfSe) analysis at the family level with the threshold set to 3.5.

major phyla of cecal microbiota, comprising of up to 90% of the total microbiota (59, 60). The Firmicutes primarily synthesizes butyrate and propionate, which are the major sources of energy for intestinal cells (61). Evidence showed that the abundance of Firmicutes was positively correlated with weight gain (62). There is a mutualistic relationship between Bacteroidetes and the animal host. Bacteroidetes helps the body to gain energy from the degradation of carbohydrates (63, 64) and maintain a healthy gut (65, 66). In this study, Bacteroidetes and Firmicutes accounted for more than 90% of intestinal bacteria in 40-day-old Wenchang chickens, which was in agreement with the literature (67). In addition, there were seven species of bacteria with a significant difference at the family level, according to the LDA effect size analysis. *Lactobacillaceae* can protect the gut by inhibiting pathogen colonization (68, 69) and have a positive effect on the feed efficiency of chickens (70). *Bifidobacteriaceae* have shown beneficial effects in an animal, such as promoting nutrient absorption and improving immunity (71). *Akkermansiaceae* belong to the phylum *Verrucomicrobia* and are considered anti-inflammatory bacteria (72). The abundance of *Akkermansiaceae* is negatively correlated with obesity, which in turn promotes the occurrence of inflammatory diseases (73). As a corollary, increasing the abundance of *Akkermansiaceae* could reduce inflammation (74). *Staphylococcaceae* belong to the Firmicutes and most of them are nonpathogenic, but a few species can cause disease. These bacteria are widespread in farmed chickens and are capable of undergoing fermentative metabolism (75, 76). This study demonstrates that dietary supplementation with dandelion tannins or soybean isoflavones regulated the gut microbiota composition related to the growth performance of Wenchang chickens.

5. Conclusions

This study demonstrates that dietary supplementation with dandelion tannins or soybean isoflavones improved the growth performance, serum biochemical indexes, serum antioxidant capacity, and intestinal health of 40-day-old Wenchang chickens. Based on the results, this study recommends that the dosage of dandelion tannins and soybean isoflavones added to diets of 40-day-old Wenchang chickens be 500 and 300 mg/kg, respectively. Our findings provide new insights into the potential of using natural plant extracts as feed additives.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found at: http://www.ncbi.nlm.nih.gov/bioproject/893339.

Ethics statement

The animal study was reviewed and approved by the Experimental Animal Ethics Committee of Animal Husbandry and Veterinary Research Institute, Hainan Academy of Agricultural Sciences.

Author contributions

XL prepared the manuscript and collected some data. RS, YG, YO, QQ, YX, XW, CH, and SJ collected the samples. GZ and LW were responsible for the design and direction of the experiment. All authors have read and agreed to the published version of the manuscript.

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Conflict of interest

XW was employeed by Hainan (Tanniu) Wenchang Chicken Co., Ltd.

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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Yeast derivatives as a source of bioactive components in animal nutrition: A brief review

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With a long history of inclusion within livestock feeding programs, yeast and their respective derivatives are well-understood from a nutritional perspective. Originally used as sources of highly digestible protein in young animal rations in order to offset the use of conventional protein sources such as soybean and fish meal, application strategies have expanded in recent years into non-nutritional uses for all animal categories. For the case of yeast derivatives, product streams coming from the downstream processing of nutritional yeast, the expansion in use cases across species groups has been driven by a greater understanding of the composition of each derivative along with deeper knowledge of mechanistic action of key functional components. From improving feed efficiency, to serving as alternatives to antibiotic growth promoters and supporting intestinal health and immunity while mitigating pathogen shedding, new use cases are driven by a recognition that yeast derivatives contain specific bioactive compounds that possess functional properties. This review will attempt to highlight key bioactive categories within industrially applicable yeast derivatives and provide context regarding identification and characterization and mechanisms of action related to efficacy within a range of experimental models.

KEYWORDS

yeast, bioactives, livestock, health, nutrition

Introduction

Yeast are single celled, eukaryotic organisms that belong to the fungi kingdom. With size ranging from 3 to 4 μm, they possess cell walls, nuclear membranes, but unlike plant cells do not contain chloroplasts (1). Yeasts are key member of nutrient recycling as they rely on other organisms, be it alive or dead for nutrients and obtain these through the production and secretion of proteolytic, glycolytic and or lipolytic enzymes to digestion organic matter into usable nutrients (2). Reproduction occurs through budding and fission (3), whereby budding is the process of parent cell expansion into a protrusion or "bud" along the cell wall that separates from the parent or expands further into a ribbon structure of yeast buds. Fission is the process where a parent cell expands and divides into two daughter cells (4). Yeast can survive in aerobic

or anaerobic environments and are therefore described as facultative anaerobes (5). However, yeasts tend to prefer aerobic conditions for propagation, as the production of carbon dioxide and energy from oxygen and sugars is more efficient than under anaerobic conditions where the end product of metabolism tends to be ethanol (6, 7).

The inclusion of yeast into livestock feeds has occurred under many use cases for many decades (8, 9). Traditionally, yeast, either whole or derivations thereof, were included into livestock feeds as protein sources due to their high digestibility and optimal ratio of essential amino acids (10). These yeast streams had many origins including from primary yeast production factories as well as being by-products from ethanol production. Inclusion into livestock rations was a logical endpoint for these co-streams as they generally had high nutritional value, were inexpensive and had good palatability profiles which would improve feed consumption. However, over time, a greater knowledge base has developed pertaining to the functional properties of intact yeast as well as yeast derivatives which has led to a non-nutritive use case for supplementing yeast into livestock rations in recent years (11, 12). Here, the focus has been on using the cell wall fraction of yeast cells (13, 14) or on using the nutrient rich cytosol fraction (15), both of which are streams that originate after the lysis of whole, live or inactivated, yeast cells. It has been estimated that the combined value of the feed yeast market will reach $3.96 billion US dollars by 2026 (https://www.reportlinker.com/p06320094/Feed-Yeast-Global-Market-Report.html?utm_source=GNW), with the majority of growth and value coming from the yeast derivative market segments. Given the interest and continued usage and adoption of these novel technologies, this paper will focus on reviewing bioactive compounds derived from yeast cell wall and yeast cytosol fractions in order to provide better context for participants in the animal nutrition industry.

Yeast processing

Primary yeast cultures are typically proprietary strains that are developed for specific purposes. For example, select strains of *Saccharomyces cerevisiae* have been developed for the production of breads that are excellent producers of carbon dioxide, which is important in the leavening process of dough production (16). Similarly, *Pichia pastoris*, which is used in the industrial production of biotherapeutics, is grown under specific conditions in order to maximize expression of target compounds (17). These strains require propagation through a series of lab scale growth steps prior to large scale propagation (Figure 1). When considering yeast for application into the food and beverage industries the final product can take the form of cream yeast or dried yeast, depending on the application of the final user, while in pharmaceutical and research based uses, it is the expression products from a given yeast organism that is of interest.

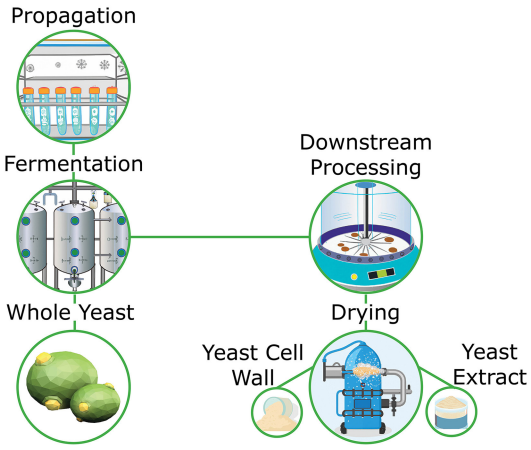

FIGURE 1
Overview of whole yeast and yeast derivative production.

As previously mentioned, downstream processing can be applied during the propagation steps of yeast in order to inactivate live yeast or lyse whole yeast into its constituent parts. Typically, initial processing steps involve manipulation of temperature, pH and osmotic balance in order to facilitate cell lysis (18, 19). Once cells are lysed, centrifugation can be applied to split fractions into insoluble carbohydrate rich cell wall fractions and soluble protein rich cytosolic fractions. Filtration can be used in place of or in conjunction with centrifugation in order to further partition each phase depending on the desired purity or composition of each fraction (20). Finally, water is removed thereby increasing the concentration of bioactive compounds within each fraction. Spray drying is typically used for dewatering, however, box and drum drying can also be utilized depending on the throughput and compositional integrity requirements.

Proximal composition of intact nutritional yeast

The composition of nutritional yeast and yeast derivatives are considerably different and have been reviewed extensively (21). From a nutritional standpoint, intact nutritional yeasts such as brewers and torula, which are commonly referred to as nutritional yeasts in the context of animal nutrition, typically possess high levels of crude protein and low levels of crude fat and fiber. For context, and as was described previously, yeasts are single celled organisms which is a critical characteristic as it differentiates them from many other nutritional feedstuffs that originate from multicellular plant or animal sources. For example, plant proteins usually originate in the seed or fruit structure and as such contain not only protein but fractions of the seed hull and cotyledon which are high in fibers

such as cellulose, hemi-cellulose, pectin and lignin (22). As single cell organisms, yeast have a cell wall in place of a hull and are therefore lower in fiber as compared to plant based feedstuffs. Furthermore, as yeast rely on external nutrient sources, they possess limited intracellular fat storage, which contrasts to plant based feed ingredients which store fat for germination within oleosomes (23). Thus, nutritional yeasts, be they brewers, bakers or torula tend to be high in crude protein and low in crude fat and fiber, respectively. Although conventional fiber measurements such as crude fiber, result in low values compared to plant based feedstuffs, yeast do contain substantial amounts of what could be described as dietary fiber, which are predominantly mannan glucose based non-starch polysaccharides (NSP; Table 1). Although fiber within intact yeasts is typically low for the aforementioned reasons, the variability within and between yeasts is notable. From Table 1 it can be seen that on average, mannan and glucose account for all NSP residues in bakers and brewers yeast and combine for >95% of the total NSP fraction in torula yeast. The standard deviation for mannose and glucose is >25% of the mean in bakers/brewers yeast and <8% in torula yeast, indicating a greater degree of variability in the former as compared to torula yeast. Given that bakers /brewers yeast has greater use cases and therefore has a wider breadth of production conditions as compared to torula yeast, the described differences for each intact yeast source with respect to fibrous constituents is as expected.

The protein and mineral content of nutritional yeasts is relatively consistent across groupings. Table 1 shows that bakers/brewers and torula yeast have average crude protein levels of 44.75 and 52.04%, respectively. For mineral content, as measured by ash, bakers/brewers yeast has on average 5.19% while torula has 8.44%, with both categories having relatively little variability associated with this measurement (Table 1). Crude protein variability of bakers/brewers yeast is greater than that of torula when looking at standard deviation as a percent of the mean, which as detailed previously likely relates to broader application of the former. It is worth noting that typically, crude protein is determined by calculating total nitrogen content of an ingredient and multiplying this value by 6.25, however, a coefficient of 5.8 has been reported to be used for yeasts which could be a source of variability seen in the literature (24). Amino acid profiles of each yeast category are relatively similar with lysine being 4.54% in bakers/brewers and 4.88 in torula as an example. Additionally, it should be pointed out that crude ash encompasses key minerals such as phosphorus and calcium and given the importance of these minerals it follows that total mineral content variability would be low. For example, phosphorus, the major mineral component of nutritional yeasts, is required for energy metabolism as a component of ATP (7). Furthermore, calcium, along with phosphorus, is used within numerous cell signaling pathways (25). Although propagation techniques will lead to differences in the nominal amount of each nutrient category, it is not surprising that these nutrients are quantitatively consistent due to the critical functions they are a part of within the yeast cell.

Although the intestinal benefits of yeast cell are well-recognized, as the biotechnology progresses, more attention has been paid to value-added yeast-derived products (26, 27). The bioactivity of yeast cell components can be substantially enhanced by enzymatic modification of the cell wall polysaccharides, or by releasing and separating bioactive fractions, such as nucleic acids (28). The enzymatic hydrolysis of long-chain polysaccharides of the cell wall will result in shortening of the chain and production of more potent water-soluble derivatives, which can therefore be more effective in the gut environment than the intact yeast (29). The extracted intracellular components of yeast might exert more pronounced bioactivities, mainly due to their superior bioavailability when released from encapsulation within the cell wall. Additionally, the yeast cell derivatives can be more effectively utilized by targeting specific purposes.

Proximal composition of nutritional yeast derivatives

In the context of this paper, yeast derivatives are those fractions of the yeast that derive from lysing, centrifuging, filtering and other applications done to intact yeast cells through the activities associated with downstream processing. Although there are nearly limitless types of derivatives that are possible for livestock nutrition, the principle streams are in the form of yeast cell wall and yeast cell content.

Compared to its parent stream, that being intact or nutritional yeasts, the cell wall fraction has a lower crude protein and ash content and a proportionately higher amount of carbohydrate constituents and represents between 26 and 32% of the dry weight of intact yeast cells (30). The protein in yeast cell wall tends to be in the form of glycoproteins and associated with the residual NSP and other carbohydrates present in the cell wall structure (31). However, due to incomplete processing techniques a portion of the protein in yeast cell wall originates from the cytosol of the upstream source yeast. This can be observed with the protein related standard deviation being approximately 25% of the mean and the maximum value being nearly three times that of the minimum (Table 2). Conversely, although the variability of the key carbohydrate portion of the cell wall portion has inherent variability, the relationship of standard deviation to mean value as expressed as a percentage is lower (17 vs. 25%) than that of protein. Mannan and glucan-based residues represent upwards of 95% of the non-starch polysaccharides in yeast cell wall with variability in maximal concentrations for the mannan and glucan fractions being associated with the relative degree of downstream processing, such as acid or enzyme hydrolysis, applied to any given output stream (30, 32).

TABLE 1 Nutrient composition of intact nutritional yeast sources[a].

Nutrient, % as is	Mean	Std Dev	Min	Max
Bakers and brewers yeast (*Saccharomyces cerevisiae*)				
Dry matter	95.10	1.34	93.43	96.90
Crude protein (*N* x 6.25)	44.75	5.50	39.75	56.41
Starch	4.51	2.89	n.d.[b]	17.5
Ether extract	1.62	0.61	0.35	2.26
Ash	5.19	0.30	4.87	5.86
Crude fiber	1.90	1.30	0.1	4.4
Neutral detergent fiber (NDF)	10.05	9.40	n.d.	20.7
Acid detergent fiber (ADF)	5.87	2.50	n.d.	5.70
Non-starch polysaccharides	20.21	6.54	14.47	33.19
Glucan/glucose	10.38	2.90	6.46	14.34
Mannan/mannose	7.81	4.17	0.55	14.80
Lysine	4.54	0.90	4.60	7.60
Methionine	1.05	0.30	1.30	2.20
Cysteine	0.62	0.60	0.33	1.9
Threonine	3.18	0.70	3.70	5.60
Tryptophan	0.79	0.20	1.00	1.40
Calcium	0.29	0.11	0.10	0.54
Phosphorus	1.31	0.24	0.96	2.00
Nucleotides	0.06	0.08	n.d.	0.11
Torula yeast (*Candida utilis*)				
Dry matter	94.83	0.62	94.00	95.83
Crude protein (*N* x 6.25)	52.04	0.66	51.44	53.48
Ether extract	0.16	0.10	0.07	0.36
Ash	8.44	0.60	7.04	8.88
Crude fiber	2.07	2.79	0.08	6.2
Non-starch polysaccharides	21.83	1.75	19.17	23.87
Glucan/glucose	11.82	0.76	10.60	13.32
Mannan/mannose	9.28	1.37	7.17	10.46
Arabinose	0.73	0.23	0.43	0.98
Lysine	4.88	1.36	3.45	6.68
Methionine	0.76	0.34	0.38	1.14
Cysteine	0.74	0.27	0.49	1.02
Threonine	3.26	1.10	2.35	4.55
Tryptophan	0.57	0.07	0.52	0.62
Calcium	0.23	0.09	0.13	0.47
Phosphorus	2.43	0.61	1.78	0.61
Nucleotides	1.44	1.81	0.04	3.49

[a]Adapted from: CBS Bio-Platforms Inc. internal analyses 2020-2022; Evonik AMINODat 6.1 2021; Feedipedia.org 2022.
[b]Not detected.

Generally speaking, the intracellular components of lysed yeast cells are referred to as yeast extracts. These extracts typically contain higher levels of both crude protein and non-protein nitrogen and lower carbohydrate concentrations than either intact yeast cells or yeast cell wall fractions (Table 2). The protein tends to be highly digestible (33), as unlike the cell wall fraction, tends to not form carbohydrate complexes, however, the presence of mannose and glucose-based polysaccharides is likely due to incomplete removal of the cell wall during downstream processing.

Containing a nitrogenous base, a ribose sugar and between one and three phosphates, nucleotides serve as the base units of the nucleic acid polymers DNA and RNA. Nucleotides are categorized by their nitrogenous base, those being guanine,

TABLE 2 Nutrient composition of yeast derivatives[a].

Nutrient, % as is	Mean	Std Dev	Min	Max
Yeast cell wall (from: *Saccharomyces cerevisiae*)				
Dry matter	95.81	1.62	91.91	99.71
Crude protein (*N* x 6.25)	32.53	8.13	15.81	47.22
Ether extract	0.69	1.01	n.d.[b]	5.44
Ash	3.79	1.11	2.17	5.66
Non-starch polysaccharides	37.88	6.42	27.77	54.55
Glucan/glucose	22.28	4.17	11.69	36.59
Mannan/mannose	15.49	4.68	6.74	27.30
Arabinose	0.33	0.16	0.13	0.67
Nucleotides	n.d.	n.d.	n.d.	n.d.
Yeast extract (from: *Saccharomyces cerevisiae*)				
Dry matter	96.47	0.45	95.87	97.23
Crude protein (*N* x 6.25)	53.22	11.77	43.09	69.21
Ether extract	0.28	0.18	0.03	0.65
Ash	19.12	6.36	10.35	25.79
Non-starch polysaccharides	9.32	2.25	6.77	11.05
Glucan/glucose	6.64	5.3	0.56	10.26
Mannan/mannose	2.61	3.12	0.79	6.21
Nucleotides	15.06	10.88	3.05	40.20

[a]From: CBS Bio-Platforms Inc. internal analyses 2020–2022.
[b]Not detected.

adenine, cytosine and thymine in DNA, while in RNA uracil is present in place of thymine. Nucleotides play a central role in cellular metabolism, by providing energy (ATP, GTP) as well as being involved in protein synthesis and contributing to cell signaling [cGMP; (34)]. Compared to torula yeast, brewers yeast contains trace amounts of nucleotides (0.06 vs. 1.44%) with variability being high in the former as in some cases nucleotides are undetectable (Table 2). It should be noted that the variability of nucleotides in torula yeast is also high with the standard deviation being in excess of 100% of the mean as well as the maximum value being 87 times greater than the minimum (3.47 vs. 0.04%). This variability in both brewers and torula is due in part to differences of applied methods of analyses as well as factors related to production and extraction (34, 35). In addition, the means by which nucleotides are degraded from parent DNA and RNA, harvested and the conditions by which the parent cells were cultivated contribute to total and relative amounts present in any given yeast extract preparation (36).

Targeting bioactive compounds within yeast carbohydrate fractions

The carbohydrate fraction of yeast cell wall has long been used as a source of bioactive compounds for the livestock feeding industry. Colloquially referred to as MOS (mannan-oligosaccharide), various yeast cell wall preparations have been developed that claim that the provision of MOS will increase and/or improve various aspects of health and production. However, as outlined above, mannan-based carbohydrates are present within yeast cell wall isolations along with other molecules including glucan-based polysaccharides such as beta-1,3;1,6 glucans and to a lesser extent chitin (37). Additionally, mannan exist as long chain water insoluble polysaccharides rather than an oligosaccharides (38), so the use of term MOS is a misnomer as yeast cell wall preparations contain little, if any, mannan based oligosaccharides.

A plethora of beneficial attributes have been observed and assigned to the dietary provision of yeast cell wall preparations into livestock, poultry, and aquaculture feeds. Amongst these are improvements in body weight gain and feed efficiency (39, 40) as well as augmentation of health and immunity (12, 41, 42). Other use cases have focused on the ability of yeast cell wall carbohydrates to prevent enteric infection (43) while also mitigating pathogen shedding (29) thereby bridging the gap between the classical growth and health use case into a food safety assurance technology. Looked at as a whole, it is clear that the carbohydrate portion of yeast cell wall possess bioactive properties that have the potential to beneficially augment livestock across multiple application scenarios.

As outlined above, targeting the carbohydrate fraction of yeast cells means targeting the cell wall as this is where the majority of carbohydrates reside within the yeast cell (30, 31). Within the yeast cell wall, carbohydrates exist as glycoproteins along with various minerals such as phosphorus, calcium, sodium and others. The carbohydrates are typically large polysaccharides, 10,000+ subunits in length, and composed predominantly of mannose and glucose subunits which are assembled as mannan and glucan polysaccharides. Mannose subunits within mannan polysaccharides link at locations 1,6 and 1,2- and 1,3 in alpha configurations, thereby making alpha 1,6 mannans and to a limited extent alpha 1,2 polysaccharides (44). The mannans found in yeast cell walls differ from those found in plant ingredients such as soybeans as well as palm and coconuts fruits which are also linked at the 1,6 location but are in a beta configuration and are therefore described as beta-mannans (45). Conversely, the glucose fractions are linked together at the 1,3 and 1,6 locations in beta configurations (46), thereby making beta-1,3; 1,6 glucan subunits that exist as extended beta-glucan polysaccharides. These beta-glucans differ from those found in grains such as barley, oats and rye which are connected at the 1,4 and 1,6 locations (47) but are otherwise identified as beta-glucans. The unique structure of yeast based mannans and beta-glucans impart a greater potential for biological activity compared to their plant based counterparts, the latter of which, although possessing some prebiotic properties, are generally viewed in the field of animal nutrition as an impediment to the nutritional value of their respective ingredient (37).

One commonality that all carbohydrate constituents have in common is their purported health benefits, however the degree and consistency that each inparts on the receiving animal is debatable (11, 48). To this, *Hooge* reviewed the response of broiler chickens fed yeast cell wall preparations containing both mannan and glucan components and reported that in 44 studies, an average positive increase in body weight of 1.75% was observed 79.5% of the time, while 13.6% of studies reported a decrease (49). Furthermore, when mortality was analyzed, it was reported that 40.9% of studies observed an average decrease of 16.4 while 22.7% of studies observed increases in mortality. Relatively similar results were observed for nursery pigs where a meta-analysis of 54 studies reported that an average body weight increase of 2.25% was observed in 66.6% of studies while 31.8% observed a decrease (50). Although the reported increases from these meta-analyses are promising in terms of the ability of the test preparations to improve rate of growth and reduce mortality, the fact that such high percentages reported negative responses likely relates to incomplete or incorrect targeting of the bioactive mannan and glucan fractions present within the dietary test articles under evaluation in each respective study.

Broadly speaking, the contents, or digesta, of the gastrointestinal tract can be partitioned into two sections, those being the water and water insoluble phases (51). Actions critical to digestion and enteric function, such as enzymatic breakdown of nutrients and proliferation of beneficial bacteria occur within the water-soluble phase (52). The water insoluble phase is populated by compounds such as non-emulsified fats, undigested protein complexes and various dietary fiber components (22), which although important to the overall health of the GIT, are less impactful with respect to potential benefits associated with yeast carbohydrate bioactive compounds. Thus, if a bioactive compound of carbohydrate origin is to elicit broadscale beneficial effects, it must be able to enter the water soluble phase as this is where it is able to exert the aforementioned beneficial effects. Failure to achieve entry into the water-soluble phase will limit the degree to which a bioactive compound can exert beneficial effects as it will have similar functional properties as dietary fiber (13, 22). When considering yeast carbohydrates as bioactive candidates, a critical limitation that is likely responsible for the inconsistent responses observed in the literature is that many of these preparations are highly water insoluble. For example, it was reported that a yeast cell wall preparation fed to broiler chickens that containing mostly insoluble mannans and beta-glucans had no effect on body weight gain or feed efficiency compared to the control group (53). Although the lack of improvement was not directly attributable to the structural composition of the test article, it is possible that a lack of solubility prevented a performance effect as the test article was observed to have increased intestinal villus height, which is a known end point for yeast carbohydrate bioactivity (54). The insolubility of the aforementioned test article as well as preparations in other studies that failed to show a beneficial response is related to many structural characteristics of the yeast cell wall including an extended chain length of both the mannan and glucan-based polysaccharide fractions along with the presence of associated proteins and O- and N-glycans which collectively work to reduce overall water solubility (31). Overcoming this limitation is important if functional bioactives are to be isolated and developed from the carbohydrate fraction of yeast cell wall.

It has been proposed that enzyme technology could be utilized to overcome the previously outlined solubility challenges that to date have limited the efficacy of many yeast carbohydrate bioactive materials (29, 55). There is precedence for enzyme technology application where, along with other processing technologies, carbohydrase enzymes have been successfully applied to co-products of corn starch processing resulting in the production of highly purified fructose containing oligosaccharides (FOS) possessing bioactive properties (56, 57). Similarly, exogenous enzymes have been used to generate bioactive galacooligosaccharides (GOS) from soybean and canola meal, respectively (58). With respect to yeast carbohydrates, enzymes specific to the major polysaccharides are required, namely beta 1,3-1,6 glucanase and alpha mannanase, in order to generate low molecular weight bioactive compounds *via* hydrolysis. This concept recently demonstrated the effectiveness

of this approach when a purified beta 1,3-1,6 glucanase enzyme preparation was incubated along with yeast cell wall biomass in order to generate an enzymatically derived bioactive preparation composed of highly soluble, low molecular weight beta-1,3 glucan compounds along with soluble but intact mannans, that later of which have been released from the extended glucan core (29). The efficacy of this preparation was evaluated in adult laying hens where *Salmonella enteritidis* shedding within an oral challenge model was used as an endpoint and it was observed that compared to control birds as well as birds fed non-enzymatically modified yeast carbohydrates, those fed the bioactive compound had significantly reduced shedding (87 vs. 62 vs. 37%) 6 days post-inoculation as well as quantitatively reduced cecal counts of *Salmonella enteritidis* (4.30 vs. 2.95 vs. 1.90 Log10 cfu/g). When a similarly produced enzymatically produced preparation of yeast carbohydrates was fed to broiler breeder hens, it was observed that eggs laid from hens fed the bioactive material had statistically greater concentrations of IgA within the yolk (7.9 vs. 7.7 ug/ml) compared to control hens (54). Furthermore, in this same study, chicks receiving dietary supplementation of the bioactive preparation for 9 days post-hatch had longer jejunal villus heights than control chicks, but did not show any significant differences in terms of growth performance. Contrasting these largely positive outcomes on pathogen mitigation, a similar study found that supplementation of an intact mannan preparation with low water solubility, reduced quantitative Salmonella populations, non-significantly, within the ceca of 10-day old broiler chicks (27). Similar outcomes have also been observed with *E. coli* where it was demonstrated that yeast β1,3-1,6 glucans provide protection from the effect of challenge in broiler chickens (59). For both challenge models described above, it has been proposed that one potential mode of action involves yeast carbohydrates competing with pathogens that possess mannose-specific fimbriae such as *Salmonella* for specific enteric binding sites, thus decreasing attachment and colonization. In addition to this, yeast-based products can improve gut health by providing favorable conditions for intestinal *Bifidobacterium* and *Lactobacillus* spp. thus supporting their beneficial properties, including mucus production and protection of the gut integrity. Thus, the chain length and water solubility of the carbohydrate preparation being supplied directly impacts the ability of said preparation to impact pathogen control in broiler chickens.

The underlying mechanism of action of the previously described health and immunity benefits can potentially be explained at the cellular level in a study where a chicken B cell line (DT40) was incubated along with intact or enzymatically hydrolyzed yeast carbohydrates in an *in vitro* immunity evaluation model (28). Here, the authors observed that exposure to the enzymatically derived bioactive preparation compared to the unprocessed material resulted in significant increases in the expression of toll-like receptor 2b (TLR2b) and interferon gamma (INF-ɤ) within the cell line, both of which are cytokines that are involved, along with other key immunological functions (60, 61), the detection of and defense against pathogens (62, 63). Likewise, significant increases in the expression of interleukin 4 (IL-4) and 12 (IL-12) were observed in response to cellular exposure of enzymatically produced bioactive carbohydrates. This is noteworthy as both of these cytokines have roles in the management of innate immunity *via* promoting the growth and differentiation of B-cells in the case of IL-4 (64) and *via* promotion of T-cell function for IL-12, respectively (61, 65). These shifts toward a more robust innate immune response dovetail with the recently proposed concept of trained immunity and the role beta 1,3-glucans have therein (66, 67). Here, cells associated with the innate immune system recognizes beta-1,3-glucans as microbe associated molecular patterns *via* pathogen recognition receptors (66, 68). Following recognition, beta-1,3 glucans are phagocytosed and processed by innate immune systems cells present within upper intestinal lymphatic tissues, are then broken down into soluble particles and released by immune cells in order to facilitate a more robust and efficient immune response to pathogenic challenges (66, 67).

Taken together the above studies indicate that the beta-1,3 glucan and mannan fractions contained within the carbohydrate fraction of yeast cell walls have immunomodulatory properties that are in part facilitated through direct interaction with immune cell receptors that in turn promote the upregulation of cytokines that are responsible for strengthening cellular immunity. These results, combined with other similar observations (13, 27, 69), indicate the adequacy of categorizing yeast derived beta-1,3 glucan and mannans as bioactive compounds while also confirming the efficacy of said carbohydrate fractions within livestock feeds in order to promote and maintain growth performance, health and immunity.

Bioactive targets within yeast intracellular fluid

Historically, the intracellular components of yeast have been used in the food industry as flavoring agents (70) and in the industrial microbiology and pharmaceutical industries as ingredient inputs for fermentation (71, 72). However, when used in animal nutrition the components that were of interest in other use verticals have parallel uses as bioactive targets. Chief among these are bioactive peptides (73) and nucleic acids, for which in the case of the latter, a host of research has been performed as it applies to livestock feeding programs in terms of improving growth (74) and strengthening immunity (15).

Peptides are short chains of amino acids linked by peptide bonds (75). Dipeptides, tripeptides along with chains of upto 20 amino acids are colloquially referred to as oligopeptides. From a nutritional standpoint, peptides are important as key

sources of amino acids that are used in lean tissue accretion as well as in growth, development and maintenance of animals (33). However, research has shown that many peptides possess bioactive properties such as being antimicrobial (76) or being stimulative to enteric tissue development (77). However, the origin of bioactive peptides tends to be either recombinant (78) or from the hydrolysis of animal proteins such as bovine milk or fishmeal (79). Thus, given that bioactive peptides do not tend to originate in yeast cytosol, targeting this fraction of yeast for these compounds is not recommended.

As outlined previously, nucleic acids are key components of yeast extracts and typically are present as mono-, di-, or triphosphoric nucleotides (80). From a functionality point of view, nucleotides have been described as being conditionally essential (81) in that under normal metabolic conditions, *de novo* synthesis is adequate to supply biological demand. However, under conditions of stress, poor health or environmental insult, demand outstrips supply, and an exogenous dietary supply of nucleotides is required to maintain homeostasis (82). Thus, from an application standpoint, the use of yeast-derived nucleotides as bioactive compounds can become situational within a nutritional program.

To date, a number of studies have focused on best practices associated with the dietary supplication of yeast-derived nucleotides. In pigs, studies have shown that dietary supplementation of nucleotides can increase feed consumption and rate of gain of piglets (74, 83). It is thought that these improvements in performance are driven by multiple factors including improvements to intestinal morphology (15, 84) leading to increased nutrient absorption (85, 86) and improved intestinal immunity (87, 88). However, improvements tend to only be observed under unfavorable production conditions (85, 89) which narrows the applicability of yeast-based nucleotides as a dietary bioactive supplement for pigs. This narrow window whereby efficacy can be detected is likely related to the fact that the highest demand for exogenous nucleotides occurs in tissues such as the GIT which experience rapid cellular turnover immediately post-weaning and/or during infection (89, 90). Therefore, the efficacy associated with feeding yeast derived nucleotides can be optimized when provided to newly weaned piglets or preventatively to those pigs with a likelihood of encountering a stress or disease event.

Poultry species do not have the same evolved requirement for dietary nucleotides during the first weeks of life as mammals do (91). However, modern strains have been bred for rapid growth and tissue accretion and experience multiple stressor during their production cycles compared to their ancestors. It follows that due to these genetic improvements modern broiler chickens, turkeys and laying hens have an increased responsiveness to exogenous supplies of dietary yeast based nucleotides. For example, feed efficiency of broiler chickens was improved when diets were supplemented with a nucleotide containing yeast extract preparation (92, 93). In both studies it was observed that supplementation of the yeast extract led to improved GIT maturation as measured by greater ileal secretion of the digestive enzyme alkaline phosphatase as well as jejunal and ileal crypt depth. However, for contrast it was also reported that supplementing broiler chicken diets with nucleotides for 21 days had no impact on performance but led to a significant increase in ileal goblet cells (68). These results are inline with previously described observations in young pigs with the exception that in broiler chickens the benefits of nucleotide supplementation can be seen in both newly hatched and fully grown animals. Regarding impacts on health and immunity, in a study where broiler chickens were fed a nucleotide rich yeast extract and challenged with live *Eimeria* it was observed that those birds receiving the supplement had improved feed efficiency pre-challenge along with improved indices of gut function post-challenge (94). However, this same lab in a separate study observed that the provision of the same nucleotide rich yeast extract to broiler chickens had no effect on growth performance at 7 days of age but had increased bursa weight at day 35 following an *Eimeria* challenge (95).

The aquaculture industry has a long history of using yeast derived nucleotides in various feeding programs (96, 97). Originally included as a source of dietary protein (98), supplementation of yeast derived nucleotides into young fish rations has become routine practice in recent years (99). Here, the inclusion of nucleotides coincides with known periods of stress such as smoltification in salmonids (100, 101) or during early development when fish are susceptible to infection (102, 103). Compared to terrestrial animals there is strong recognition of yeast based nucleotides as bioactive compounds and adoption into commercial feeding programs is widespread.

When evaluating the efficacy of yeast-derived nucleotides purity of the preparation must always be kept in mind. This is due in most part to inconsistencies in how the extracts are manufactured which results in wide ranges in the amount and ratio of nucleotides in any given preparation. In addition, few yeast derived nucleotide preparations are manufactured solely for use in livestock nutrition, and as described above, have parallel applications in other industries. Therefore, reported results can be attributed to the presence of nucleotides within the test article but can also be linked to the presence of other yeast components such as mannans and beta 1,3-1,6-glucans which are known to be bioactive. Additionally, studies rarely declare the amount, ratio or method of analysis used to quantify each nucleotide present in a test article, which may be a contributing factor to mixed results reported to date. This inherent variability justifies the case for further identification and possible purification of yeast nucleotides in order to prove their use case as bioactives compounds.

Future implications

Yeast derivatives have the potential to serve as sources of bioactive compounds that will have beneficial properties for livestock feeding programs. Given the accelerated movement to reduce and in most cases remove antibiotic growth promoters from livestock feeds, there is an obvious need for suitable replacement technologies. Additionally, there is a growing realization that, unlike antimicrobial compounds, such replacements are likely not to be broad-spectrum in their application strategy and that more targeted approaches will ultimately reap optimal outcomes. From this mindset one could envision multiple bioactive compounds being used to manage various challenges in any given production operation. For example, bioactive carbohydrates from yeast cell wall derivatives could be incorporated into poultry rations at certain points in the production cycle to improve body weight gain and bolster immunity and at other points to manage pathogen colonization and shedding. Additionally, nucleic acids originating in the cytosol of yeast could be incorporated into young swine and calve diets in order to support intestinal development and minimize infection related mortality and morbidity during susceptible stages of production.

Conclusion

The challenge of understanding how best to use novel bioactive compounds will frame how this technology category incorporates itself into modern livestock production. Research that is focused on identifying and characterizing novel bioactive compounds will be required to better understand their potential. This base level knowledge capture will then require validation in multiple *in vivo* experimentation models that assess growth performance as well as broad and narrow effects on health and immunity at the cellular, tissue and systemic levels. Only once this level of comprehensive research is performed can one truly understand the beneficial potential of bioactive compounds originating from yeast derivatives.

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

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

Conflict of interest

RP is employed by CBS Bio-Platforms Inc.

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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Adding a polyphenol-rich fiber bundle to food impacts the gastrointestinal microbiome and metabolome in dogs

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Introduction: Pet foods fortified with fermentable fibers are often indicated for dogs with gastrointestinal conditions to improve gut health through the production of beneficial post-biotics by the pet's microbiome.

Methods: To evaluate the therapeutic underpinnings of pre-biotic fiber enrichment, we compared the fecal microbiome, the fecal metabolome, and the serum metabolome of 39 adult dogs with well-managed chronic gastroenteritis/enteritis (CGE) and healthy matched controls. The foods tested included a test food (TF1) containing a novel pre-biotic fiber bundle, a control food (CF) lacking the fiber bundle, and a commercially available therapeutic food (TF2) indicated for managing fiber-responsive conditions. In this crossover study, all dogs consumed CF for a 4-week wash-in period, were randomized to either TF1 or TF2 and fed for 4 weeks, were fed CF for a 4-week washout period, and then received the other test food for 4 weeks.

Results: Meaningful differences were not observed between the healthy and CGE dogs in response to the pre-biotic fiber bundle relative to CF. Both TF1 and TF2 improved stool scores compared to CF. TF1-fed dogs showed reduced body weight and fecal ash content compared to either CF or TF2, while stools of TF2-fed dogs showed higher pH and lower moisture content vs. TF1. TF1 consumption also resulted in unique fecal and systemic metabolic signatures compared to CF and TF2. TF1-fed dogs showed suppressed signals of fecal bacterial putrefactive metabolism compared to either CF or TF2 and increased saccharolytic signatures compared to TF2. A functional analysis of fecal tryptophan metabolism indicated reductions in fecal kynurenine and indole pathway metabolites with TF1. Among the three foods, TF1 uniquely increased fecal polyphenols and the resulting post-biotics. Compared to CF, consumption of TF1 largely reduced fecal levels of endocannabinoid-like metabolites and sphingolipids while increasing both fecal and circulating polyunsaturated fatty acid profiles, suggesting that TF1 may have modulated gastrointestinal inflammation and motility. Stools of TF1-fed dogs showed reductions in phospholipid profiles, suggesting fiber-dependent changes to colonic mucosal structure.

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Discussion: These findings indicate that the use of a specific pre-biotic fiber bundle may be beneficial in healthy dogs and in dogs with CGE.

KEYWORDS

metabolomics, microbiome, fiber, dogs, post-biotics, polyphenols, pre-biotics

1. Introduction

The gut microbiome is a critical immune and metabolic organ that consists of bacteria, archaea, viruses, and a variety of eukaryotic organisms that are symbiotically associated with the host. Bacteria constitute the largest fraction of these intestinal microorganisms, with metagenomic sequencing demonstrating that >98% of reads from fecal samples assigned to bacteria in both dogs and cats (1–3). A well-functioning gut microbiome modulates the immune system, helps defend against intestinal pathogens, and provides the host with vitamins and nutrients.

Intestinal bacteria coexist in a symbiotic relationship with their mammalian hosts, exerting its influence through conversion of dietary and host-derived substances and molecules into bioactive metabolites known as post-biotics (2). Short-chain fatty acids (SCFAs), metabolites of dietary tryptophan, and secondary bile acids represent examples of post-biotics arising from host-microbiota interactions that influence gut health status, including energetics, permeability, and immunity (4, 5). Gut bacteria ferment undigested fibers and carbohydrates into straight SCFAs that nourish epithelial cells and regulate intestinal motility and cytokine production (5, 6). Certain proteolytic bacteria also generate branched SCFAs and ammonia from excess protein putrefaction (7). The gut microbiota influences tryptophan metabolism into the kynurenine pathway, serotonin pathway, and indole-containing aryl hydrocarbon receptor ligands, directly and indirectly impacting the inflammatory response, immune homeostasis, epithelial function, and gastrointestinal signaling (8, 9).

The impact of dietary fibers on the gastrointestinal health of dogs has been well-established (10). Historically, fibers have been classified according to their solubility, dispersibility in water, and fermentation profiles (11, 12). There are numerous publications regarding the use of fibers to normalize canine stool firmness (13–15); however, research into the impact of different fibers on the microbial processes of proteolysis and saccharolysis and the accompanying production of antioxidants and anti-inflammatory post-biotics is limited (16–18). While many fibers exhibit similarities based on these characteristics (19), not all provide the same health benefits. In fact, physicochemical characteristics, surface area, and hydration properties may be better indicators of the health benefits associated with fibers. The impact of additional properties of fibers, such as the presence of bound bioactive compounds, is also gaining recognition (12, 20). Foodstuffs and compounds that promote the growth of beneficial microbes and serve as substrates for post-biotic metabolism are known as pre-biotics. For example, fruits and vegetables are rich pre-biotic sources of plant fibers and polyphenols (21), which are a group of compounds that include flavonoids, tannins, and phenolic acids and their derivatives (22). Plant-derived resistant starches that bypass digestion in the small intestine and reach the colon intact also represent a source of fiber. Abundance of resistant starch in dry pet foods is roughly inversely proportional to the intensity of extrusion processing, which involves mechanical shear forces and heat (23). The gut microbiota of dogs is capable of fermenting a wide range of plant fibers and metabolizing polyphenol conjugates and oligomers into more bioavailable forms, while the presence of dietary polyphenols can also influence the composition and function of bacterial populations in the gut microbiota (24, 25). Dietary polyphenol supplementation has been proposed as an intervention for acute diarrhea in dogs, due to the antioxidant, anti-inflammatory, and microbiome-sculpting properties of polyphenols and their catabolites (26).

While variations in dietary composition can alter the composition and function of the gut microbiome of dogs (16, 27, 28), the optimal characteristics of dietary fiber in terms of fermentability, physiochemistry, and bound metabolites are under investigation (29). Consequently, a novel pre-biotic fiber bundle that consists of fibers specifically selected for their pre- and post-biotic activity, water-holding, and stool-bulking capacities was developed. Our previous work has demonstrated that dietary interventions with this pre-biotic fiber bundle have improved stool scores, shifted fecal microbial metabolism from deleterious putrefactive to desirable saccharolytic metabolism, and increased fecal anti-inflammatory and antioxidant metabolites compared with control foods in both dogs and cats (16, 17, 30–33). In a study of dogs with chronic large bowel diarrhea, a therapeutic food that included the pre-biotic fiber bundle significantly and rapidly improved stool consistency, resolved clinical signs of chronic diarrhea, and improved stooling behaviors and quality of life (30). The food also shifted the functional capacity of the GI microbiome and its metabolism toward saccharolytic fermentation from proteolytic putrefaction, increased the abundance of bioavailable post-biotics, and modulated inflammatory and colonic mucosal signatures compared to the pre-intervention baseline state (16).

We hypothesize that differences in fiber content and composition in foods for dogs with gastrointestinal disorders may lead to differences in the fecal and systemic impact of these foods, differences that may be mediated by the dogs' microbiome and metabolism. To better understand how microbiome and metabolism interact to impart the therapeutic benefit of the novel pre-biotic fiber bundle and to determine how differences in fibers alter their fecal and systemic impact, we conducted a randomized, controlled crossover study investigating fecal composition, microbiome, and metabolomic signatures in adult dogs consuming a food containing the pre-biotic fiber bundle, a control food lacking the bundle, or another fiber-based therapeutic food for dogs with gastrointestinal disorders.

2. Materials and methods

The study was a randomized, crossover design conducted between August and December 2016 that comprised four phases, each of which lasted 28 days (Supplementary material 1). In Phase 1, all dogs received the control food. In Phase 2, dogs were randomized into Group 1 and received Test Food 1 (TF1), which included the pre-biotic fiber bundle, or Group 2 and received another therapeutic food. In Phase 3, all dogs again received the control food (CF). Finally, in Phase 4, dogs received the test food that they did not receive in Phase 2 [Test Food 2 (TF2) in Group 1 and TF1 in Group 2]. All dogs were fed twice daily, once in the morning and again in the afternoon. All study protocols were reviewed and approved by the Institutional Animal Care and Use Committee (CP693a) of Hill's Pet Nutrition, Inc., in Topeka, KS, USA, and all methods were carried out in accordance with relevant institutional and national guidelines and regulations.

2.1. Study dogs

This study subject population consisted of adult beagles housed at the Hill's Pet Nutrition Center (HPNC), as previously described (17), with both healthy dogs and those diagnosed with naturally occurring chronic gastroenteritis, diagnosed by the HPNC veterinarian based on attitude/activity, appetite, vomiting, stool consistency, stool frequency, weight loss, albumin levels, and histopathological analysis by endoscopy and microscopic assessment by an independent veterinarian pathologist when it was deemed in the best interest of the dog (e.g., dog not of advanced age). Healthy control dogs were matched to dogs with CGE by age, weight, and gender. Demographics data for study participants are presented in Table 1. Dogs with CGE had been under the care of the HPNC veterinarian for >15 months and their disease was considered well-managed at the time of the trial. This contrasted with the previous study in which the CGE dogs presented with active signs at the start of the trial (17). Dogs were considered healthy when there was no evidence of chronic systemic disease from physical examination, complete blood count, serum biochemical analyses, urinalysis, or fecal examination for parasites; exclusion criteria were recorded instances of gastrointestinal upset (vomiting, diarrhea) or abnormally low appetite. All dogs were pair-housed in spacious indoor rooms with natural light at HPNC and had the opportunity for behavioral enrichment by interacting with each other, as well as through play time with caretakers, daily runs outside, and access to toys. Dogs were fed twice daily and had *ad libitum* access to water. All dogs were immunized against canine distemper, adenovirus, parvovirus, Bordetella, and rabies, were monitored for parasites, and received routine heartworm preventative. Symptoms of CGE dogs were managed with bismuth subsalicylate, prednisolone, cobalamin, and omeprazole as needed to maintain their quality of life.

TABLE 1 Demographic data for dogs enrolled in study.

Characteristic	Group 1	Group 2	*P*-value
Age, years, mean ± SD	7 ± 3.7	8 ± 3.6	0.47
Weight, kg, mean ± SD	12 ± 2.6	11 ± 2.1	0.42
Sex, *n* (%)			
Females	5 (23)	6 (30)	0.73
Males	17 (77)	14 (70)	

2.2. Study foods

The nutrient profiles of the 3 dry foods used in the study are listed in Table 2. The CF and TF1 differ only in that TF1 included the pre-biotic fiber bundle. The pre-biotic fiber bundle in TF1 (Hill's® Prescription Diet® Gastrointestinal Biome dry dog food) includes ground pecan shells, flaxseed, dried beet pulp, dried citrus pulp, pressed cranberries, and psyllium seed husk, while the fiber components of TF2 (Royal Canin® Veterinary Diet Gastrointestinal High Fiber dry dog food) includes powdered cellulose, rice hulls, dried beet pulp, psyllium seed husk, and fructooligosaccharides. In CF, cornstarch was used in lieu of the TF1-containing fiber bundle. TF2 is a commercially available therapeutic product designed to help manage fiber-responsive gastrointestinal conditions with a similar nutrient profile and different functional ingredients. All 3 foods represent complete and balanced dry nutritional pet foods and met 2017 Association of American Feed Control Officials guidelines for maintenance of adult dogs (34). All dogs were offered an appropriate number of calories to maintain body weight, with pre- and post-weights recorded to assess intake. Caloric needs were also calculated, in part, using an activity factor, to ensure that the dogs' individual nutritional needs were met throughout the trial. The nutritional needs, health, and

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TABLE 2 Compositional analysis for foods administered in study.

Nutrient	CF	TF1	TF2
Protein, %	18.1	19.8	23.3
Fat, %	11.6	12.3	18.5
Crude fiber, %	2.5	6.6	10.1
Soluble fiber, %	1.6	2.6	3.7
Insoluble fiber, %	5.7	13.7	18.8
Nitrogen free extract, %	53.3	47.2	31.8
Ash, %	5.6	6.0	8.3
Calcium, %	0.96	1.08	1.04
Magnesium, %	0.07	0.10	0.08
Phosphorus, %	0.67	0.65	0.89
Potassium, %	0.83	0.93	0.74
Sodium, %	0.34	0.37	0.35
Eicosapentaenoate (EPA), %	0.26	0.27	0.17
Docosahexaenoate (DHA), %	0.19	0.21	0.08
Sum $n-3$ fatty acids, %	0.79	1.41	0.51
Sum $n-6$ fatty acids, %	3.11	3.28	3.42

wellbeing of the dogs included in the study were overseen by the veterinarian and animal care technician team at HPNC.

2.3. Study procedures

Animal care staff recorded food intake and completed a stool diary, documenting stool frequency, consistency, and characteristics, beginning on study Day 1. Intake was measured twice daily for each animal in each 4-week period. The two daily measurements were summed to provide a total daily intake estimate for each animal. The daily totals were then averaged over each 4-week period to provide an average daily intake for each animal in each period.

Body weight, fecal scores, fecal composition, SCFAs, and fecal and serum metabolomics data were collected at four time points: (1) at the end of the 4-week wash-in period prior to Period 1 (Day 29); (2) at the end of Period 1 (Day 56); (3) at the end of the 4-week washout period between periods 1 and 2 (Day 84); and (4) at the end of Period 2 (Day 122). The consistency and characteristics of feces were assessed using a 5-point scale, from 1, where the dog's stool was liquid in form, to 5, in which the dog's stool was solid and >80% firm. To ensure the welfare of the dogs, any dog who consumed <60% of their maintenance energy requirements was removed from the study.

Whole feces were collected, homogenized by planetary centrifugal mixer until visually uniform, snap-frozen in liquid nitrogen, and stored until processing at −80°C. Proximate analyses and analyses of pH, vitamins, amino acids, and SCFAs were conducted using certified official compendial methods in commercial laboratories accredited by the International Organization of Standards. Moisture of fecal samples was assessed by spreading feces in an aluminum pan and allowing it to dry for ∼3 h . Ash values were evaluated by weighing a portion of the fecal sample in a small ceramic crucible and heating the sample to 600°C for ∼2 h.

Serum chemistry and complete blood count profiles were obtained from fasted dogs at the end of each washout period (Days 29 and 84) and the end of Period 1 and 2 (Days 56 and 122) to monitor the health and response of dogs throughout the study.

2.4. Metabolomic profiling

Fecal and serum metabolomics were performed by Metabolon, Inc (Morrisville, NC), as previously described (35). Briefly, samples were subjected to a methanol-based extraction and divided among four methods for analysis on exact-mass Q-Exactive mass spectrometers (MS): "early" and "late" reverse phase (RP)/ultra-high performance liquid chromatography-tandem-MS (UPLC-MS/MS) in positive ion mode, RP/UPLC-MS/MS in negative ion mode, and polar hydrophilic interaction chromatography/UPLC-MS/MS in negative ion mode. Metabolites were identified and elution peaks were integrated using provider-developed software that matched features to an in-house library of authentic and *in silico* standards, followed by automatic and manual quality control. Compounds with missing values (i.e., those measured in some but not all animals) were imputed with the observed minimum for that biochemical. Metabolites that lacked authentic standards, but still demonstrated high confidence in chemical identification are marked with an asterisk (*) in the Supplementary material. Metabolites with identical masses (isobar) that could not be resolved by chromatography were named accordingly (e.g., mannitol/sorbitol).

2.5. Microbiome evaluation

Total DNA was extracted from frozen fecal samples using the PowerFecal DNA isolation kit (MOBIO, Carlsbad, CA), following the manufacturer's instructions. A sonication step was performed before vortexing the bead tubes with fecal samples horizontally for 15 minutes, as described by Jackson and Jewell (17). PCR amplification was then conducted using the primer pairs 341F and 806R spanning the V3–V4 hypervariable regions of the 16S RNA gene along with Illumina adapters. Amplicon sequencing was performed according to the Illumina 16s metagenomic sequencing library preparation protocol (15044223 Rev. A). Resulting sequences were de-multiplexed

based on the dual index sequences, using the Miseq built-in metagenomics workflow to generate FASTQ files. FASTQ files were processed using Mothur (version 1.39.5) with standard parameters (36, 37). All retained sequences were aligned to Green Genes Database and classified using the naive Bayesian classifier within Mothur with a minimum confidence of 80% for each assignment. Operational taxonomic units were identified based on taxonomic hierarchy and further processed using the Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt) protocol (38) to correct 16s gene copy numbers followed by predicting functional attributes using the Kyoto Encyclopedia of Genes and Genomes (KEGG) database.

The interaction of fecal metabolites and hindgut microbial enzyme functions was assessed by co-mapping those metabolites having compound identifiers and PICRUSt-derived functions having KEGG orthology (KO) identifiers related to tryptophan metabolism, as previously described (16). The resulting relative abundances were projected onto a single space (ko00380; tryptophan metabolism) (36, 37, 39).

2.6. Statistical analysis

2.6.1. Evaluation of the effect of health status

Preliminary analyses of the microbiome, metabolome, and SCFA data were performed that included disease group (healthy vs. CGE dogs) and a disease group x diet interaction term as fixed effects in the models. In comparing the fecal metabolites for TF1 and TF2, the disease group main effect was statistically significant for one out of the 376 metabolites analyzed, while disease group x diet interaction was statistically significant for 26 metabolites (rejection rate of 6.9%, which is only slightly higher than the Type I error rate), distributed randomly among eight different metabolomic categories. For the serum metabolites, the disease group main effect was statistically significant for two out of 201 metabolites (1%) analyzed, found in two different metabolomic categories, while a statistically significant disease group × diet interaction was observed for 16 metabolites (8.0%), occurring among eight different metabolomic categories. In comparing TF1 and CF, the disease group main effect was not significant for any fecal metabolites and was statistically significant for only one serum metabolite. The disease group x diet interaction term was statistically significant for 12 fecal metabolites (3.2%), distributed among eight different metabolomic categories, and four serum metabolites (2.0%), which were found in four different categories. Among fecal SCFA, no disease main effects were observed comparing either diet, while a significant disease x diet interaction was observed for propionic acid in comparing TF1 and CF. The lack of a clear and consistent disease effect from these data streams prompted the removal of healthy-CGE status from the final analysis.

2.6.2. Microbiome analysis

Fecal microbiome count data were analyzed at the phylum, family, and genus levels, whereas the alpha-diversity indices and PICRUSt-predicted KO functional data were analyzed at the genus level only. The alpha-diversity indices were analyzed using the Wilcoxon signed-rank test. The 16S copy number-corrected operational taxonomic unit counts and KO functional data were first filtered by prevalence, needing to pass 80% prevalence in at least one of the diet-health experimental groups. KO functions were secondly filtered by an internally curated list of pathways to be considered for further statistical analysis. The counts of individual operational taxonomic units and predicted KO functions were analyzed by negative binomial mixed models (40) with fixed diet and health group effects and random animal effect. Permutational multivariate analysis of variance (PERMANOVA) based on Manhattan distance was used to compare microbial relative abundance compositions and pathway functional compositions between diets (41). *P*-values were false discovery rate-adjusted according to the Benjamini and Hochberg procedure (42). All the statistical microbiome analyses were carried out in R-3.3.3 (43).

2.6.3. Metabolomics analysis

Fecal and serum metabolites were categorized into 39 and 20 functional groups, respectively. Two separate analyses were conducted: (1) comparison of TF1 ($n = 39$) vs. TF2 ($n = 39$) utilizing the crossover design, and (2) comparison of TF1 vs. CF. CF samples ($n = 78$) were treated as repeated measures after combining wash-in and washout measurements. Next, the results from the crossover analysis were reviewed for a significant sequence effect, as an indication of a possible carryover effect. A significant sequence effect was detected in 11 of 373 (2.9%) fecal metabolites examined, and in three of 201 serum metabolites examined (1.5%), both of which were below the 5% null hypothesis rejection rate expected by chance alone, indicating that by the end of each 4-week feeding period there was little or no carryover effect from the previous feeding period present in the fecal metabolites.

For TF1 vs. TF2, the data were analyzed using a linear mixed-model with sequence, treatment, and period as fixed-effects, and animal nested within sequence as a random-effect. For TF1 vs. CF, the data were analyzed using a linear mixed-model with treatment (TF1 and CF) as a fixed-effect and animal and animal × diet as random effects. This analysis treats the measurements taken during the wash-in period and washout period as repeated measurements and fits a compound symmetry covariance structure to the data to account for correlations between the repeated measurements. The Kenwood-Roger adjustment was used to adjust the denominator degrees-of-freedom of the *F*-test for the test of fixed-effects. The NOBOUND option was used in situations where the animal (sequence) [TF1 vs. TF2], the animal or animal × diet [TF1 vs. CF] variance component was negative

TABLE 3 Mean fecal scores, body weight, and food intake measurements for canines in study.

Measurement	CF ± SE	TF1 ± SE	TF2 ± SE	TF1–CF ± SE	TF1–TF2 ± SE	TF2–CF ± SE
N	78	39	39			
Fecal score	4.3 ± 0.1	4.8 ± 0.1	4.6 ± 0.1	0.5 ± 0.1**	0.1 ± 0.1	0.4 ± 0.1*
Body weight (kg)	11.9 ± 0.4	11.7 ± 0.4	11.9 ± 0.4	−0.27 ± 0.05***	−0.27 ± 0.06***	−0.01 ± 0.05
Intake (g)	212 ± 9	211 ± 9	220 ± 9	0 ± 4	−8 ± 6	8 ± 4
Intake (Kcal)	722 ± 29	706 ± 32	732 ± 32	−15 ± 14	−26 ± 22	11 ± 15

*$P < 0.05$; **$P < 0.001$; ***$P < 0.0001$. CF, control food; SE, standard error; TF1, test food 1; TF2, test food 2.

to allow for negative estimated values so that the degrees-of-freedom associated with this variance component were not pooled with the residual error degrees-of-freedom. Treatment least-squares means and mean differences were estimated using this linear model.

Functional metabolomic groups were analyzed using a multivariate analysis of variance (MANOVA) option in PROC GLM in SAS®. The same linear mixed-models described above were used except that PROC GLM does not allow for the use of the Kenward-Roger option. With the crossover analysis of TF1 vs. TF2, the sequence fixed-effect was tested using animal (sequence) as the error term and the treatment and period fixed-effects were tested using the residual error term. With the comparison of TF1 vs. the CF, the residual error term was used to test for a significant diet effect. The Wilk's lambda test statistic was used to identify significant treatment differences. The analysis was performed on scaled variables; therefore, the data were not log-transformed prior to analysis.

Principal component (PC) analysis was performed using PROC PRINCOMP using the correlation matrix to standardize the variables. Scores for the top 9 PCs ranked by percentage of variance were analyzed individually using the linear mixed models described above. Separate analyses were performed for fecal and serum metabolites. All analyses were performed using SAS® PROC MIXED, version 9.4.

3. Results

3.1. Demographics, study disposition, stool quality, and stool composition

A total of 42 dogs were enrolled and 3 were removed from the study during the initial wash-in period due to megaesophagus on Day 1, pica on Day 15, and intestinal hemorrhage on Day 18. Because these dogs were removed from the study prior to the initial crossover treatment period, data are available on the 39 dogs (healthy, $n = 21$; CGE, $n = 18$) that participated in a randomized crossover design study.

Although study dogs consumed a similar amount of each food, in terms of both grams and calories (Table 3), consumption of TF1 resulted in ∼0.3 kg loss of body weight (∼2.5%) compared to either CF or TF2 ($P < 0.0001$). Stool scores were evaluated as proxy measurements for gastrointestinal health. Both TF1 ($P = 0.0002$) and TF2 ($P = 0.01$) significantly improved fecal scores relative to CF, while TF1 and TF2 had similar stool scores.

Because we did not match a control for TF2 lacking the pre-biotic fiber bundle, we directly compared CF and TF2 only for macroscopic measurements and not for assessments of stool composition, metabolomics, or the microbiome. An analysis comparing the impact of CF, TF1, and TF2 in healthy dogs compared to those with managed CGE revealed no differences in stool quality and very few significant differences in the microbiome, metabolome, or SCFA response to foods by health status (see Materials and methods). Consequently, results for the subgroups of dogs with managed CGE and healthy dogs are not presented in this manuscript. Additionally, a statistical analysis of the metabolomics data from feeding the CF during the wash-in period and washout period indicated the absence of a carryover effect, allowing the wash-in and washout feedings to be combined in the CF analysis.

TF1 had no effect on either moisture content (Figure 1A) or fecal pH (Figure 1B), compared to CF; however, dogs consuming TF2 defecated stools of significantly lower moisture content ($P < 0.0001$) and significantly higher pH ($P = 0.001$) compared to TF1. TF1 consumption significantly reduced total fecal ash compared to both CF and TF2 ($P < 0.0001$, Figure 1C). Further inspection of the mineral contributions to the ash indicated that, compared to CF, TF1 significantly reduced fecal calcium, copper, iron, magnesium, manganese, phosphorus, and zinc, while significantly increasing fecal sodium levels (Supplementary material 2). Compared to TF2, however, TF1 had significantly higher levels of calcium, copper, potassium, magnesium, and zinc, while manganese and phosphorus levels were significantly lower, and iron and sodium levels were not statistically different.

3.2. Fecal microbiome characterization and high-level metabolomics analysis

For fecal microbiome abundance data, five phyla, 41 families and 80 genera passed the prevalence filter and were considered for statistical analyses. The results of principal coordinate

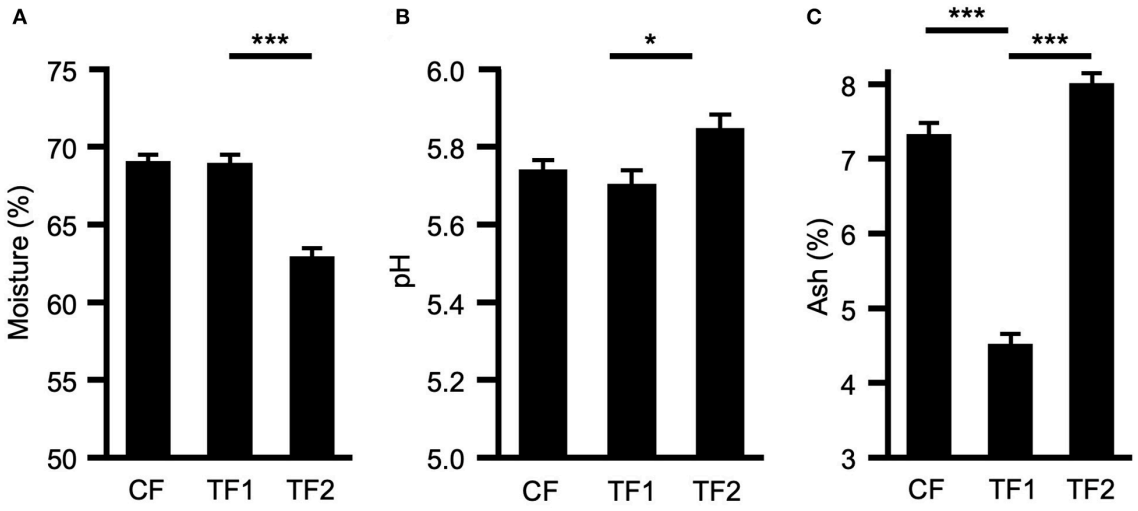

FIGURE 1
Macroscopic fecal composition comparisons. Fecal measurements for **(A)** percent moisture, **(B)** pH levels, and **(C)** percent ash for dogs consuming control food (CF), test food 1 (TF1), and test food 2 (TF2). Significant differences among CF vs. TF1 and TF1 vs. TF2 are indicated (*$P < 0.05$, ***$P < 0.0001$).

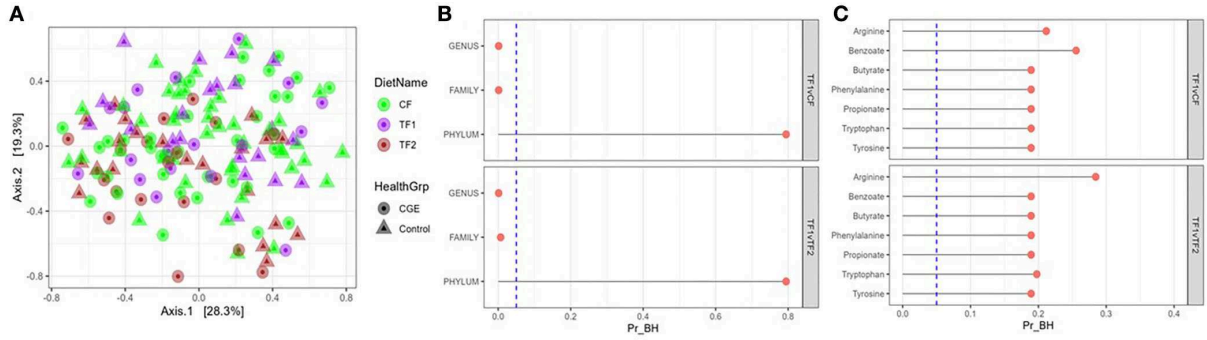

FIGURE 2
Fecal microbiome analysis. **(A)** Principal coordinate analyses of the fecal microbiome at genus level of dogs consuming different foods used in this study. **(B)** PERMANOVA analyses showing false discovery rate-corrected bacterial taxa (genus, family, and phylum level) and **(C)** KEGG pathways in feces from dogs that consumed the study foods TF1 vs. CF and TF1 vs. TF2. Vertical dotted line represents $P = 0.05$. KEGG, Kyoto Encyclopedia of Genes and Genomes; PERMANOVA, permutational multivariate analysis of variance; Pr_BH, *p*-value corrected by the Benjamini–Hochberg method; CF, control food; TF1, test food; TF2, test food 2.

analyses based on beta diversity measures using Manhattan distance matrix are shown in Figure 2A; no visible clustering was observed for food type (CF, TF1, and TF2) or health status (healthy and CGE). By contrast, PERMANOVA analysis revealed significant compositional differences by treatment between both TF1 and CF ($P = 0.02$) and TF1 and TF2 ($P = 0.005$), at the family and genus levels, but not at the phylum level (Figure 2B).

Analysis of individual phyla indicated that Fusobacteria significantly increased ($P = 0.01$) in dogs fed TF1 compared with CF and significantly decreased ($P < 0.0001$) in dogs fed TF1 compared with TF2 (Supplementary material 3). Similarly, analyses of individual families and individual genera showed that the relative abundances of family Pseudomonadaceae, and genus *Pseudomonas*, which belong to the phylum Proteobacteria, significantly decreased in the dogs fed TF1 compared to both CF and TF2, as well as family Clostridiaceae and genera *Clostridium* and 02d06, which belong to phylum Firmicutes. Additional Firmicutes family Ruminococcaceae and genus *Faecalibacterium* were significantly increased in dogs fed TF1 compared with CF and TF2. Families such as Bacteroidaceae, Turicibacteraceae, Peptostreptococcaceae, and Fusobacteriaceae, as well as genera *Bacteroides*, *Turicibacter*, and *Megamonas* were significantly increased in dogs fed TF1 compared with CF. The Proteobacteria family Helicobacteraceae and genus

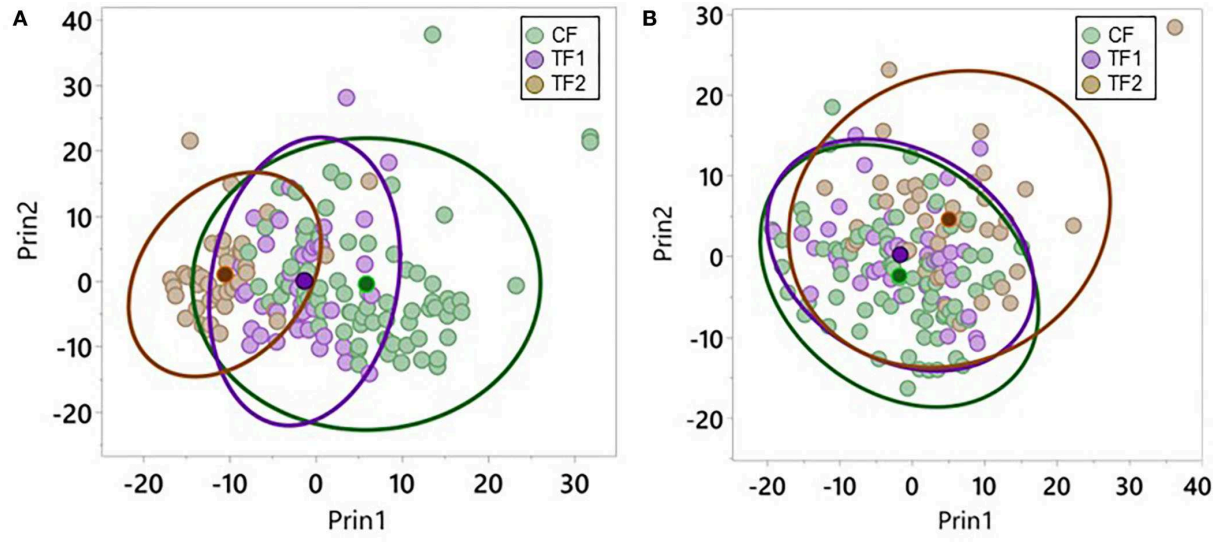

FIGURE 3
Principal component analysis for metabolomics data. Individual dogs consuming CF (green), TF1 (violet), and TF2 (brown) were plotted according to their aggregate PC1 and PC2 scores for **(A)** feces and **(B)** serum. Diet group centroids and 95% confidence intervals are indicated by darker dots and ovals, respectively.

Helicobacter, as well as the Firmicutes genera *Blautia* and *Dialister* were significantly increased in dogs fed TF1 compared with TF2.

All calculated bacterial -diversity indexes at the genus level, including the Shannon diversity index and the Simpson index, showed no significant differences among either CF and TF1 or TF1 and TF2. Richness analysis indicated that the number of species within a given sample were significantly reduced in TF1 vs. either CF ($P = 0.005$) or TF2 ($P = 0.004$). PERMANOVA analyses of microbial KO functional compositions showed no significant differences between the food comparisons TF1 vs. CF and TF1 vs. TF2 for all curated pathways (Figure 2C).

We performed PC analysis on both the fecal and serum metabolomics datasets for all collected samples. Eigenvalues and proportion of variation explained by the top 9 PCs for the fecal and serum datasets are presented in Supplementary material 4. Overall, PC1 accounted for 12.4 and 10.6% of the variation in fecal and serum metabolites, respectively, while PC2 accounted for 9.9 and 7.5% of the total fecal and serum metabolites, respectively. Approximately half of the metabolites with relatively high loadings contributing to PC1 were from the amino acid superpathway, while about two thirds of those contributing to PC2 were from the lipid superpathway.

Plotting the top 2 PCs in the fecal dataset by the diets each individual animal consumed revealed significant ($P < 0.0001$) separation in PC1 when comparing CF and TF1 and when comparing TF1 and TF2; however, such differences were not observed in PC2 (Figure 3A). Similar analyses performed using the serum dataset indicated significant ($P < 0.0001$) separation in both PC1 and PC2 between TF1 and TF2, but only the difference between CF and TF1 in PC2 reached statistical significance ($P < 0.0001$; Figure 3B).

3.3. Impact of fiber inclusion on microbial metabolism

3.3.1. Saccharolytic metabolism

Fecal straight SCFA quantitative measurements (Figure 4A), as well as monosaccharide metabolomic relative abundances, were analyzed as surrogates for microbial saccharolytic processes. TF1-fed dogs showed significantly increased fecal acetic acid levels ($P = 0.001$) while significantly reducing propionic acid ($P < 0.0001$), and an overall decrease in total straight SCFA levels compared to CF-fed dogs ($P = 0.03$). Compared to dogs in the TF2 group, dogs in the TF1 group showed significantly reduced fecal propionic acid levels ($P = 0.03$) but significantly higher butanoic acid levels ($P < 0.0001$), ultimately resulting in higher total straight SCFA levels in the TF1 group vs. the TF2 group ($P = 0.04$).

TF1 ingestion significantly affected both fecal (Table 4) and serum (Table 5) carbohydrates ($P < 0.0001$ by MANOVA) compared to CF, yet the directionality was metabolite dependent. In feces (Supplementary material 5), consumption of TF1 resulted in significantly elevated arabinose (Figure 4B), galacturonate, and ribulose/xylulose [isobar] levels compared to CF (all $P < 0.0001$). Fecal levels of erythritol ($P = 0.004$), lactate ($P = 0.009$; Figure 4C), mannitol/sorbitol [isobar] ($P = 0.03$), ribose ($P = 0.0001$), and xylose ($P < 0.0001$) were all decreased with TF1 vs. CF. Serum assessments of these metabolites

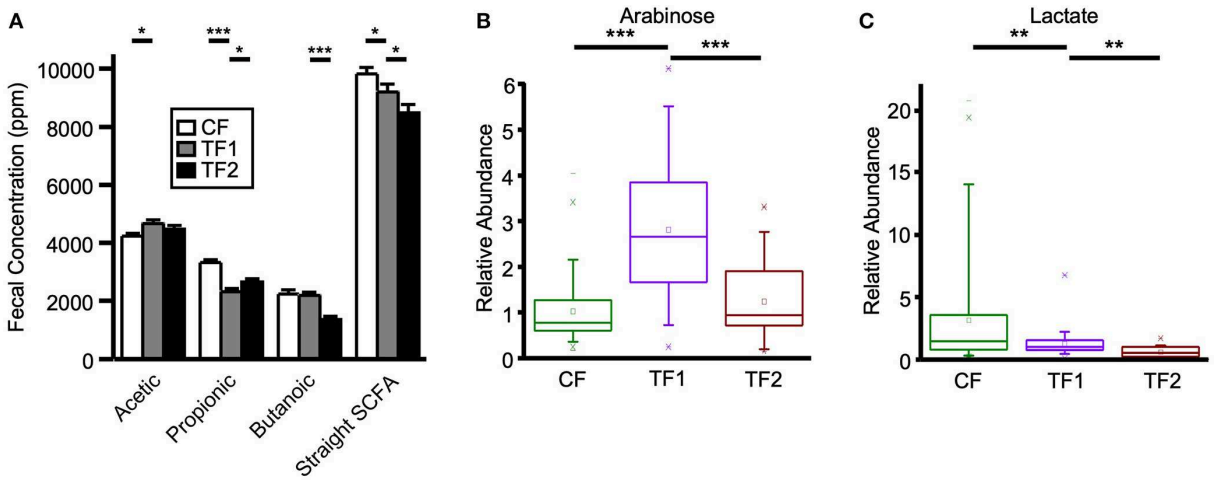

FIGURE 4
Fecal markers of saccharolytic microbial metabolism. **(A)** Straight short chain fatty acid measurements for dogs consuming CF (white), TF1 (gray), and TF2 (black). Relative abundance levels for **(B)** arabinose and **(C)** lactate in healthy dogs consuming CF (green), TF1 (violet), and TF2 (brown). Significant differences among CF vs. TF1 and TF1 vs. TF2 are indicated (*$P < 0.05$, **$P < 0.001$, ***$P < 0.0001$).

(Supplementary material 6) revealed significant reductions in circulating erythritol ($P = 0.0008$), lactate ($P < 0.0001$), and ribose ($P = 0.001$) in TF1-fed dogs vs. CF. Glucuronate ($P = 0.02$) and xylose ($P = 0.02$) were significantly elevated with TF1 consumption in serum vs. CF, while mannitol/sorbitol was unchanged. Reductions in serum erythronate ($P = 0.004$) and pyruvate ($P < 0.0001$) were observed, yet the addition of the fiber bundle in TF1 had no appreciable effect on these metabolites in feces.

Compared to TF2, TF1 consumption resulted in significantly higher levels of both fecal and serum carbohydrate metabolites ($P < 0.0001$ by MANOVA). Both fecal and serum analyses revealed that consumption of TF1 increased erythronate and lactate, while significantly reducing levels of mannose, compared to TF2. Other fecal monosaccharides higher in TF1-fed dogs included arabinose, all galactose-related compounds, glucose, glycerate, maltose, mannitol/sorbitol [isobar], pyruvate, and ribulose/xylulose [isobar]. Xylose was the only monosaccharide lower in dogs consuming TF1 compared to TF2.

3.3.2. Putrefactive metabolism

To evaluate dietary contributions to putrefactive microbial metabolism, quantitative measurements of fecal branched SCFAs and ammonium, as well as relative abundances of amino acids, dipeptides, and polyamines were considered. TF1 consumption significantly reduced fecal isobutyric ($P < 0.0001$) and 2-methylbutyric ($P < 0.0001$) acids from CF levels, and both branched SCFAs were also significantly lower in the stool of TF1-fed dogs compared to TF2-fed dogs (Figure 5A). Total fecal branched SCFAs were significantly reduced in dogs consuming TF1 compared to CF ($P = 0.0008$). Fecal total branched SCFAs were lower in TF1-fed dogs relative to TF2-fed dogs, although this difference was not statistically significant ($P = 0.08$). Fecal ammonium concentrations (Figure 5B) in dogs fed the fiber bundle in TF1 were lower than those in CF-fed dogs ($P = 0.0001$) and in TF2-fed dogs ($P < 0.0001$).

Compared to CF, TF1 was associated with significant reductions in both fecal (Table 4) and serum (Table 5) levels of amino acids, as well as fecal polyamines (all $P < 0.0001$), as indicated by MANOVA. The amino acids alanine, cysteine, methionine, and proline were reduced in both feces (Supplementary material 5) and serum (Supplementary material 6) in dogs fed TF1 vs. CF, while isoleucine, lysine, threonine, and valine were only reduced in feces. Significant differences in fecal and serum amino acids were also observed between TF1 and TF2 (Table 4; all $P < 0.0001$). Among the 21 fecal amino acids evaluated, 18 were significantly elevated among dogs fed TF1 compared to TF2, while levels of aspartate, proline, and taurine were similar. Yet when comparing circulating amino acids in dogs fed TF1 vs. TF2, 9 were higher with TF1, including aspartate ($P = 0.0006$), and five were lower, including taurine ($P = 0.0008$).

Among the 18 fecal dipeptides, seven were higher and six were lower in stools of dogs fed TF1 vs. CF. When fecal dipeptide levels were compared between TF1 and TF2, the levels of 14 were significantly higher in TF1-fed dogs.

Almost all measured fecal polyamines and associated metabolites were significantly reduced in dogs fed TF1 vs. CF, including cadaverine, putrescine, and spermidine (Figure 5C; all $P < 0.0001$). Fiber inclusion in TF1 also significantly reduced the N-acetylated metabolites of these primary polyamines, including N1, N12-diacetylspermine vs. CF, while spermine was not observed in feces. Exceptions included ornithine, the precursor

TABLE 4 Overall fecal metabolomics data[a].

Metabolite classification		TF1 vs. CF	TF1 vs. TF2
		MANOVA *p*-value	MANOVA *p*-value
Sugars		<0.0001	<0.0001
Putrefactive metabolism	Amino acids	<0.0001	<0.0001
	Dipeptides	<0.0001	<0.0001
	Polyamines	<0.0001	<0.0001
Collagen metabolism		0.0001	<0.0001
Tryptophan metabolism	Indole pathway	<0.0001	<0.0001
	Kynurenine pathway	<0.0001	<0.0001
	Serotonin pathway	0.0003	<0.0001
Plant-based compounds	Alkaloids	<0.0001	<0.0001
	Benzoate metabolism	<0.0001	<0.0001
	Phenolics	<0.0001	0.0003
	Post-biotics	<0.0001	<0.0001
	Terpenoids	<0.0001	<0.0001
Endocannabinoids		<0.0001	<0.0001
Free fatty acids	Polyunsaturated n3	<0.0001	<0.0001
	Polyunsaturated n6	0.0184	<0.0001
	Saturated & monounsaturated	<0.0001	<0.0001
Acylglycerols	Monoacylglycerols	<0.0001	<0.0001
	Diacylglycerols	<0.0001	<0.0001
Linolenate metabolism		<0.0001	<0.0001
Sphingolipids	All	<0.0001	0.0006
	Ceramides	<0.0001	<0.0001
	Dihydroceramides	0.0046	0.0004
	Dihydrosphingomyelins	0.0686	0.0016
	Hexosylceramides (HCER)	<0.0001	<0.0001
	Lactosylceramides (LCER)	<0.0001	<0.0001
	Sphingolipid synthesis	0.0019	0.3686
	Sphingosines	0.0144	<0.0001
	Sphingomyelins (SM)	0.0003	0.0026
Phospholipids	Lysophospholipids	<0.0001	<0.0001
	Phosphatidylcholines (PC)	<0.0001	<0.0001
	Phosphatidylethanolamines (PE)	<0.0001	0.0002
	Phospholipid Metabolism	<0.0001	<0.0001
Vitamins and cofactors	Hemoglobin Metabolism	<0.0001	0.1841
	NAD Metabolism	<0.0001	<0.0001
	Tocopherol Metabolism	<0.0001	<0.0001
Bile acids	Primary	<0.0001	0.0149
	Secondary	0.0002	0.0069
Redox active couples	Oxidized	0.0042	0.0052
	Reduced	0.0085	<0.0001

[a]For each test, blue and red indicate metabolites within the pathway significantly increased or decreased in the dogs consuming TF1 relative to the comparator, respectively, as determined by summation of the numerical values for individual metabolites within the overall class. Yellow indicates the pathway was significantly altered but overall directionality was inconclusive. Full numerical data are delineated in Supplementary material 5. CF, control food; MANOVA, multivariate analysis of variance; TF1, test food 1; TF2, test food 2.

TABLE 5 Overall serum metabolomics data[a].

Metabolite classification		TF1 vs. CF	TF1 vs. TF2
		MANOVA *p*-value	MANOVA *p*-value
Sugars		<0.0001	<0.0001
Amino acids		<0.0001	<0.0001
Tryptophan indole pathway		0.0004	<0.0001
Free fatty acids	Eicosanoids	0.4516	0.8588
	Polyunsaturated n3	<0.0001	<0.0001
	Polyunsaturated n6	0.0009	<0.0001
	Saturated & monounsaturated	0.0012	<0.0001
Sphingolipids	All	<0.0001	<0.0001
	Ceramides	<0.0001	<0.0001
	Dihydroceramides	0.1211	0.0096
	Dihydrosphingomyelins	0.0006	<0.0001
	Hexosylceramides (HCER)	0.0003	<0.0001
	Lactosylceramides (LCER)	0.2302	<0.0001
	Sphingosines	0.0687	0.2188
	Sphingomyelins (SM)	0.0001	<0.0001
Phospholipids	Lysophospholipids	<0.0001	<0.0001
	Phosphatidylcholines (PC)	<0.0001	<0.0001
	Phosphatidylethanolamines (PE)	0.0001	<0.0001
	Phosphatidylinositol (PI)	<0.0001	<0.0001
Tocopherol metabolism		<0.0001	<0.0001
Collagen metabolism		<0.0001	<0.0001

[a]For each test, blue and red indicate metabolites within the pathway significantly increased or decreased in the dogs consuming TF1 relative to the comparator, respectively, as determined by summation of the numerical values for individual metabolites within the overall class. Yellow filled cells indicate the pathway was significantly altered but overall directionality was inconclusive. Full numerical data are delineated in Supplementary material 7. CF, control food; MANOVA, multivariate analysis of variance; TF1, test food 1; TF2, test food 2.

to putrescine, and the primary polyamine agmatine, which were both unchanged. Differences in fecal polyamines were inconsistent among TF1 and TF2; the metabolic intermediates 4-acetamidobutanoate, 5-methylthioadenosine, and ornithine were significantly higher in TF1-fed dogs, as were N-acetyl-cadaverine and N-acetylputrescine, while carboxyethyl-GABA and [N(1) + N(8)]-acetylspermidine levels were significantly lower. The primary polyamine spermidine was significantly lower in the stools of TF1-fed dogs vs. TF2-fed dogs ($P = 0.0001$), yet this decrease (0.30) was less pronounced than the difference between CF- and TF1-fed dogs (2.00, $P < 0.0001$; Figure 5C).

3.3.3. Tryptophan metabolism

Metabolism of the essential amino acid tryptophan was implicated as a potential driving factor in TF1 alleviating clinical diarrhea in our previous longitudinal, single-arm intent-to-treat study (16). In this study, TF1 feeding did not appreciably affect fecal tryptophan levels vs. CF, yet it significantly reduced tryptophan-related metabolites in the kynurenine ($P < 0.0001$), serotonin ($P = 0.0003$), and indole ($P < 0.0001$) subpathways in feces (Table 4, Supplementary material 5). Levels of the kynurenine-related metabolites 2-aminophenol ($P < 0.0001$), anthranilate ($P = 0.0001$), N-formylanthranilic acid ($P = 0.01$), picolinate ($P < 0.0001$), and quinolinate ($P = 0.03$) were all significantly reduced in TF1- vs. CF-fed dogs, while kynurenine, kynurenate, and xanthurenate levels were unchanged. Serotonin ($P = 0.04$) and its catabolite 5-hydroxyindoleacetate ($P = 0.001$) were both significantly reduced with TF1 from CF-fed levels. Significantly reduced fecal indoles in TF1-fed dogs vs. CF-fed dogs included 2-oxindole-3-acetate ($P < 0.0001$), indole ($P = 0.01$), and tryptamine ($P = 0.004$), as well as indolelactate ($P = 0.006$) and indolin-2-one ($P = 0.0001$). Levels of the latter two compounds, as well as 3-indoxyl sulfate ($P = 0.006$) and

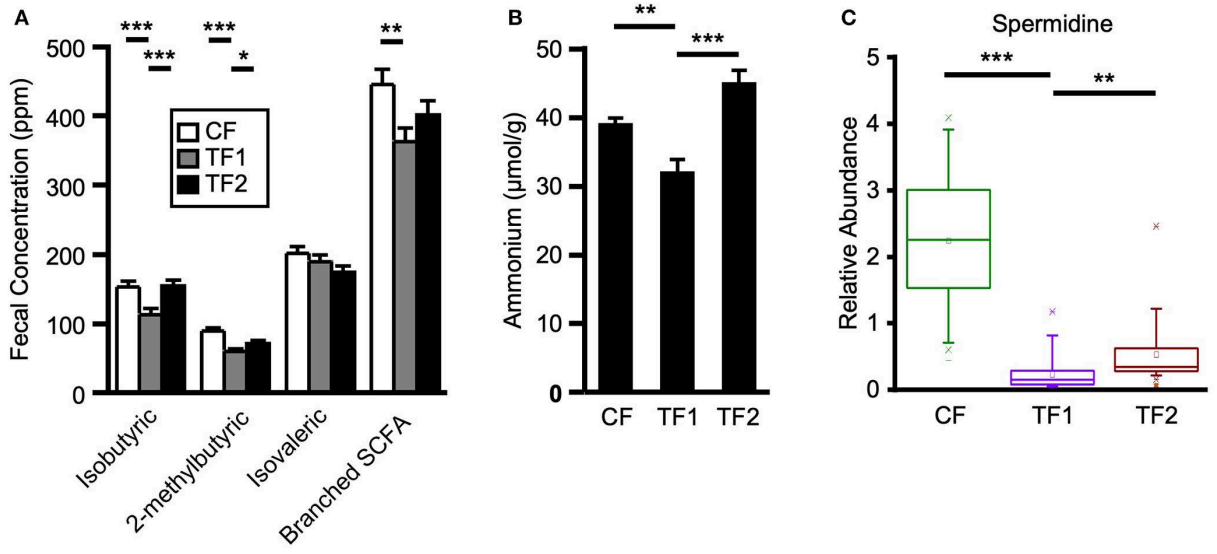

FIGURE 5
Markers of proteolytic microbial metabolism. **(A)** Branched short chain fatty acid measurements for dogs consuming CF (white), TF1 (gray), and TF2 (black). **(B)** Ammonium levels and **(C)** spermidine relative abundance levels for adult dogs consuming CF (green), TF1 (violet), and TF2 (brown). Significant differences among CF vs. TF1 and TF1 vs. TF2 are indicated (*$P < 0.05$, **$P < 0.001$, ***$P < 0.0001$).

tryptophan ($P < 0.0001$), were among indoles measured in both feces and serum that were also reduced in circulation, while metabolites in the tryptophan indole pathway were significantly reduced ($P = 0.0004$) in dogs consuming TF1 relative to CF (Table 5, Supplementary material 6).

We integrated fecal inferred microbial enzyme functions from PICRUSt and tryptophan metabolites to project relative abundance differences between CF and TF1 consumption onto a simplified tryptophan metabolic pathway map (KO00380, Figure 6, Supplementary material 7). The projection illustrates how TF1 feeding particularly impacted microbial metabolism of tryptophan through kynurenine metabolism to nicotinamides and energy substrates (e.g., 2-oxoglutarate dehydrogenase, K00164, $P < 0.0001$) in the tricarboxylic acid cycle. TF1 consumption resulted in increased kynurenine-3-monooxygenase (K00486, $P < 0.0001$) and 3-hydroxyanthranilate 3,4-dioxygenase (K00452, $P < 0.0001$) catabolism of kynurenine, as well as arylformidase (K07130, $P = 0.02$) catabolism of N-formylanthranilic acid, while activity of downstream enzymes kynureninase (K01556, $P = 0.01$) and aminocarboxymuconate-semialdehyde decarboxylase (K03392, $P = 0.0001$) were downregulated. In the indole pathway, tryptophanase (K01667, $P < 0.0001$) activity was elevated in TF1-fed dogs, as were nitrilase (K01501) and amidase (K01426, $P = 0.008$) functions, while tryptophan 2-monooxygenase (K00466) abundance was downregulated.

Fecal tryptophan levels were elevated and activity in all three of its metabolic subpathways were significantly upregulated in dogs consuming TF1 compared to dogs consuming TF2 ($P < 0.0001$). Notable fecal tryptophan metabolites that were increased in TF1-fed dogs include kynurenate ($P = 0.02$), kynurenine ($P < 0.0001$), serotonin ($P < 0.0001$), 2-oxindole-3-acetate ($P < 0.0001$), indoleacetate ($P < 0.0001$), indoleacetylglutamine ($P = 0.005$), and indolepropionate ($P = 0.0001$). Circulating levels of indoleacetylglutamine and indolepropionate were also significantly higher in dogs consuming TF1 vs. TF2, and serum indoles as a class were significantly different ($P < 0.0001$), despite serum tryptophan levels being statistically similar. Levels of the fecal tryptophan metabolites N-formylanthranilic acid ($P = 0.0008$), quinolinate ($P = 0.0002$), and indolin-2-one ($P = 0.008$) were significantly lower in TF1-fed dogs vs. TF2-fed dogs. Circulating 3-indoxyl sulfate ($P = 0.001$) and indolin-2-one ($P = 0.004$) levels were also significantly reduced in dogs fed TF1, compared to those fed TF2.

3.4. Impact of the pre-biotic fiber bundle on the availability of inflammatory mediators and anti-oxidative plant-derived compounds

3.4.1. Plant-derived phenolics, alkaloids, and terpenoids, and corresponding post-biotics

Nutritional plant components measured in feces were delineated as phenolics, alkaloids, and terpenoids (Supplementary material 5). MANOVA indicated that phenolic

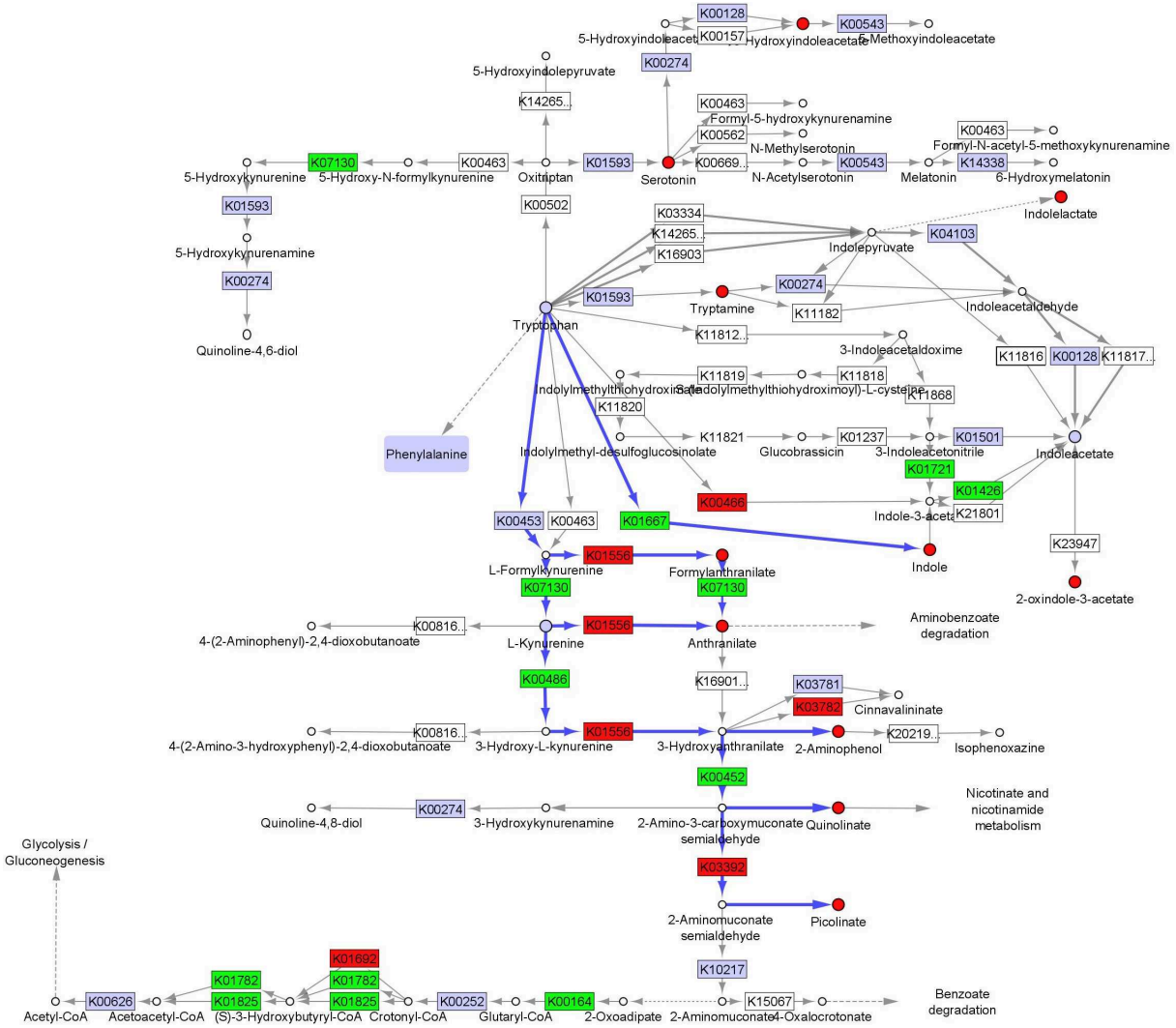

FIGURE 6
Fecal microbiome and metabolomic pathway analysis of tryptophan. Simplified projection of fecal metabolite and inferred function from microbiome measurements on the KEGG tryptophan pathway (KO00380) (36, 37, 39). Significantly increased and decreased enzymatic functions and metabolites are colored green and red, respectively, while observed but unchanged functions and metabolites are rendered gray. Likely catabolic paths discussed in the text are indicated with blue arrows.

compounds were significantly enriched ($P < 0.0001$) in the feces of dogs consuming TF1 compared to both CF and TF2 (Table 4). Most of these biochemicals were increased by roughly the same amount in dogs fed TF1 relative to those fed CF or TF2, which likely reflects their inclusion in the fiber bundle. Among the citrus-derived flavone and flavonoid polyphenols enriched in the stool of TF1-fed dogs vs. TF2- and CF-fed dogs were apigenin, chrysoeriol, diosmetin, sinensetin, and eriodictyol, as well as the flavanone glycosides hesperidin, and narirutin. The dihydrochalcone phloretin was also similarly significantly elevated ($P < 0.0001$) in the stool of dogs fed TF1 relative to both CF and TF2. Similarly, the flaxseed-rich lignan secoisolariciresinol diglucoside was also elevated in the stool of TF1-fed dogs ($P < 0.0001$), relative to CF and TF2. Conversely, levels of the soy-sourced isoflavones daidzein and genistein were similar among CF and TF1 fecal samples but were significantly reduced in the stools of TF1-fed dogs compared to those of TF2-fed dogs, while glycitein levels were similar between TF1- and CF-fed dogs and between TF1- and TF2-fed dogs. Additionally, fecal levels of the phenolic acids sinapate, syringic acid, and vanillate were similar in TF1- and CF-fed dogs and significantly elevated in dogs consuming TF1 relative to TF2, indicating these were also likely components of CF.

Multivariate analysis indicated that fecal alkaloid ($P < 0.0001$) and terpenoid ($P < 0.0001$) levels were both significantly

different among TF1-fed dogs compared to dogs fed either CF or TF2 (Table 4). Piperidine and stachydrine levels were significantly higher ($P < 0.0001$) with TF1 relative to both CF and TF2, pointing to their enrichment in TF1. Deoxymugineic acid levels were higher in stools of TF1-fed dogs compared to TF2-fed dogs but were similar to those observed in the CF group. Conversely, both TF1- and CF-fed dogs had similar fecal levels of pyrraline; however, TF1-fed dogs had significantly lower levels ($P < 0.0001$) than TF2-fed dogs (Figure 7A). Dogs in the TF1 group also showed significantly reduced fecal levels of DIMBOA (2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one), 1-methyl-beta-carboline-3-carboxylic acid, and nicotianamine relative to CF, while fecal levels of DIMBOA and 1-methyl-beta-carboline-3-carboxylic acid in TF-1 fed dogs were significantly higher than those in TF2-fed dogs. TF1 feeding significantly reduced all the fecal steroidal terpenoids (beta-sitosterol, campesterol, ergosterol, lanosterol, and stigmasterol) and the fecal carotene diols relative to CF. By contrast, levels of these compounds were significantly higher in TF1-fed dogs vs. TF2-fed dogs, with the exception of ergosterol, which was significantly lower for TF1, and lanosterol, which showed no significant differences between groups. Fecal limonoids limonin and nomilin were both significantly enriched in TF1-fed dogs relative to either CF-fed or TF2-fed dogs, as was the case for the chlorophyll metabolite pheophorbide A.

We separately conducted a multivariate analysis on fecal post-biotics known to arise from the microbial metabolism of plant biochemicals (Table 4). These post-biotics were significantly enriched ($P < 0.0001$ by MANOVA) in TF1-fed dogs relative to dogs fed either CF or TF2. These included the deglycosylated flavonoids hesperetin (Figure 7B), naringenin, and ponciretin, the deglycosylated lignan secoisolariciresinol, and the phytoestrogen enterodiol (Supplementary material 5). In contrast, levels of the other phytoestrogens, enterolactone, and equol, were similar among dogs consuming the three foods. Diaminopimelate and 2-piperidinone were the only compounds in this class that were lower in TF1-fed dogs than in CF-fed dogs but these levels were similar among dogs consuming TF1 and TF2. Beta-guanidinopropanoate levels were lower in TF1-fed dogs vs. TF2-fed dogs, yet similar in TF1-fed dogs vs. CF-fed dogs.

Fecal benzoate metabolism was significantly different ($P < 0.0001$ by MANOVA) in TF1-fed dogs relative to either CF- or TF2-fed dogs (Table 4). Notably, the flavonoid metabolite 2,4,6-trihydroxybenzoate was significantly enriched ($P < 0.0001$) in TF1-fed dogs by roughly the same amount when compared to either CF- or TF2-fed dogs (Figure 7C). To a lesser extent, levels of the related flavonoid metabolite 3,4-dihydroxybenzoate were also significantly higher in stools of TF1-fed dogs relative to CF-fed dogs ($P < 0.0001$) or TF2-fed dogs ($P = 0.006$). Conversely, 3-(2-hydroxyphenyl) propionate levels were similar among dogs consuming TF1 and TF2, but were significantly lower ($P = 0.009$) among TF1-fed dogs vs. CF-fed dogs. Levels of other benzoate-related catabolites were similar among TF1- and CF-fed dogs but were significantly higher in TF1-fed dogs relative to TF2-fed dogs.

3.4.2. Fatty acid lipid mediators

We considered the fecal N-acyl amino acids, including arachidonoyl ethanolamide (AEA) and its N-acylethanolamide (NAE) congeners as endocannabinoids, molecules with diverse functions in the mammalian gut. As a group, fecal levels of these endocannabinoids were significantly reduced ($P < 0.0001$) in TF-fed dogs relative to either CF- or TF2-fed dogs (Table 4). By univariate analysis, TF1 feeding significantly decreased each of the 14 measured fecal NAEs compared to CF, but only one of the six measured taurine- and serine-containing species (Supplementary material 5). Among TF1 and TF2, 11 NAEs were significantly decreased with TF1 vs. TF2, including AEA ($P < 0.0001$), the primary cannabinoid receptor agonist, which showed the largest magnitude reduction in relative abundance (2.84), as were four out of six taurine- and serine-linked fatty acids.

Polyunsaturated fatty acids (PUFAs) are food-derived precursors to lipid mediators, and here we separated n3 and n6 PUFAs for multivariate analysis. We separately analyzed linolenate-containing lipids since the n3 vs. n6 isomerism was not resolved. Linolenate-containing lipids were significantly elevated ($P < 0.0001$) in stools and circulation among TF1-fed dogs compared with either CF- or TF2-fed dogs. TF1 was associated with significantly increased levels of n3 PUFAs compared with CF or TF2 (both $P < 0.0001$, Table 4). Among the eight measured n3 PUFAs in feces, levels of six were significantly higher among TF1-fed dogs vs. CF-fed dogs and five were significantly higher with TF1 than with TF2 (Supplementary material 5), including docosahexaenoate and eicosapentaenoate. Circulating eicosapentaenoate and linolenate were significantly higher in the TF1 group than in either the CF or the TF2 groups.

TF1 feeding also significantly increased both fecal ($P = 0.02$) and serum ($P = 0.0009$) n6 PUFAs relative to CF. Circulating and fecal levels of hexadecadienoate (16:2n6) and linoleate (18:2n6) were significantly elevated among TF1-fed dogs compared to CF-fed dogs, while arachidonate (20:4n6) was only higher with TF1 in feces. Levels of docosapentaenoate (22:5n6) were elevated in stools of TF1-fed dogs relative to CF-fed dogs, yet levels were reduced in TF1-fed dogs vs. CF-fed dogs in circulation. Fecal n6 PUFAs indicated levels were significantly different among the test foods ($P < 0.0001$ by MANOVA). By univariate analysis, fecal hexadecadienoate (16:2n6) levels were lower in TF1-fed dogs vs. TF2-fed dogs, while levels of all other detected n6 PUFAs were similar in the TF1 group relative to either CF or TF1. Circulating n6 PUFAs were significantly higher in TF1-fed dogs vs. CF-fed dogs ($P = 0.0009$) and lower vs. TF-2 fed dogs by MANOVA ($P < 0.0001$).

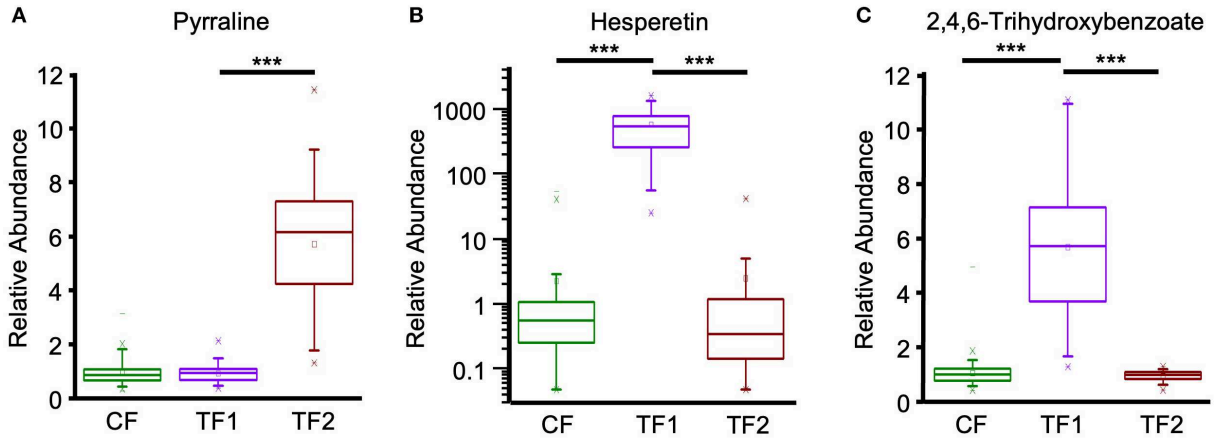

FIGURE 7
Fecal plant-derived compounds and microbial post-biotics metabolism. Relative abundance levels for **(A)** pyrraline, **(B)** hesperetin, and **(C)** 2,4,6-trihydroxybenzoate in dogs consuming CF (green), TF1 (violet), and TF2 (brown). Significant differences among CF vs. TF1 and TF1 vs. TF2 are indicated (***$P < 0.0001$).

Four of eight circulating n6 PUFAs were significantly lower in TF1-fed dogs ($P < 0.0001$), including arachidonate (20:4n6) and docosapentaenoate (22:5n6). Levels of circulating eicosanoids were comparable among TF1 vs. either CF or TF2.

3.4.3. Sphingolipids

Ingestion of the pre-biotic fiber bundle profoundly impacted fecal sphingolipids, reducing this class of metabolites in TF1-fed dogs vs. CF-fed dogs ($P < 0.0001$), including significant reductions in the ceramide ($P \leq 0.0001$), dihydroceramide ($P = 0.005$), hexosylceramide ($P < 0.0001$), lactosylceramide (LCER, $P < 0.0001$), sphingosines ($P = 0.01$), and sphingolipid synthesis metabolite ($P = 0.002$) subclasses, as indicated by MANOVA (Table 4). By univariate analysis, 15 of these 18 fecal metabolites showed significant reductions with TF1 vs. CF (Supplementary material 5). Fecal sphingomyelins (SM) as a class were significantly altered ($P = 0.0003$) with the addition of fiber in TF1 vs. CF, yet an increase in tricosanoyl sphingomyelin (d18:1/23:0)* levels was the only difference to reach significance by univariate analysis ($P < 0.05$) compared to CF. Dogs consuming TF1 showed significantly reduced circulating levels of SM, dihydrosphingomyelins, and HCERs compared to CF, as well as significantly altered ceramides and sphingolipids levels overall with inconclusive directionality (Table 5). Circulating LCER levels were similar with TF1 and CF.

While TF1 consumption resulted in an almost universal reduction in fecal sphingolipids compared to CF, multivariate analysis indicated fecal sphingolipid levels were significantly higher in the stool of TF1-fed dogs compared to TF2-fed dogs overall ($P = 0.0006$) and in most subcategories, including SM and dihydrosphingomyelin (Table 5). In contrast to the results in stool, circulating sphingolipids were significantly lower in TF1-fed dogs compared to TF2-fed dogs ($P < 0.0001$ by MANOVA). Such differences were evident in all sphingolipid subcategories except sphingosines, for which sphingosine 1-phosphate levels were unchanged (Supplementary material 6). In fact, univariate analysis revealed that 18 out of 23 detected circulating sphingolipids were significantly lower in the TF1 group compared to the TF2 group. Levels of serum SM were significantly different among dogs consuming TF1 and TF2 ($P < 0.0001$), yet the directionality was metabolite dependent. Of the 27 detected circulating SMs, 13 were lower and 4 were higher among TF1-fed dogs vs. TF2, respectively.

3.5. Phospholipids associated with the gut mucosal layer

Multivariate analysis (Table 4) indicated that TF1 feeding significantly decreased fecal phosphatidylcholines (PCs, $P < 0.0001$) and fecal phosphatidylethanolamines (PEs, $P < 0.0001$) compared to CF, and significantly altered other phospholipid metabolites ($P < 0.0001$). Eight of the 11 fecal PCs showed significant reductions with TF1 vs. CF, and all six fecal PEs were also significantly lower when the fiber bundle was included (Supplementary material 5). On the other hand, TF1 consumption significantly increased levels of fecal lysophospholipids ($P < 0.0001$), with 6/13 individual lysophospholipids higher in T1-fed dogs compared to CF-fed dogs. TF1 was associated with significantly increased levels of circulating PCs ($P < 0.0001$), PEs ($P = 0.0001$), and phosphatidylinositols (PIs, $P < 0.0001$), and significantly decreased serum lysophospholipid levels ($P < 0.0001$) compared to CF. Among the 19 circulating PCs, levels of 10 were lower

and four were higher in the TF1 group than in the CF group. Notably, nine of the serum PCs that were reduced in TF1-fed dogs were also reduced in feces compared to CF (Supplementary material 6).

Levels of fecal phospholipids were also largely significantly lower with TF1 feeding vs. TF2. TF1 consumption led to significantly lower levels of fecal PCs ($P < 0.0001$) and PEs ($P = 0.0002$) by multivariate analysis, whereas univariate analysis indicated that eight of 11 individual PCs and four of six PEs were significantly reduced with TF1 vs. CF. Circulating PCs and PEs were also significantly different between TF1 and TF2 on multivariate analysis. Of the 19 measured serum PCs, seven were higher and 10 were lower in TF1-fed dogs compared to TF2-fed dogs. Intriguingly, 12 of these significantly altered PCs had the same directionality in comparing TF1 and TF2 as when comparing CF and TF1. A similar effect was observed for PEs, whereby three of the five significantly altered lipids showed the same directionality when TF1-fed dogs were compared to either TF2- or CF-fed groups. Multivariate analysis indicated significantly lower levels of circulating PIs and lysophospholipids among TF1-fed dogs compared to TF2-fed dogs. By univariate analysis, consumption of TF1 resulted in significant reductions in five of six PIs and 11 of 22 lysophospholipids, compared to consumption of TF2, while two additional lysophospholipids were significantly elevated in the TF1 group vs. the TF2 group.

4. Discussion

4.1. Distinct macroscopic, microbiome, and metabolomic signatures associated with dietary fiber

A veterinary program of managed care for dogs with CGE typically involves nutritional interventions intended to relieve symptoms and relieve colonic dysbiosis. We surmised that the efficacy of therapeutic foods for CGE could be driven by the specific effects of the novel pre-biotic fiber bundle on the gastrointestinal microbiome of the adult dogs consuming the food. This crossover study was designed to test this hypothesis by evaluating the clinical, metabolic, and metabolomic effects of a food containing this novel pre-biotic fiber bundle, a commercially available therapeutic food with traditional fibers, and a control food in healthy adult dogs and dogs with well-managed CGE.

Stool characteristics indicated that TF1 and TF2 improved GI function of the dogs in the study compared to CF, which lacked the novel pre-biotic fiber bundle. Both TF1 and TF2 significantly increased fecal scores compared to CF, which is consistent with the designated indication of these therapeutic foods. The pre-biotic fiber bundle technology comprises both soluble and insoluble fibers, specifically chosen and proportioned for their unique properties, including prebiotic, water holding, stool bulking, and antioxidant characteristics (16, 30). Inclusion of the pre-biotic fiber bundle had no effect on moisture content relative to CF, yet it still improved stool scores, which is reflective of the selected fiber properties. Furthermore, TF2 also significantly decreased total mineral ash, including all measured inorganic compounds, except the fecal osmolytes sodium and potassium, which were increased and unchanged, respectively. These results suggest that the fiber enhanced mineral bioavailability in healthy dogs and dogs with managed CGE, relative to a food not enriched with fiber. This is consistent with the impact of pre-biotics included in foods [reviewed in Whisner and Castillo (44)], fiber supplementation in general (13), and our previous investigation of the same pre-biotic fiber bundle supplementation with CGE dogs in hydrolyzed foods enriched with either grain or meat (17). In contrast, although TF1 consumption reduced total fecal ash relative to TF2, the differences were less consistent than for TF1 vs. CF, with some minerals increased and others decreased. The reduced fecal moisture content associated with TF2 likely reflects the higher percentage of insoluble fiber included in the food vs. TF1.

4.2. Pre-biotic fiber bundle impacted microbial metabolism in the absence of major compositional changes

Our previous studies investigating the pre-biotic fiber bundle in different food backgrounds (17) and dogs with chronic large bowel diarrhea (16) showed evidence for a shift away from putrefactive toward saccharolytic microbiome activity using the same analytical tools and signatures investigated here in canine stool. In dogs consuming TF1 compared to CF, reductions in fecal total branched SCFA, ammonium, and MANOVA of amino acids and polyamines corroborated our previous observations supporting reduced proteolytic activity upon fiber ingestion. Furthermore, TF1 feeding resulted in reduced fecal abundance of the proteolytic genus *Clostridium* compared to both CF and TF2. Reduced microbial putrefactive activity in the lower gastrointestinal tract is largely considered a positive nutritional outcome for the host (45). Indeed, dogs with acute diarrhea present with increased levels of *Clostridium* (26, 46, 47), and the pathogenic bacterium *Clostridium difficile* is largely proteolytic (48). *Helicobacter* sp. are commonly found in canine feces, regardless of health status (49). High fiber foods, which would include TF1 and TF2, are known to negatively impact protein digestibility (17, 50). The pre-biotic fiber bundle appears to affect protein digestion by preventing fully digested amino acids from either reaching the colon or shifting microbes away from further metabolizing incompletely digested protein, which could explain why the universally decreased fecal amino acid signature was not

fully consistent among dipeptides. The various fibers added to TF1 and TF2 clearly impact protein digestion and absorption differently. While TF2 contains slightly more protein by weight, consumption of TF1 resulted in higher fecal amino acid and dipeptide levels, as well as circulating amino acid levels. Despite the increased protein availability with TF1, microbial proteolysis was suppressed such that fecal ammonium levels were lower in TF1-fed dogs, while primary polyamine and branched SCFA levels were largely in line with those measured in the TF2 group.

Compared to CF, dogs consuming TF1 showed reduced signatures of saccharolytic metabolism, namely reduced straight SCFAs, possibly because TF1 contained 11% less total nitrogen-free extract, and possibly due to a reduction in residual resistant starch in TF1. In TF1, cornstarch was replaced with the fiber bundle, although it is not expected that cornstarch contributes more resistant starch given the extrusion conditions used to produce the food (23). Consuming the fiber bundle led to increased acetic acid but less propionic acid and lactate production. Intriguingly, the pH of feces from CF- and TF1-fed dogs were similar, even though CF resulted in higher overall SCFA and lactate levels, the largest contributors to fecal pH (51). Increased fecal levels of arabinose and ribulose/xylulose were consistent with our previous findings in dogs (16, 17) and cats (32, 52), and thus may represent a biochemical marker of ingesting this particular pre-biotic fiber.

Among the therapeutic foods, TF1 imparts a stronger fecal saccharolytic signature than TF2, including reduced pH and increased levels of lactate and monosaccharides, propionic and butanoic acids, and total straight SCFA. The higher fecal pH of dogs fed TF2 compared with TF1 could be attributed to a reduction in *Bifidobacterium* sp. abundance in combination with increased abundance of families Veillonellaceae, Enterobacteriaceae, as well as Clostridium and 02d06 genera within Clostridiaceae, all of which have been associated with elevated fecal pH in human infants (53). Members of the Ruminococcae family, including genus *Faecalibacterium*, generate butyrate from acetate, and their fecal abundances were higher among TF1-fed dogs compared to either CF or TF2. Lower levels of these taxa and the acetate producing *Bacteroidetes* were reported in dogs with acute diarrhea (46, 47, 54). Colonocytes consume microbiota-produced butanoic acid as their primary energy source through mitochondrial respiration (55). Both *Faecalibacterium* sp. and *Bacteroides* sp. play an important complementary role in maintaining colonic epithelial homeostasis by influencing the production of mucin glycans (56). Higher fecal lactate and straight SCFA levels, reduced pH, reduced ammonium, and reduced branched SCFAs have also been observed in dogs fed pomegranate pulp extract (51), while similar results were also reported in dogs consuming foods produced with higher levels of resistant starch (17, 57). Given that feces of TF2-fed dogs showed reduced butanoic acid and elevated pyrraline, a Maillard reaction product and marker of the thermal treatment of sugars in food processing (58), the TF2 food may have undergone more intense extrusion processing compared to TF1, in addition to including different pre-biotic fibers. Taken together, these results confirm that the composition and the preparation of TF1 and TF2 have differential effects on microbial fermentation and its subsequent beneficial post-biotic outputs in canine hindguts.

Mammalian hosts, including dogs, and their resident gut microbes readily exchange dietary tryptophan for a host of bioactive catabolites, such that these metabolites represent a noteworthy currency in assessing gut health, including barrier integrity and immune function, as well as systemic toxicity (8). Compared to control, TF1 consumption decreased most fecal monomeric amino acids, but not tryptophan. Integrated metabolomics and microbiome showed that enzymatic degradation of kynurenine by kynurenine-3-monooxygenase (K07130) function in feces was elevated, but downstream enzyme activity was reduced, possibly representing metabolic bottlenecks that ultimately resulted in decreases in 2-aminophenol, picolinate, and quinolinate. Suppression of most fecal nicotinamide adenine dinucleotide (NAD)-related metabolites could partly be due to reduced *de novo* NAD synthesis via quinolinate. Indole generation by tryptophanase is attributed to microbial activity, as mammals lack this enzyme functionality (59). Tryptophanase (K01667), as well as downstream nitrilase (K01501) and amidase (K01426) functions all were elevated in feces, while indole and indoleacetate levels were decreased and unchanged, respectively. These results imply that excess indole was quickly further catabolized to other indole species and/or indole and its catabolites were absorbed locally, as circulating levels were largely unaffected. By contrast, most fecal amino acid levels, including tryptophan, were higher with TF1 vs. TF2, which largely resulted in higher levels of other fecal TF1 tryptophan metabolites. The suppression of fecal quinolinate levels seen with the fiber bundle in TF1 was also evident vs. TF2; however, other NAD-related fecal metabolites were elevated with TF1 vs. TF2, suggesting the NAD salvage pathway more than compensated for any loss in *de novo* synthesis.

Analysis of circulating indole-related compounds provides clues into systemic health via gut microbiome metabolism. Compared to CF and TF2 consumption, TF1 consumption resulted in reduced serum levels of the indole sulfates 3-indoxyl sulfate, 5-hydroxyindole sulfate, and 7-hydroxyindole sulfate, as well as indolin-2-one (also known as oxindole), which was also lowest in feces of TF1-fed dogs, and these results were consistent with our previous study (16). Indoxyl sulfates are products of host-microbe biochemistry that result from the conversion of dietary tryptophan to indole by microbiota. Indole then enters the circulation and is hydroxylated and sulfated by the liver. Indoxyl sulfates are largely considered mammalian uremic toxins associated with acute and chronic kidney injury (60). By contrast, serum indoxyl glucuronide, another potential indole uremic toxin (61), was higher with TF1 vs. TF2 but not vs. CF. Indolin-2-one production may

depend on multiple gut commensals and was implicated as a potent neurodepressive compound in a rat study (62). Others have reported that the inclusion of increasing levels of protein in pet foods fed to healthy dogs correlated with increasing serum levels of indole sulfates and fecal levels of indolin-2-one (7). Through a combination of reducing indole production and possible improved barrier function, consumption of the fiber bundle largely suppressed the production of several tryptophan-associated uremic toxins.

4.3. Fiber altered the anti-oxidative and anti-inflammatory capacity in the canine gastrointestinal tract

Metabolite components of the plant materials comprising the fiber bundle, as well as their microbe-metabolized post-biotics, were clearly evident in the feces of TF1-fed dogs, given the similar abundance differences between TF1 and both CF and TF2 fecal measurements. A previous study investigating TF1 as an interventional therapy in dogs with severe diarrhea also uniquely identified these plant-derived compounds in stool within 48 hours (30). In fact, several of the citrus pulp-derived phenolic (e.g., diosmetin, hesperidin, narirutin, phloretin, and sinensetin) and terpenoid (e.g., limonin and nomilin) components were presumably below their limits of detection in many of the samples from CF- and TF2-fed dogs, and comparison statistics were derived from imputation (data not shown). While the putative health benefits of phenolic phytonutrients are largely derived from *in vitro* studies, a growing body of clinical evidence in companion dogs corroborating the prebiotic, antioxidant, and immunomodulatory mechanisms of these benefits is emerging [reviewed extensively in Tanprasertsuk et al. (63)]. The inclusion of diverse phenolic bound fibers in dog food and their associated bioactive effects in canine hindguts may impart additional metabolic benefits for the host.

Post-biotics arising from microbiota-directed catabolism often are more bioavailable than their parent phenolic compounds, and thus may more strongly potentiate disease modification (25, 64). The gut microbiota readily converts the flavanone poncirin to ponciretin, and while both compounds attenuated chemically induced colitis and associated inflammation *in vivo*, ponciretin demonstrated more pronounced inflammasome inhibition *in vitro* (65). Here, only TF1-fed dogs showed increased ponciretin in feces; however, the presence of both compounds in citrus and absence of poncerin means we cannot definitively associate its presence in the TF1 group with microbiota metabolism. Similarly, the flavanone hesperetin is more bioactive than its glycoside parent compound hesperidin in its anti-inflammatory and radical scavenging properties (64). Hesperetin supplementation reduced cytokine secretion and improved epithelial barrier integrity in a chemically induced colitis model (66). The feces of TF1-fed dogs uniquely contained abundant hesperidin and hesperetin, yet the microbiome-directed contribution of the latter is unclear. Finally, orange juice rich in the flavanones hesperidin, narirutin, and their metabolites hesperetin and naringenin was shown in an *ex vivo* human feces fermentation study to act as a positive prebiotic, decreasing ammonium production and stimulating butyric, acetic, and propionic SCFA production and increasing antioxidant activity while affecting microbiota characteristics (67). The increase of ponciretin and hesperetin levels in the feces of dogs fed TF1 corroborates with the increased abundance levels of *Bacteroides* sp. and *Fusobacterium* sp. due to their potential alpha 1- rhamnosidase and beta-glucosidase activity in converting poncirin and hesperidin into ponciritin and hesperetin, respectively (68).

In a previous clinical study of TF1, the fiber-associated phenolics and their post-biotics were hypothesized to contribute to the resolution of chronic diarrhea resolution (16, 30). A recent murine study found significant, dose-dependent alleviation of colitis upon ingestion of eriodictyol, which was enriched in stools of TF1-fed dogs (69). A similar study investigating phloretin, which was also abundant in TF1-fed dog feces, reported the flavanone ameliorated colitis disease through a mechanism involving the regulation of the gut microbiota, including inflammatory modulation, restoration of barrier integrity, and increased anti-oxidative capacity (70). Phloretin may represent an intermediate post-biotic of flavonoid (e.g., apigenin and naringenin) microbial metabolism to 3-(4-hydoxyphenyl)-propionate (71). The gut microbiota can further metabolize the A and B rings of flavonoids into different stable phenolic acid end products. B-ring products include 3,4-dihydroxybenzoate and 4-hydroxybenzoate, while 2,4,6-trihydroxybenzoate can be derived from the A ring. TF1 consumption resulted in the fecal enrichment of 2,4,6-trihydroxybenzoate and 3,4-dihydroxybenzoate compared to both CF and TF1, and the fecal enrichment of 3-(4-hydroxyphenyl)-propionate and 4-hydroxybenzoate compared to TF2. The flavonoid post-biotic 2,4,6-trihydroxybenzoate was recently shown to have potent anti-colorectal cancer activity *in vitro* through inhibition of cyclin dependent kinase (72). Collectively, these results suggest that the citrus-derived phenolics and their post-biotics provided by the pre-biotic fiber bundle may have beneficial effects on the gastrointestinal health of dogs.

The lignans enterodiol and enterolactone and the isoflavone equol are bioactive phytoestrogens generated in mammals via the gut microbial metabolism of plant polyphenols (73, 74) and have been shown to provide anti-inflammatory, antioxidant, and free radical scavenging properties for the host (75). The TF1 ingredient flaxseed includes high levels of the lignan secoisolariciresinol diglucoside, which is metabolized by gut microbes to secoisolariciresinol and enterodiol. All

three metabolites were uniquely enriched in stools of TF1-fed dogs in this study and in previous studies (17, 18). Consumption of TF1 compared to either CF or TF2, however, resulted in similar fecal levels of enterolactone and its parent lignan matairesinol, suggesting flaxseed largely contributes to microfloral production of enterodiol. While the soy isoflavones daidzein and genistein were present at higher levels in the stool of TF2-fed dogs compared to TF1-fed dogs and measured in all test samples, equol and glycitein levels were comparable among the therapeutic foods. In our previous study that switched dogs from various foods to TF1, all four isoflavones were significantly reduced (16). Given that the foods in this study appear to have trace soy-based ingredients, differences in downstream equol present in stools may have been negligible.

Dietary fiber is known to have profound effects on lipid digestion and absorption, depending on the composition, viscosity, and other physicochemical properties of the fiber (76). All three study foods contained a roughly equivalent percent weight of fish oil, a rich source of n3 PUFAs. TF1 also contained flaxseed, which likely contributed to elevated fecal levels of linolenate and linolenate-containing fatty acids in the TF1-fed group relative to the CF- or TF2-fed groups. Consumption of the pre-biotic fiber bundle in TF1 appeared to enhance lipolysis and mediate absorption of its free fatty acid substituents, resulting in higher levels of fecal monoacylglycerols and lysophospholipids, fecal and circulating free n3 and n6 PUFAs, and medium- and long-chain free fatty acids, while decreasing fecal levels of non-linolenate-containing diacylglycerols. Unfortunately, triacylglycerides were not measured in the metabolomic analysis, limiting the extent to which the dietary effects of the fiber bundle on lipolysis could be investigated. Among the therapeutic foods, fecal and serum n3 PUFAs as a class were higher in TF1-fed dogs, while circulating n6 PUFAs were lower, suggesting an improved n3/n6 ratio in healthy dogs consuming TF1, a finding consistent with the results of our previous intervention study in dogs with active large bowel diarrhea (16). Targeted MS/MS analysis would confirm such ratios. Serum levels of the inflammatory mediating eicosanoid lipid classes after TF1 consumption previously had reflected changes in their n3 and n6 precursors; however, in this study, these levels were similar regardless of diet, which is likely a reflection of the absence of active, uncontrolled disease.

Targeting the endocannabinoid system and related NAE biochemicals represents an emerging opportunity to impact gastrointestinal health, given their roles in mediating inflammation, gut motility, barrier function, and microbiota composition. While technically only AEA (also known as anandamide) and 2-arachidonoylglycerol (not measured here in feces) are endocannabinoid receptor agonists, the additional NAEs are non-selectively generated alongside AEA by the same enzymatic pathways, usually in higher yields, and are also implicated in gastrointestinal health and disease (77). The broad reductions in fecal NAEs with consumption of the pre-biotic fiber bundle corroborate our previous reports and appear independent of the disease status of the subjects (16, 17). Among the two fiber-containing foods tested, TF2 showed considerably higher fecal levels of AEA and other N-acylated amino acids, for example N-taurine- and N-serine-linked species, suggesting the phenomenon may be unique to the fiber blend in TF1 and not reflective of fiber supplementation overall. Investigations of NAE levels vary by gastrointestinal disease state, but broadly speaking, AEA and other NAEs are often inversely related to beneficial outcomes, whereas increased AEA and reduced palmitoylethanolamide and oleoylethanolamide have been associated with increased food intake, an increased risk of metabolic syndrome, and reduced barrier integrity (78). In a diet-driven obesity murine model, an activated endocannabinoid system influenced by the gut microbiome promoted barrier permeability and treatment with a pre-biotic restored AEA levels and barrier function (79). It is tempting to speculate that the novel pre-biotic fiber bundle modulates NAE generation and/or degradation to some degree, given the consistent and near-universally lower fecal levels associated with its intake; however, additional research is needed to support such a conclusion.

Sphingolipids and the enzymes involved in their metabolism modulate cell proliferation and immunity through mechanisms that can involve the gut microbiota (80). Obtained primarily from animal dietary sources as SM, most digestion occurs in the small intestine, which harbors the enzyme alkaline sphingomyelinase (alk-SMase), a potent degrader of SM to ceramide. Ceramidases hydrolyze ceramide to sphingosine, which is readily absorbed (81). Thus, circulating SM and ceramide are synthesized by the host and are unrelated to their respective levels in feces, which would reflect undigested sphingolipid. Consumption of the pre-biotic fiber bundle largely suppressed fecal and circulating sphingolipid levels compared to CF, with the exception of fecal SMs and serum ceramides, for which mixed directionality was observed. Since these foods presumably contained equivalent dietary sphingolipids, the data could reflect efficient alk-SMase and ceramidase activity and/or improved absorption by host tissues, as most sphingolipids were reduced in circulation. Gene expression or proteomics experiments may shed additional light on the fiber's modulation of sphingolipid metabolism. Alk-SMase activity correlates with improvements in inflammatory bowel disease and can be activated by probiotics in humans and mice (80). TF1 consumption largely resulted in higher fecal but lower serum levels of sphingolipids than TF2, including ceramides, but it did not affect fecal markers of sphingolipid synthesis (e.g., 3-ketosphinganine and sphinganine) or serum SM. TF1 may have contained higher dietary SM or TF2 may have improved sphingolipid absorption, given the similarities in markers of sphingolipid synthesis between groups and the generally higher sphingolipid levels in TF2.

4.4. Possible colonic mucosal layer restructuring upon pre-biotic fiber consumption

Phospholipids contribute to a hydrophobic barrier separating colonocytes from resident microbes, a critical function of the colonic mucosal layer. The phospholipid composition of the canine hindgut roughly follows its distribution along the entire gastrointestinal tract; PC and PE constitute approximately two thirds of the bulk, with ∼10% each of SM, PI, and PS, and <5% lysophosphatidyl choline (82). In cases of inflammatory bowel disease in humans (83) and in dogs (84), irregularities in phospholipid levels at the tissue level and systemically have been documented. In fact, delayed release PC has been investigated as therapy for human ulcerative colitis (85).

Consumption of TF1 largely suppressed fecal PC, PE, SM, and lysophospholipids in dogs compared to CF and TF2, and while circulating phospholipids were also impacted, an overall decrease was evident in only SM and lysophospholipids. These results were more pronounced than those in our previous study in dogs with chronic large bowel diarrhea, in which PC and SM were significantly decreased in feces but were generally increased in serum (16). Taking both studies into account and acknowledging that the present study design controlled for environmental and breed variations, it appears the pre-biotic fiber bundle reduces phospholipid elimination in stool, regardless of disease state, while its impact on circulating phospholipids is more disease dependent. A study of circulating metabolites in children with ulcerative colitis or Crohn's disease also found irregularities in phospholipids vs. controls, with differing signatures associated with the specific inflammatory bowel disease (86). Interestingly, that study identified that the abundance of a single ceramide, LCER (18:1/16:0), could differentiate Crohn's disease from ulcerative colitis and that the level of serum LCER (18:1/1:0) was higher in Crohn's disease or ulcerative colitis than in controls. In our current study, fecal LCER (18:1/16:0) levels were lower, while serum levels were unchanged in TF1 vs. CF. In our previous intervention study, LCER levels increased in serum and were not measured in feces (16). Finally, n3 PUFA fatty acids (including those found in fish oil) are known to integrate into intestinal epithelial cells *in vivo* and have been associated with gut barrier and inflammatory protection (87). TF1 increased fecal and circulating n3 and n6 levels, but it is unknown whether gut epithelial tissue assimilated these protective lipids into membranes.

Two major factors limited the scope of this study. First, TF2 lacked a proper positive control, and since it was a commercially available food, we could not develop an appropriate control food for TF2 lacking its fiber supplement, as we did for TF1. We therefore limited our analysis to the effect of the fiber bundle under investigation (i.e., TF1 vs. CF) and the therapeutic foods themselves (i.e., TF1 vs. TF2). Thorough statistical analysis of the metabolomics data indicated the absence of period effects, which prompted us to combine the wash-in and washout feedings as controls. Second, the metabolomics measurements are quantitative but not absolute (i.e., do not provide concentrations), meaning we cannot properly compute metabolite ratios (e.g., n3/n6) or determine whether metabolite levels reached physiologically relevant concentrations. We therefore treated this study as exploratory and largely focused on metabolite pathway changes in our interpretation.

5. Conclusion

In summary, we identified gut microbiome structure and metabolism signatures associated with pre-biotic fiber supplementation in dogs. In our previous study investigating the impact of TF1 on the clinical resolution of chronic large bowel diarrhea, we inferred changes from baseline in dogs with active disease (16, 30). In this study, we largely corroborated those findings by comparing TF1 to a control food lacking the novel pre-biotic fiber bundle and to a different pre-biotic fiber-supplemented food, TF2, and further extended the results of the previous trial to both healthy dogs and dogs with controlled chronic gastroenteritis/enteritis. We demonstrated that consumption of the polyphenol-rich pre-biotic fiber blend in TF1 resulted in distinct fecal and circulating metabolomes from CF and TF2-fed dogs. While both TF1 and TF2 improved stool quality, TF1 feeding uniquely promoted microbial saccharolytic and post-biotic metabolism, while suppressing putrefactive processes, and further altered fecal and circulating lipid metabolism with positive implications for gut function and inflammation. This suggests TF1 is effective in both the early and continuing stages of veterinary management of gastroenteritis and demonstrates the value of comprehensive microbiome and metabolomic profiling in understanding how fiber interventions contribute to pet gastrointestinal health.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation. The raw sequence data presented in this study are deposited in the National Center for Biotechnology and Information (NCBI) repository, accession number PRJNA918369. The data can be found here: https://www.ncbi.nlm.nih.gov/bioproject/?term=prjna918369.

Ethics statement

The animal study was reviewed and approved by Institutional Animal Care and Use Committee (CP693a) of Hill's Pet Nutrition, Inc.

Author contributions

DF, MJ, SW, and KG participated in the design and execution of the study. All authors participated in the data analysis and interpretation, drafted and revised the manuscript, and endorse the content of the work. All authors contributed to the article and approved the submitted version.

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Conflict of interest

DF, MJ, SW, DB, C-YC, and KG were employed by Hill's Pet Nutrition, Inc. GF and JB are consultants for 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.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2022.1039032/full#supplementary-material

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The impact of curcumin on livestock and poultry animal's performance and management of insect pests

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Plant-based natural products are alternative to antibiotics that can be employed as growth promoters in livestock and poultry production and attractive alternatives to synthetic chemical insecticides for insect pest management. Curcumin is a natural polyphenol compound from the rhizomes of turmeric (*Curcuma spp.*) and has been suggested to have a number of therapeutic benefits in the treatment of human diseases. It is also credited for its nutritional and pesticide properties improving livestock and poultry production performances and controlling insect pests. Recent studies reported that curcumin is an excellent feed additive contributing to poultry and livestock animal growth and disease resistance. Also, they detailed the curcumin's growth-inhibiting and insecticidal activity for reducing agricultural insect pests and insect vector-borne human diseases. This review aims to highlight the role of curcumin in increasing the growth and development of poultry and livestock animals and in controlling insect pests. We also discuss the challenges and knowledge gaps concerning curcumin use and commercialization as a feed additive and insect repellent.

KEYWORDS

curcumin, feed additive, insects, plant-based natural products, livestock and poultry animals

1. Introduction

Development of animal performance without disease is great prominence in increasing livestock and poultry production. Relying on antibiotics to enhance the quantity and quality of meat of food animals has been widely depended upon for a long time. However, scientists claim that conflict over antibiotic residues and resistance have also emerged as an outcome of the overuse of antibiotics (1, 2). In this context, finding alternatives to antibiotics to improve animal performance by promoting gut health has gained interest. Using plant-derived natural products and their analogs instead of antibiotics for improving animal performance and wellbeing are widely accepted eco-friendly strategies. It is also an effective alternative to synthetic chemical insecticides for insect pest management. Previously, plant-derived natural products were used in animal nutrition for their numerous beneficial effects such as antimicrobial activity, ability to promote gut health of the animals, flavoring agents in feed due to their aromatic value, etc. (1, 2). A humongous number of research reveals the efficacy of plant products, especially the productive reuse of "waste" parts such as citrus peels as feed for livestock, which can also be considered

a strategy to recycle the peels (3). Another approach is to isolate the natural substances from plants and their by-products used as traditional medicines in the past (4), including flavonoids, polyphenols, anthocyanins, etc. (1, 5, 6).

Curcumin, also known as diferuloylmethane ($C_{21}H_{20}O_6$), is a hydrophobic polyphenolic phytocompound present in the rhizomes of the turmeric (*Curcuma. spp*) belonging to the family of Zingiberaceae which is commonly found in Asian countries. It is the major constituent of turmeric powder, widely used as a culinary spice and traditional drug. Turmeric isolated from the rhizomes of the plant *C. longa*, has also been very well-known for its medicinal benefits for decades. Curcumin is the active ingredient of turmeric that owes its yellow color (4). Turmeric consists of 60–70% carbohydrates, 6–8% proteins, 5–10% fat, 3–7% minerals, and 6–13% moisture (7). Although, 3–5% of curcuminoids, include more than 50 structurally related compounds. Three main compounds include, curcumin, demethoxycurcumin, and bisdemethoxycurcumin (8). Curcumin is biosynthesized from two molecules of feruloyl-CoA and one molecule of malonyl-CoA *via* two enzymatic conversions, catalyzed by DIKETIDE-CoA SYNTHASE (DCS) and CURCUMIN SYNTHASE (CURS). Both DCS and CURS belong to the type III polyketide synthase family (9–11). Curcumin is reported to have effects as an antioxidant and anti-inflammatory agent (12–14). As a consequence, it has been used to treat oxidative and inflammatory disorders, metabolic syndrome, arthritis, anxiety, hyperlipidemia, cancers (i.e., Lung cancer, bladder cancer, and breast cancer), and neurological disorders (12, 15–20). Curcumin also possesses nutritional and insecticide properties improving poultry and livestock animal's production performances and broad-spectrum activity against insects that damage the agricultural crops and can transfer diseases to human.

Curcumin is recently being increasingly preferred by animal nutritionists as an alternative to chemical additives such as chemotherapeutic drugs and antibiotics in animal feed because animal production industries are under pressure to improve the animal production performances, decrease the economic losses, and confirming the safety of products for human consumption (21, 22). The usage of curcumin has made remarkable advancements in a wide range of nutritional aspects across growth, reproductive capacity, digestibility, stress response, immune functions, and histopathology in different age stages of monogastric animals such as pigs, poultry, and fish (23). Curcumin supplementation reduces absolute and abdominal fat weights by regulating lipid metabolism in broiler chickens (24). The dietary addition of curcumin improve meat quality, alleviate oxidative stress, and reduce fat deposition in pigs as well (25). Curcumin also owns broad-spectrum activity against insect pests. Arthropod vectors are responsible for driving and spreading the diseases such as malaria, dengue, chikungunya to human (26) and also for causing significant damage to agricultural crops (27). The climate changes may have impact on insect mutation (25, 26). But, the prevalent use of chemical insecticides on large populations of insects over space and time quickly demonstrated the direct relevance of the mutation process to insect control with the rapid development of insecticide resistance. Also, Insects very quickly evolved heritable, stable, qualitative or quantitative changes in their genomes that rendered many chemicals largely ineffective. In this context, the environment friendly approach to insect management include the use of natural compound based botanical insecticides (28). The bioactive compounds in the form of essential oil from turmeric extracts have insecticidal properties, with curcumin being the most active chemical and acting as a natural insecticide (29). The larvicidal activity of curcumin make them appropriate environment friendly vector control agent (30). Curcumin was reported to induce autophagic cell death in *Spodoptera frugiperda* cells *in vitro*, reported the first cytotoxic effect of curcumin on insect cells (31). Similarly, curcumin and its derivatives (demethoxycurcumin, curcumin-BF2 complex, and a monocarbonyl tetramethoxy curcumin) exhibited larvicidal activities in *Culex pipiens* and *Aedes albopictus* mosquitoes vectors even though no specific structure-activity relationship was clear enough to describe the effect of curcumin (32).

Despite the overwhelming therapeutic research on curcumin, little work has been done to describe curcumin's role in improving poultry and livestock production and controlling insect pests. This review mainly discusses how curcumin plays a role in improving the life quality of poultry and livestock animals and managing insect pests. We also mention challenges and research gaps related to curcumin use and issues concerning commercialization as a feed additive and insect repellent.

2. Chemical structure and various benefits of curcumin

Curcumin is a symmetrical molecule with IUPAC name (1E,6E)-1,7-bis (4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione and a molecular weight of 368.38 (33). Curcumin has a structure with two phenols and potentially enolizable β-diketone moieties, which are conjugated by two allylic double bonds (34). Curcumin exists mostly in a hydrogen-bond-stabilized keto-enol state. In polar solvents, curcumin exists in diketo form, and in non-polar solvents, it exists in the enol form. In basic media, the enol form of curcumin dominates and acts as an electron donor, whereas in neutral and acidic media, the keto form of curcumin is dominated and acts as a proton donor. Curcumin is soluble in dichloromethane, chloroform, dimethyl sulfoxide, acetone, and ethyl acetate, but it is insoluble in water and other diethyl ethers. Curcumin possesses light sensitivity and is unstable in alkaline solutions (8). Along with curcumin, other naturally occurring curcuminoids, such as demethoxycurcumin (15%) and bisdemethoxycurcumin (5%), are also biologically active turmeric constituents with significant health benefits (35, 36). While curcumin is naturally derived, its derivatives are generally produced by a chemical reaction between aryl-aldehydes and acetylacetone (37). Benefits of using curcumin are summarized in Figure 1.

The anti-inflammatory and antioxidant properties of curcumin are extensively studied by researchers. Curcumin primarily modulates its anti-inflammatory activities by quenching the free radicals *via* NF-κB, TGF-β, and mitogen-activated protein kinase pathways and performs anti-oxidation behavior *via* the Nrf2 pathway (38, 39). In addition to the Nrf2 pathway, curcumin increase the antioxidant capacity by altering antioxidant enzymes (i.e., CAT, SOD1, and GPX1), along with other channel proteins and chaperones (Figure 2).

Curcumin and other curcuminoids are well-known for their functioning on stomach disorders with a remarkable impact on

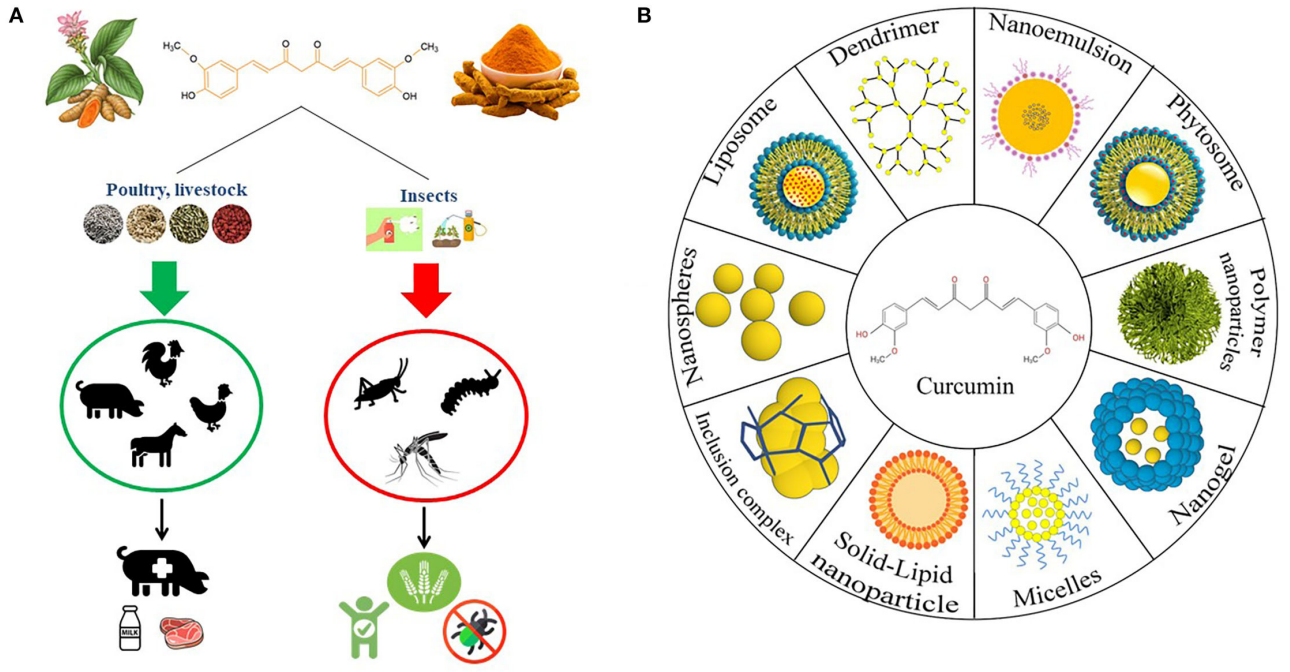

FIGURE 1
(A) Curcumin effects on livestock, poultry and insects when supplemented or used as a feed additive and insecticides. **(B)** The different types of curcumin nanoformulations.

healing various gastric-related difficulties, including ulcer healing (40). A large number of *in vitro*, *in vivo*, and *in silico* studies have shown that curcumin interactions at the molecular level in multiple signaling pathways (i.e., NF-κB, MAPK/ERK, and STAT) associated with cancers (41). Curcumin is a potential antimicrobial agent. It inhibits the activity of microorganisms such as bacteria, fungi, and viruses by targeting and eventually inactivating growth regulatory genes (42–45). Improving animal nutrition by enhancing the meat quality and increasing weight of the animals, as well as by accelerating their immune system as an initiative to remain disease-free in poultry and livestock animals is also an emerging aspect of curcumin research (46–48). Curcumin's effect on animals is not limited to enhancing growth and improving their physical health but also improves their reproductive health by increasing reproductive performance-related gene expression, which is also a crucial factor in increasing production. For instance, Jiang et al. (49) reported that dietary curcumin supplements resulted an increase in bcl-2 and decrease in caspase-3 gene expression, which in turn alleviate the testicular cell apoptosis in sheep production.

The insecticidal activity of curcumin is another important topic to be explored because curcumin may excellently contribute to the agricultural sector in crop protection and access to non-harmful insecticides from natural products. Curcumin has shown its insecticidal potential by inducing various growth inhibitory activities in insect pests (50, 51). Curcumin and its derivatives may be a possible solution to the protection of vector-borne diseases, particularly diseases spread by mosquitoes such as dengue fever and malaria, is an approach explored by several scientists by evaluating its efficacy *in vivo* (32, 52).

3. Curcumin nanoformulations

To overcome curcumin's limitations, such as low bioavailability, poor absorption, and rapid metabolism followed by systemic elimination, scientists have developed various strategies to administer curcumin in the form of a drug and to evaluate its efficacy. Many studies showed that curcumin nanoformulations can be a more effective therapeutic agent than curcumin itself both *in vitro* and *in vivo* (53, 54). Different curcumin nanoformulations are summarized in Figure 1. To begin with, a polymeric nanoparticle encapsulated formulation of curcumin, commonly known as polymeric nanoparticle-encapsulated curcumin ("nanocurcumin"), was reported to have therapeutic efficiency *in vitro* human pancreatic cell lines in a study by Bisht et al. They confirmed that the mechanistic specificity of curcumin remains unchanged in nanocurcumin while reducing the levels of pro-inflammatory cytokines (55). Liposomes are efficient drug delivery agents that enable the transportation of encapsulated compounds to their target sites while minimizing systemic toxicity (56). Nanogel formulations of curcumin are another mode of the highly advantageous delivery system.

Reeves et al. proposed that the curcumin nanogel formulations prepared by directly mixing the curcumin solution in DMSO with aqueous NG127 dispersion and simple post-treatment had excellent tumor cell killing capacity in MDA-231 breast cancer cell lines (57). Curcumin loaded in polymeric micelles is a promising approach to stabilize the compound and utilize it for further pharmacological studies (58). By taking into account the property of magnetic targeting of nanoparticles and Polyethylene glycol (PEG) conjugated drug, Ayubi et al. developed magnetic nanoparticles in which PEGylated curcumin (MNP@PEG-Cur) was used as a surface modifier as a drug delivery system. As a result of various

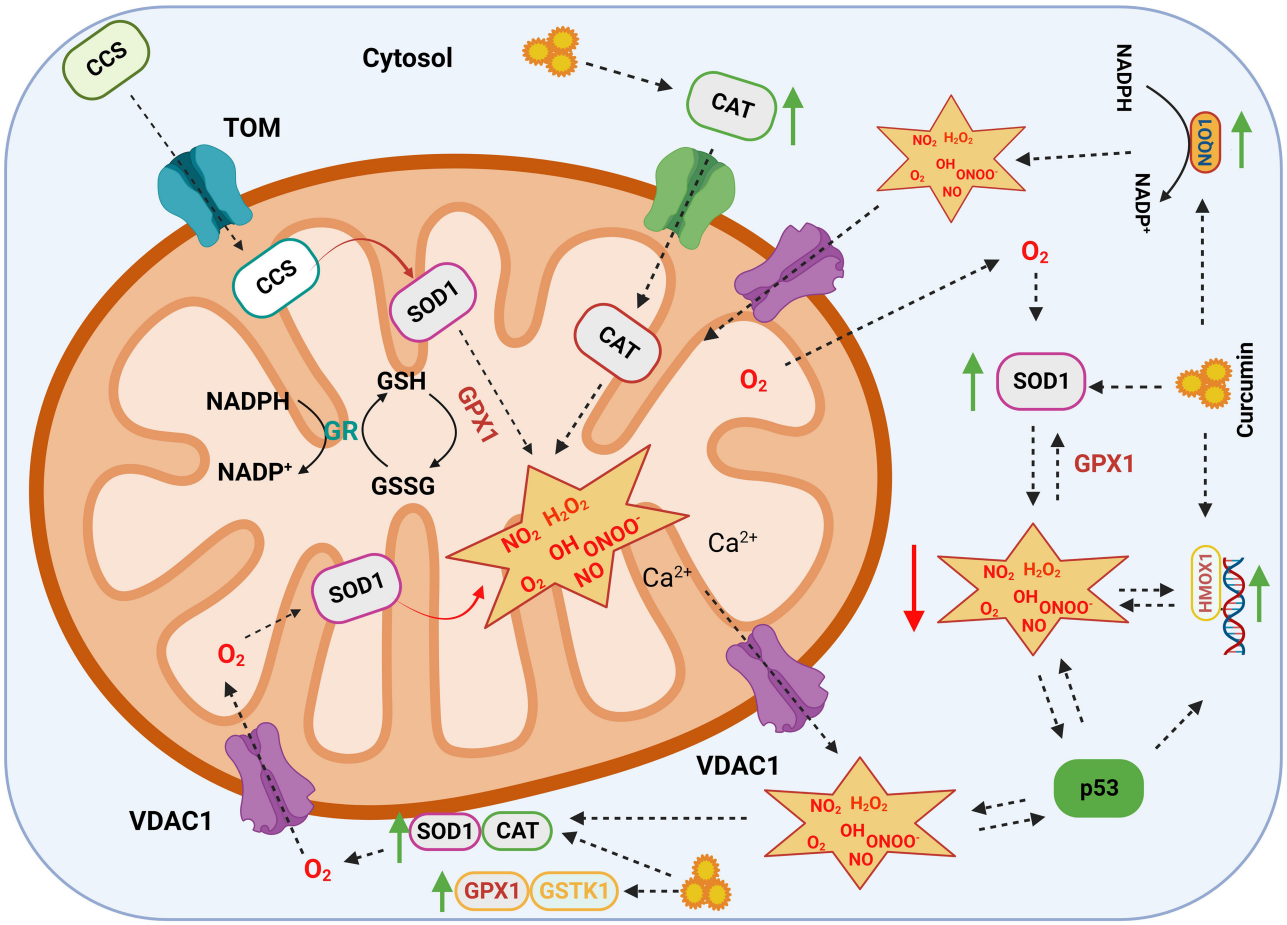

FIGURE 2
Antioxidant mechanism of curcumin. Schematic diagram summarizing a network of antioxidant enzymes regulating the formation of reactive oxygen species (ROS), which are involved in a number of cellular reactions. Curcumin upregulates antioxidant enzymes such as GPX1, GSTK1, SOD1, CAT, NQO1, and HMOX1. The ROS present in the cytoplasm are transported to the mitochondrion through gatekeeper proteins such as VDAC1 where they are scavenged by antioxidant enzymes. SOD1, superoxide dismutase; CAT, catalase; GPX1, glutathione peroxidase; GSTK1, glutathione-s-transferase kappa; HMOX1, heme oxygenase; NQO1, NAD (P) H quinone dehydrogenase; VDAC1, voltage-dependent anion channel; TP53, cellular tumor antigen p53; CCS, copper chaperone for SOD; GR, glutathione reductase; NO_2, nitrogen dioxide; H_2O_2, hydrogen peroxide; OH, hydroxyl radical; O_2, superoxide; NO, nitric oxide; $ONOO^-$, peroxynitrite. The green arrow represents upregulation and the red arrow represents downregulation.

assays and treating mice with different doses of the MNP@PEG-Cur, they concluded that MNP@PEG-Cur is a non-hemolytic, non-toxic material, and all mice were alive without any abnormal behavior (59). The stability and bioactivity of curcumin were reported to be maintained in polysaccharides-based complex particles (60). Kim et al. proved that the nanosphere loaded with curcumin blocks the mitochondrial apoptotic signaling pathway induced by the bacterium *Vibrio vulnificus* in human gastrointestinal epithelial HT-29 cells.

Similarly, nanospheres loaded with curcumin were determined to have cellular uptake by human gut epithelial HCT116 cells and mouse models of gut epithelial migration. It was observed in this study that these nanospheres significantly increased the motility of HCT116 cells and showed much higher migration efficacy than curcumin. Most importantly, nanospheres loaded with curcumin trigger PKC-dependent JNK phosphorylation and were found to regulate the transcriptional activation of NF-κB (61, 62). Curcumin-loaded dendrimers, which are highly branched with potential to manipulate and control separate components, make them suitable candidates for several biomedical applications (63). Similarly, nanoformulations of curcumin with metals and metal oxide particles are a progressing synthesis field of inorganic nanomaterials (64).

Gold nanoparticles are considered excellent drug carriers because of their high loading capacity, stability, and hydrophilicity of drugs (65). Muniyappan et al. synthesized curcumin-capped gold nanoparticles (CUR-AuNPs) through green synthesis. The CUR-AuNPs exhibited antioxidant, anti-inflammatory, and antibacterial properties effectively, which were confirmed with the help of treating the CUR-AuNPs on hydrogen peroxide, human red blood cells, and four different bacteria species, respectively (66). Recently, a study by Piwowarczyk et al. used a modified thin-film hydration method to embed curcumin and a mixture of other polyphenols in the liposomal nanoformulation to study its stability and anti-cancer potential (67). Despite the extensive researches on curcumin nanoformulation in biomedical research, no research has been performed about the effect of curcumin nanoformulation on livestock and animal production, with a few notable exceptions wherein curcumin nanospheres were supplemented in the diet of weaned piglets (23).

4. Curcumin's role in increasing livestock and poultry production performances

In recent years, livestock and poultry industries have mainly targeted the introduction of balanced diets to enhance the growth of animals and increase the resistance to diseases, over-all health conditions of animals, and environment-friendly production. In this view, plant-based natural feed additives improve livestock and poultry production performance, does not cause any side effects for animals, and ensure the safety of products for human consumption. Researchers suggest that plant-based natural products like curcumin are promising replacement additives to antibiotics (68). In this section, we discuss how curcumin contributes to improving livestock and poultry animal production.

4.1. The impact of curcumin on livestock performance

Researchers suggest that farmers prioritize the minimization of health issues in livestock. At the same time, farmers want the animals to be naturally healthy with their own individual characteristics (69). While taking into account this demand of farmers, as a matter of fact, natural compounds as a substitute for improving livestock health can be a practical step to provide a better safety profile while taking care of animal health (70).

4.1.1. Antimicrobial, antioxidant and anti-inflammatory effects

Curcumin reduces *E. coli*-induced generation of inflammatory mediators such as IL-1 while increasing antibody secretion at modest doses (300 mg/kg) (71). In addition, Gan et al. (72) showed that curcumin decreases copy number of pathogenic bacteria *E. coli* in the gut, downregulates TLR4 signaling pathways, adjust of interleukin levels, and elevates immunoglobulin levels in weaned piglets. These properties are all advantageous for piglet's growth and development during weaning when curcumin and resveratrol are ingested orally in the feed (68). *Besnoitia besnoiti* is a protozoan parasite causing a reduction in cattle's, reducing fertility and productivity and consequently, leading to economic losses. The antiparasitic efficacy of curcumin checked in *in vitro* bovine epithelial cell lines demonstrated that curcumin pretreatments of tachyzoites resulted in a dose-dependent reduction of host cell invasion and is safe and beneficial to be included in the cattle feed (73).

In Hu sheep (rare localized Chinese sheep breed), a dietary curcumin supplement was investigated, and it was found to promote lipid metabolism, antioxidant status, reproductive performance and improve immune ability by increasing the concentrations of IgA, IgM, and IgG in plasma (49). Meanwhile, nursing lambs were examined for body weight gain through stimulating creatine kinase activity and preventing fat decrease of ATP content (74). A whole transcriptomic *in vitro* study by Pauletto et al. validated that curcumin reduces AFB1-induced hepatic toxicity by triggering molecular pathways related to anti-inflammatory and antioxidant responses in cattle (75).

The well-developed and disease-free gut systems of animals play a vital role in maintaining good health. IUGR is a major crisis to be addressed to enhance good health in the livestock industry. Based on the antioxidative ability of curcumin on livestock animals, curcumin alleviated IUGR jejunum damage in pigs through Nrf2/Keap1 pathway while given as a feed additive in such a way that oxidative stress is reduced along with intestinal development (76, 77). Likewise, Li et al. reported that curcumin improved oxidative stress conditions in jejunum cell organelles and membranes in piglets (78). A similar study by Zhang et al. suggests that curcumin improves meat quality in pigs. This finding implies that curcumin may function as a natural antioxidant in IUGR offspring diet interventions to improve the meat quality and redox status of leg muscles (79). Shi et al. showed that piglet feed had more beneficial effects when curcumin was administered with piperine (80). Curcumin nanospheres supplemented in weaned piglet diets were reported to enhance growth, feed utilization, and immunity and reduce fecal pathogenic bacteria and ammonia gas emissions (23).

4.1.2. Intestinal health

In piglets infected with *E. coli*-induced intestinal injury, curcumin repairs, and improves the morphology of ileum epithelial mucosa (71). Curcumin and resveratrol enhanced the intestinal antioxidative capacity in weaning piglets by increasing the mRNA expression levels of tight junction proteins (68). Pauletto et al. suggested that curcumin help to mitigate jejunum injury by regulating the antioxidant capacity through the Nrf2/Keap1 pathway. This pathway enhanced the jejunum function by improving the immune function and jejunal tight junction in IUGR pigs (75).

4.1.3. Animal reproductive health

The reproductive health of livestock animals includes many aspects, such as a healthy gestation period, fetal health, milk production, etc. Dairy cows supplemented with certain phytonutrients (condensed tannins, encapsulated cinnamaldehyde, capsaicin, and piperine), including curcumin, have improved milk production and nutritional status in ¾ Holstein × ¼ Gir cows (81). Dietary curcumin improved the testosterone levels and testicular volume in Baladi bucks in the non-breeding season. Baladi bucks are goat bucks, seasonal breeders with very low fertilizing potentials, and other reproductive behaviors. Therefore, curcumin can be solution to overcome breeding inabilities in such a rarely reproducing dairy animal is a further opening to a more advanced level of research (82). The reported effects of curcumin in livestock animals are summarized in Table 1.

4.2. The impact of curcumin on poultry performance

Avian meat and other consumables are the major sources of nutrients for humans. Therefore, the use of antibiotics for poultry is increased to improve poultry production. Antibiotics may cause direct and indirect negative effects on animal and human health (83). Moreover, the massive use of antibiotics to promote growth may cause the development of antibiotic resistance in animals and affect the health of animals and consumers (84). Through

TABLE 1 The described effects of curcumin supplementation in livestock animals.

S. No.	Study	Effective dosage	Animal	Main findings	References
1	Effects of curcumin on growth performance, jejunal mucosal membrane integrity, morphology, and immune status in weaned piglets challenged with enterotoxigenic *Escherichia coli*	300–400 mg/kg	*Sus scrofa domesticus* (Pig)	Curcumin acts as an alternative for the antibiotic quinocetone in diets fed to weaned piglets by improving their health and growth status	(71)
2	Curcumin and resveratrol regulate intestinal bacteria and alleviate intestinal inflammation in weaned piglets	300 mg/kg	*Sus scrofa domesticus* (Pig)	Curcumin enhances intestinal immune function by regulating the piglet gut microbiota and decreasing intestinal inflammation *via* down-regulating TLR4 signaling pathway	(72)
3	Antiparasitic efficacy of curcumin against *Besnoitia besnoiti* tachyzoites *in vitro*	5.93 μM	*Bos taurus* (Cattle)	Curcumin reduced *Besnoitia besnoiti* tachyzoites viability with up to 56% mortality. Hence curcumin has anticoccidal activity *in vitro*	(73)
4	Curcumin supplement in summer diet on blood metabolites, antioxidant status, immune response, and testicular gene expression in Hu sheep	450 and 900 mg/sheep/day	*Ovis aries* (Hu sheep)	Dietary curcumin supplementation (450 and 900 mg/per sheep daily) can promote lipid metabolism, antioxidant capacity, and immune response as well as testicular development in Hu sheep	(49)
5	Diet supplemented with curcumin for nursing lambs improves animal growth, energetic metabolism, and performance of the antioxidant and immune systems	100–200 mg/kg	*Ovis aries* (Lamb)	Curcumin enhanced enzyme activity which then lead to anti-inflammatory action and weight gain in lambs	(74)
6	Productive and physiological responses of lactating dairy cows supplemented with phytogenic feed ingredients	15 g Actifor pro mix	*Bos Taurus taurus* × *Bos primigenius indicus* (Holstein × Gir cows)	Curcumin in the presence of other phytocompounds improved milk production and enhanced nutritional status	(81)
7	Curcumin mitigates AFB1-induced hepatic toxicity by triggering cattle antioxidant and anti-inflammatory pathways: A whole transcriptomic *in vitro* study	450 mg/kg in feed 10 μM in BFH12 cell lines	*Bos taurus* (Cattle)	Curcumin reduced AFB1 induced toxicity and decreased cells mortality by 30% in bovine fetal hepatocyte-derived cell line (BFH12)	(75)
8	Curcumin alleviates IUGR jejunum damage by increasing antioxidant capacity through Nrf2/Keap1 pathway in growing pigs	200 mg/kg	*Sus scrofa domesticus* (Pig)	Dietary curcumin reduced intrauterine growth retardation jejunum damage in pigs	(76)
9	Dietary supplemented curcumin improves meat quality and antioxidant status of intrauterine growth retardation growing pigs *via* Nrf2 signal pathway	200 mg/kg	*Sus scrofa domesticus* (Pig)	Curcumin served as a natural antioxidant and improved the meat quality, redox status, and growth performance	(79)
10	Effect of the single and combined use of curcumin and piperine on growth performance, intestinal barrier function, and antioxidant capacity of weaned Wuzhishan piglets	200 and 300 mg/kg	*Sus scrofa domesticus* (Wuzhishan Pig)	Curcumin improved intestinal permeability and reduced oxidative stress	(80)
11	Evaluation of dietary curcumin nanospheres in a weaned piglet model	0.5 and 1.0 ml solutions of curcumin nanospheres	*sus scrofa domesticus* [Duroc × (Yorkshire × Landrace)]	Curcumin nanospheres reduce fecal pathogenic bacteria, ammonia gas emissions in weaned piglets along with enhancing their growth, immunity and feed utilization	(23)
12	Supplemental dietary curcumin improves testicular themodynamics, testosterone levels, and semen quality in Baladi bucks in the non-breeding season	200 mg/kg	*Capra aegagrus hircus* (Goat)	Curcumin improved reproductive factors such as testosterone levels and testicular volume	(82)
13	Effects of curcumin on mitochondrial function, endoplasmic reticulum stress, and mitochondria-associated endoplasmic reticulum membranes in the jejunum of oxidative stress piglets	200 mg/kg	*Sus scrofa domesticus* (Pig)	Curcumin prevented mitochondria-associated endoplasmic reticulum (ER) membranes (MAMs) disorder in oxidative stress piglets	(78)

dietary formulation strategies, numerous natural compounds provide the ability to support the health of poultry while improving the nutritional quality of meat and eggs as well (1). So far, different natural compounds have been evaluated as feed additives in the poultry industry (85–87). Curcumin is one of those feed additives, considered to be an excellent non-toxic feed additive that improves

the immunity, growth performance, and behavioral patterns of poultry animals (88).

4.2.1. Heat and oxidative stress

Dietary curcumin was reported to prevent growth impairment induced by heat stress in broilers (89). It was observed that curcumin supplementation increased levels of various detoxifying enzymes (Glutathione peroxidase, glutathione S-transferase, and manganese superoxide dismutase) and decrease ($P < 0.05$) of heat shock protein 70 mRNA levels in the breast muscle. Similarly, it was also shown that curcumin activated the glutathione peroxidase and Nrf2-mediated phase II detoxifying enzyme systems simultaneously in poultry to scavenge reactive oxygen species, which caused oxidative stress (90). Likewise, curcumin increased the mitochondrial manganese superoxide dismutase gene expression and mitigated the hepatic mitochondrial dysfunction in heat-stressed broilers. Nawab et al. observed increased antioxidant enzyme expression levels, similar to Zhang et al. (90), in heat-stressed laying hens when administered with curcumin-supplemented feed (91). Curcumin exhibited positive responses on antioxidant capacity, lesion score, and oocyst shedding in a dietary treatment study conducted by Yadav et al. on broiler chickens (48). Due to high stocking densities under stressful conditions in broilers, the increase in the mRNA expression levels of insulin-like growth factor-1 (IGF-1), growth hormone receptor (GHR), myostatin (MSTN), and leptin in liver tissues enhancement in curcumin supplementation were noticed by Hafez and coworkers showed curcumin's oxidative stress-relieving ability and potential of increasing poultry growth performance and immune status (88).

4.2.2. Protective effect on poultry gut microbiome

The interplay of curcumin and the gut microbiota of poultry is an increasing area of research since curcumin has protective and antagonistic effects against both beneficial and harmful microbes, respectively (47). A solid dispersion form of curcumin and boric acid has a synergistic bactericidal impact against a bacterial poultry disease potentially causing foodborne illness in humans when consumed (92). Curcumin also has a protective effect against liver oxidative injury in ducks by lipid metabolism disruption by modulating intestinal microbiota (47). According to Yadav et al. (48) certain species of gut microbiota located in different sites play a major role in maintaining intestinal integrity in poultry. They found that *Eimeria* spp (parasites that cause coccidiosis in poultry) infected broilers had a leaky gut due to infection caused by the parasite infection. However, curcumin-fed birds had lower gut permeability at 100 mg/kg doses than other experimental group birds (48). The antimicrobial activity is another primary advantage in protecting the poultry from harmful disease-causing microbes *via* ingesting curcumin in the animal feed (93).

4.2.3. Egg and meat quality

Improving meat and egg quality contribute to economically promoting the poultry sector. Zhang et al. (94) showed that curcumin, as a potential antioxidant, improved meat quality and oxidant stability of muscle in broilers. In a similar study, feed-added curcumin was investigated to decrease the total cholesterol and fat content in the breast meat of broiler chicken (95). It is well-known that curcumin has a scavenging effect over oxidative stress because of its antioxidant capability, which also becomes advantageous when supplemented in poultry feed in several ways (96–98). Liu and coworkers shows that, on heat-stressed hens, curcumin had a favorable impact on laying performance and egg quality. Curcumin supplementation improves laying performance and egg quality by significantly increasing egg production, egg shell thickness, eggshell strength, and albumen height while decreasing the feed-to-egg ratio (99). Additionally, spraying curcumin in different concentrations on hatching eggs of dokki-4 chickens (a breed of chicken native to Egypt) was examined after exposure to thermal stress. Best hatchability rates and other productive traits were improved in eggs sprayed with curcumin compared to the non-sprayed eggs (100). The addition of curcumin to broiler diets significantly improved the levels of monounsaturated fatty acids (MUFAs) and polyunsaturated fatty acids (PUFAs) in breast and thigh muscles (101). Similarly, a recent study showed that egg production in broilers benefited from improved oxidative stability and egg shell breaking force when a phytonutrient solution of *Curcuma* was supplemented in laying hens (102). Curcumin supplementation in duck feed improved the meat quality by limiting the extent of lipid oxidation during the post-mortem period (103).

4.2.4. Detoxifying effects

Aflatoxins (AFB1) are toxic metabolites that reduce productivity and reproductive performance, eventually leading to organ malfunctions (104). Curcumin's detoxifying effect against aflatoxins in poultry is a well-studied topic that has cited various protective mechanisms of the same (105, 106). In an *in vivo* exposure model study carried out upon arbor acres broiler, curcumin successfully inhibited a cytochrome P450 enzyme that mediated the bioactivation of AFB1 (107). AFB-1-induced toxicity was reported to be reduced by curcumin in combination with another cellulosic polymer in broiler chicken (108). AFB1-induced spleen damage was regulated by curcumin by activating the Nrf2 signaling pathway, upregulating the expression of antioxidant enzymes, and inhibiting the NF-κB pathway in ducklings (109). Curcumin also initiate a reduction in the toxicity of AFB1 by activating the Nrf2-ARE signaling pathway and inhibiting the NF-κB signaling pathway in a study conducted on ducks that investigated acute ileum damage (110). Meanwhile, curcumin administration attenuated the renal oxidative stress parameters induced by AFB1 in broiler chickens. The stress parameters evaluated in this study included serum antioxidant capacity and enzymatic activity of kidney superoxide dismutase, catalase, and glutathione peroxidase, which were found to be downregulated (97).

On the other hand, curcumin can also protect against AFB1-induced necroptosis and inflammation by regulating TLR4/RIPK pathway in arbor acres broilers (111). Recently, Ruan et al. reported that curcumin modulated LPS-induced homeostatic imbalance by modulating gut microbiota through the BA-FXR pathway in chickens (112). Tang et al. observed curcumin's effect on arsenic-induced toxicity by growth inhibition, reduced hyaline degradation, and distortion in duck spleen, along with suppression of various pro-inflammatory cytokines and autophagy-related genes (113). In addition to aflatoxins, curcumin may protect ducks from ochratoxin, a mycotoxin that is difficult to remove from feed and

induces impairment of intestinal barrier function and mitochondrial integrity (114).

The protective effect of curcumin against ochratoxin-A-induced liver injuries in the duck was evaluated by Zhai et al. They found that curcumin supplementation relieved the decreased abundance of butyric acid-producing bacteria which were induced by ochratoxin-A (47). The above-mentioned studies suggest curcumin potentially increase meat quality, especially in broiler chickens, the most commonly consumed avian product. Curcumin effect in poultry animals is described in Table 2.

5. Curcumin's protective effect against insect pests

Class Insecta is the most species-rich animal category on the planet. Some insects may cause deleterious effects in to humans, poultry and livestock animals, and crops. The increasing rate of mutation in insects due to insecticide resistance, frequent use of insecticides and other various climatic and environmental factors has a bearing on many aspects of human health (115–117). Advancing the subject of how insects cause turmoil to animals, insects are vectors of many contagious parasitic diseases, especially insects that feed on blood from animals can have adverse effects on the wellbeing, behavior, and productivity of livestock animals (118). In addition, economic losses caused by these behavioral changes are high (119). Chemical insecticides are extensively used to control pest insects. The detrimental aspect of chemical insecticides include harmful effects in disturb human health and the environment (120). This negative effect drives a resurgence of interest in insecticides based on natural compounds because of their minimal costs and ecological side effects (121). Nevertheless, as discussed in earlier sections, natural polyphenols provide a promising source for insecticidal applications (28). Kindly note that curcumin is one such polyphenol (30–32). Researchers have explained the anti-insect effects and safety of curcumin to different insects. In the following sections, damage caused by insect pests in the agriculture sector and animal health and wellbeing is outlined and discussed, along with how curcumin plays a role in eradicating those destructive complications.

5.1. Vector mosquitoes

The *A. aegypti* and *A. albopictus* are the most common species responsible for the transmission of the deadly vector-borne virus disease, dengue (122). The research outcome from de Souza et al. (123) indicates that curcumin in sugar formulations is highly efficient, proving it to be a promising and safe alternative to control *A.aegypti* mosquitoes. This study evaluated curcumin's photolarvicidal and ovicidal activity in sucrose and D-mannitol. According to Raman microspectroscopy results, D-mannitol showed high permeability to the larva's peritrophic membrane, causing irreversible damage to the simple columnar epithelium of the digestive tube (123). Relatedly, curcumin formulated has photodynamic activity against *A.aegypti* larvae and yielded a water-soluble, non-toxic byproducts of curcumin as a result of photodegradation (124). In a larvicidal bioassay study with the integration of *in silico* molecular structural validations in *C.pipiens* and *A. albopictus*, curcumin derivatives appeared to have larvicidal effects. Among the tested compounds, four exhibited a mortality rate ≥10% in both *C. pipiens* and *A. albopictus* larvae, followed by evaluation of larvicidal activity using various concentrations for 24 h incubation (32). An *in-silico* study followed by *in vivo* and *in vitro* validations by the research group of Rao et al. revealed curcumin's molecular interplay in controlling *C. pipiens*. Rao et al. revealed in *C. pipiens* that curcumin increase the mortality in the mosquitoes vector at an early stage in its life cycle by Acetylcholine esterase 1 (AchE1) inhibition. *In vitro* and *in vivo* inhibition assays shows that AChE inhibitor pyridostigmine bromide and malathion were used as controls for examining the AChE inhibition by curcumin from the larvicidal extract. The authors also claim that curcumin induced larvicidal activity by employing competitive inhibition of AChE, hence curcumin may be a potential replacement for carbamates and organophosphates, popular AChE inhibitor (125).

5.2. Agricultural insect pests

Agricultural insect pests controlled by botanicals from *C. longa,* including essential oils, chemical constituents, and other extracts, are known to be studied for their role as "crop protectors" (126). Hemanta Chowdhury et al. (127) isolated and characterized turmeric components and derivatives, which were shown to have a modest insect growth inhibitory effect on *Schistocerca gregaria* (Forsk) and *Dysdercus koenigii* (Walk). These conclusions were made based on the inhibitory activities of the compounds in both insect species; however, they found that turmeric oil had a nymphal mortality rate of 60%, along with 10% abnormal growth compared to the test compounds. Therefore, turmeric oil was insecticidal rather than growth-inhibiting. They concluded that curcumin-I, its dibutyl derivative (7), and the benzene extract of turmeric rhizome powder, which contains both turmerones and curcuminoids, were the most active (127).

Tetranychus cinnabarinus or carmine spider mites are also catastrophic agricultural pests. Transcriptomics and functional enrichment analysis by Liu et al. (50) revealed 23 differentially expressed genes that were functionally identical or similar to the targets of insecticide/acaricides or genes that were associated with mite detoxification and metabolism. In particular, calmodulin, phospholipase A_2, and phospholipase C were activated upon curcumin treatment suggesting that the calcium channel related genes might play important roles in mite's response to curcumin (50).

Lepidopterans are one of the most widely studied agricultural pest categories partly because of their ability to absorb and digest nutrients rapidly. Along with the fact that environmental microorganisms easily enter the gut system of lepidopterans, enhancing their crop destruction capabilities (128). Veeran et al. (31) reported the cytotoxic effect of curcumin in insect cells for the first time, wherein curcumin induced autophagic cell death in a time and dose-dependent manner in a lepidopteran *S. frugiperda in vitro* cell lines. They observed that the autophagy induction effect of curcumin using various morphological assays and cell proliferation assays in Sf9 cells. In addition, autophagy-related proteins, ATG8-I, and ATG8-II expression levels were elevated after curcumin treatment. Curcumin and avermectin synergistic effects against *S.litura* tested at *in vitro* and *in vivo* condition. It revealed that programmed cell death was may be the possible synergistic mechanism of curcumin combined with

TABLE 2 The described effects of curcumin supplementation in poultry animals.

S. No.	Study	Effective dosage	Animal	Main findings	References
1	Dietary curcumin supplementation protects against heat-stress-impaired growth performance of broilers possibly through a mitochondrial pathway	100 and 200 mg/kg	*Gallus gallus domesticus* (Broiler chicken)	Curcumin prevented growth impairment due to heat stress by improving the antioxidant defense system and enhancing the mitochondrial biogenesis	(89)
2	Effect of various levels of dietary curcumin on meat quality and antioxidant profile of breast muscle in broilers	50 and 100 mg/kg	*Gallus gallus domesticus* (Broiler chicken)	Curcumin improved meat quality and muscle oxidant stability with its antioxidative properties	(94)
3	Curcumin successfully inhibited the computationally identified CYP2A6 enzyme-mediated bioactivation of Aflatoxin B1 in arbor acres broiler	450 mg/kg	*Gallus gallus domesticus* (Arbor Acres broiler)	Curcumin inhibited an enzyme associated with AFB1 bioactivation in arbor acres broilers	(107)
4	Dual role of dietary curcumin through attenuating AFB1-induced oxidative stress and liver injury *via* modulating liver Phase-I and Phase-II enzymes involved in AFB1 bioactivation and detoxification	450 mg/kg	*Gallus gallus domesticus* (Arbor Acres broiler)	Preventive actions of curcumin against AFB1-induced liver injury in broilers	(106)
5	Curcumin attenuates heat-stress-induced oxidant damage by simultaneous activation of GSH-related antioxidant enzymes and Nrf2-mediated phase II detoxifying enzyme systems in broiler chickens	100 and 200 mg/kg	*Gallus gallus domesticus* (Broiler chicken)	Curcumin positively modulated antioxidant enzyme GSH and antioxidant activity related pathway	(90)
6	Evaluation of a solid dispersion of curcumin with polyvinylpyrrolidone and boric acid against *Salmonella Enteritidis* infection and intestinal permeability in broiler chickens: a pilot study	0.05 and 0.1% CUR/PVP	*Gallus gallus domesticus* (Broiler chicken)	Curcumin possessed antimicrobial effects against *Salmonella Enteritidis,* a bacterial disease of poultry	(92)
7	Effect of dietary curcumin on the antioxidant status of laying hens under high-temperature conditions	200 mg/kg	*Gallus gallus domesticus* (Hen)	Curcumin increased expression levels of antioxidant enzymes in laying hens under heat stress	(91)
8	Evaluation of cellulosic polymers and curcumin to reduce Aflatoxin B1 toxic effects on performance, biochemical, and immunological parameters of broiler chickens	0.2 %	*Gallus gallus domesticus* (Broiler chicken)	Detoxifying effects of curcumin from AFB1 toxins in broilers	(108)
9	Effects of curcumin on performance, antioxidation, intestinal barrier and mitochondrial function in ducks fed corn contaminated with ochratoxin A	400 mg/kg	*Anas platyrhynchos domesticus* (Pekin ducks)	Curcumin reduced enterotoxicity caused by ochratoxin A, a naturally occurring food borne mycotoxin and various other intestinal health parameters	(114)
10	The effects of different doses of curcumin compound on growth performance, antioxidant status, and gut health of broiler chickens challenged with *Eimeria* species	200 mg/kg	*Gallus gallus domesticus* (Broiler chicken)	Curcumin had effective improvement against *Eimeria* infection	(48)
11	Protective effect of curcumin on ochratoxin A–induced liver oxidative injury in duck is mediated by modulating lipid metabolism and the intestinal microbiota	400 mg/kg	*Anas platyrhynchos domesticus* (Pekin ducks)	Curcumin modulated the cecum microbiota in ducks and lessened lipid metabolism and OTA-induced injury	(47)
12	Feed added curcumin with increased solubility on plasma lipoprotein, meat quality, and fat content in broiler chicks	0.2%	*Gallus gallus domesticus* (Broiler chicken)	Curcumin reduced total cholesterol and fat content of broiler chicken breast meat	(95)
13	Effect of curcumin on laying performance, egg quality, endocrine hormones, and immune activity in heat-stressed hens	150 mg/kg	*Gallus gallus domesticus* (Hen)	Curcumin supplementation in hen diet improved egg quality and laying performance	(99)
14	Evaluation of curcumin and copper acetate against *Salmonella Typhimurium* infection, intestinal permeability, and cecal microbiota composition in broiler chickens	1:9 ratio of curcumin in polyvinylpyrrolidone	*Gallus gallus domesticus* (Broiler chicken)	Curcumin displayed its antimicrobial effect and protect broiler chickens from hazardous microbes	(93)
15	dietary curcumin alleviated acute ileum damage of ducks (*Anas platyrhynchos*) induced by AFB1 through regulating Nrf2-ARE and NF-κB signaling pathways	500 mg/kg	*Anas platyrhynchos* (Duck/Mallard)	Antioxidation and anti-inflammatory activity of curcumin protected the ileum of ducks *via* activating Nrf2-ARE signaling pathway and inhibiting NF-κB signaling pathway	(110)

(Continued)

TABLE 2 (Continued)

S. No.	Study	Effective dosage	Animal	Main findings	References
16	Dietary curcumin improves energy metabolism, brain monoamines, carcass traits, muscle oxidative stability and fatty acid profile in heat-stressed broiler chickens	100 mg/kg	*Gallus gallus domesticus* (Broiler chicken)	Curcumin increased MUFA and PUFA content in breast and thigh muscles of broilers	(101)
17	Impact of treating hatching eggs with curcumin after exposure to thermal stress on embryonic development, hatchability, physiological body reactions, and hormonal profiles of Dokki-4 chickens	250 mg/litter	*Gallus gallus domesticus* (Dokki-4 chicken)	Curcumin treatments triggered better hatchability traits	(100)
18	Curcumin supplementation protects broiler chickens against the renal oxidative stress induced by the dietary exposure to low levels of Aflatoxin B1	400 mg/kg	*Gallus gallus domesticus* (Broiler chicken)	Curcumin diminished oxidative stress caused by AFB1 in chicken kidney	(97)
19	The impact of curcumin on growth performance, growth-related gene expression, oxidative stress, and immunological biomarkers in broiler chickens at different stocking densities	200 mg/kg	*Gallus gallus domesticus* (Broiler chicken)	Curcumin increased mRNA expression levels of IGF-1, GHR, MSTN	(88)
20	Curcumin alleviates LPS-induced intestinal homeostatic imbalance through reshaping gut microbiota structure and regulating group 3 innate lymphoid cells in chickens	300 mg/kg	*Gallus gallus domesticus* (Broiler chicken)	Curcumin modulated homeostatis imbalance in gut microbiota through BA-FXR pathway	(112)
21	The association of curcuma and Scutellaria plant extracts improves laying hen thermal tolerance and egg oxidative stability and quality under heat stress conditions	1:1 ratio of CUR and SCUT	*Gallus gallus domesticus* (Hen)	Curcuma supplementation improved oxidative stability and egg shell breaking force	(102)
22	Curcumin activates the Nrf2 pathway to alleviate AFB1-induced immunosuppression in the spleen of ducklings	400 mg/kg	*Anas platyrhynchos* (Ducklings)	Curcumin activated Nrf2 signaling pathway, upregulated the expression of antioxidant enzymes and inhibited NF-κB pathway in ducklings with AFB-1 induced spleen damage	(109)
23	Protective role of curcumin on aflatoxin B1-induced TLR4/RIPK pathway mediated-necroptosis and inflammation in chicken liver	300 mg/kg	*Gallus gallus domesticus* (Broiler chicken)	Curcumin reduced inflammatory cytokines levels, oxidative stress biomarkers, inflammation genes triggered by AFB1-induced necroptosis through TLR4/RIPK pathway	(111)
24	Curcumin antagonizes infammation and autophagy induced by arsenic trioxide through immune protection in duck spleen	400 mg/kg	*Anas platyrhynchos* (Sanshui white ducks)	Arsenic-induced toxicity was controlled by reduced hyaline degradation and distortion in duck spleen, suppression of various pro-inflammatory cytokines and autophagy-related genes	(113)

avermectin (129). Curcumin elevated the expression of xanthotoxin-induced detoxification genes by regulating ROS/CncC and signaling pathway genes in *S. Litura* (130). Interestingly, CnCC is the insect ortholog of the transcription factor, Nrf2, which has been mentioned in previous sections to have been studied to be regulated by curcumin (131). Curcumin's effect on insects is depicted in Figure 3 (123, 124, 128) and Table 3.

Agricultural plant parasite nematodes are microscopic roundworms that live in the soil and feed on plant roots. Many commercially important crops are susceptible to considerable size and quality losses due to plant-parasitic nematodes (138). Infectious disease in agricultural crops due to nematodes is also increasing (139). Curcumin's nematicidal effects have not been well-explored, but there is evidence that turmeric extracts are efficacious. In a study by Rashid et al. utilizing various fractions and concentrations of turmeric, they caused larvicidal mortality in the root-knot nematode *Meloidogyne incognita* (140). Turmeric extract using methanol as a solvent at a concentration of 20% was found to have the most efficacy in suppressing *Meloidogyne* spp (141). 0.5, 1, and 2% concentrations of turmeric extract were used in the pot experiment containing tomato plants with nematode eggs. Plants treated with turmeric extracts had the lowest number eggs in their roots. Mortality rate of second-stage juvenile eggs was higher in 2% concentration of the extract (132).

6. Model insects and organisms

Interestingly, model insects and organisms including *Drosophila melanogaster*, *Apis mellifera*, and *Caenorhabditis elegans* are used to test the curcumin's various beneficial effects. Curcumin was observed to extend lifespan of honeybee. Curcumin decreased global DNA methylation levels and increased the natural age-related level in older bees, along with being an effective natural bio-stimulator, improving apian health and vitality (132). Similarly, lifespan shortening in honeybees (*Apis mellifera*) due to ethanol intake was restored by

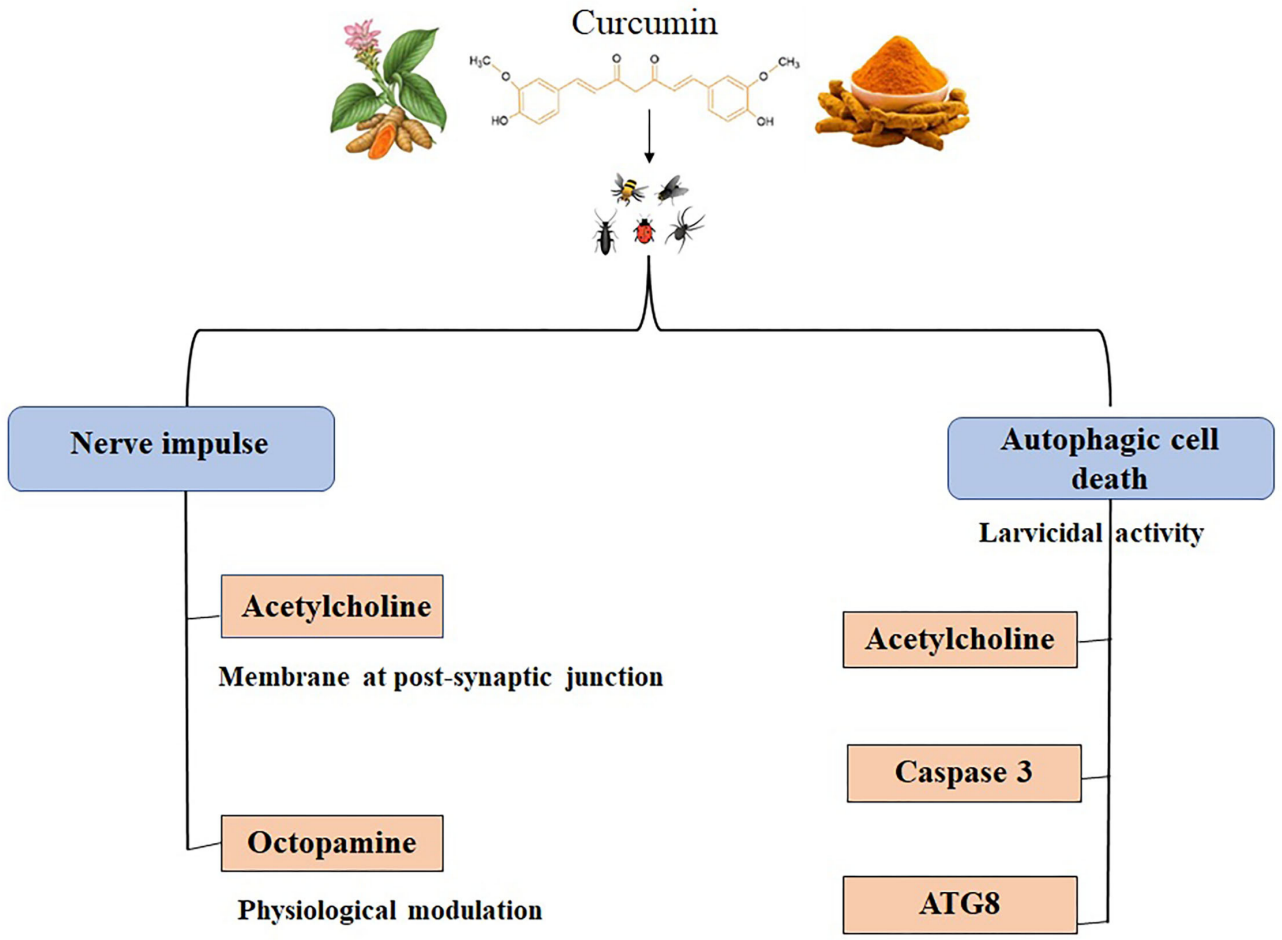

FIGURE 3
Insecticidal effects of curcumin. Acetylcholine and octopamine are the neurotransmitters in insects affected by curcumin, leading to blockage of nerve impulse. Acetylcholine, caspase 3, and ATG8 are targeted while curcumin induces autophagic cell death in insect larvae (123, 124, 128).

curcumin when co-administered with ethanol (136). Bees fed with 1:1 *v/v* sugar water with 100 ppm curcumin lowered viral loads by positively impacting the gut microbiome. Curcumin treatment lead to lower levels of *Gilliamella,* a honeybee bacterial gut symbiont (137). 0.2 mg/g curcumin diet fed Drosophila alleviated the increased oxidative stress caused by heat stress, by increasing the expression of SOD1, CAT, and PHGPx and decreasing the expression of Hsp70 and Hsp83 (133). Ten micrometer curcumin, in combination with another phytochemical thymoquinone had significant improvement in survival rate of *Drosophila* by improving locomotor functions in the adult flies (134). Aging negatively influences the circadian clock heme oxygenase is one of the enzymes under control of the circadian clock in Drosophila and mammals as well. One mg/ml medium of curcumin was fed with standard diet elevated *ho* mRNA levels (135). *Caenorhabditis elegans* is a model nematode that is optimal for quick and efficient analysis of gene function because of its evident simplicity, precise genetics, availability of whole genome sequence, and full molecular toolset (142). Therefore, *C. elegans* has been used extensively to study curcumin's expertise in several parameters. Curcumin and along with other analogs, increased lifespan, improved locomotive activity, reduced fat accumulation, modulation of oxidative stress resistance, protection from microbes, and lowering manifestation of different types of diseases (143–146). Effect of curcumin on model insects and organisms are depicted in Table 4.

7. A brief account of the safe dosage and toxicity of curcumin

Determining the safe and effective dosage of curcumin is a tedious task in livestock and poultry animals as well as insects. Looking at the aforementioned studies, we saw that researchers given different dosages of curcumin as a feed additive and observed the livestock and poultry performances. According to the research studies mentioned in previous sections, the optimal dose in weaned piglets ranged from 300 to 400 mg/kg (71, 72, 80), whereas in growing pigs, the optimal dose was found to be 200 mg/kg (76, 79). Similarly, researchers found that 100–200 mg/kg of curcumin for lamb and 450–900 mg /day for sheep were the best doses (49, 74). In poultry, under oxidative stress, the dosage was recommended to be 50–200 mg/kg, irrespective of age. In the case of heat stress, 50–200 mg/kg of curcumin has been shown to be optimal in many studies (88, 91, 101).

TABLE 3 Curcumin's protective effect against different insect pests.

S. No.	Study	Effective dosage	Insect	Main findings	References
1	Isolation, characterization and insect growth inhibitory activity of major turmeric constituents and their derivatives against *Schistocerca gregaria* (Forsk) and *Dysdercus koenigii* (Walk)	20 μg (*Schistocerca Gregaria*), 50 μg (*Dysdercus koenigii*)	*Schistocerca Gregaria* (Forsk), *Dysdercus koenigii* (Walk)	Insect growth inhibitory activity of curcumin-I against *Schistocerca gregaria* and *Dysdercus koenigii* nymphs	(127)
2	Curcumin induces autophagic cell death in *Spodoptera frugiperda* cells	5–15 μg/mL	*Spodoptera frugiperda* (Fall armyworm)	Curcumin induces autophagic cell death in *Spodoptera frugiperda* insect cell line	(31)
3	Curcumin in formulations against *Aedes aegypti*: mode of action, photolarvicidal and ovicidal activity	0.01 and 0.02 mg/l	*Aedes aegypti* (Yellow fever mosquitoes)	Photolarvicidal and ovicidal activity of curcumin by invading and injuring the intestinal epithelium of the larvae	(123)
4	Revealing the molecular interplay of curcumin as *Culex pipiens* Acetylcholine esterase 1 (AChE1) inhibitor	100 ppm	*Culex pipiens* (Northern house mosquitoes),	Curcumin inhibits AChE1 at an early life stage in *Culex pipiens* and induces mortality	(125)
5	Curcumin derivatives as potential mosquitoes larvicidal agents against two mosquitoes vectors, *Culex pipiens* and *Aedes albopictus*	6.0 ppm (*Culex pipiens*) and 9.2 (*Aedes albopictus*)	*Culex pipiens* (Northern house mosquitoes), *Aedes albopictus* (Asian tiger mosquitoes)	Curcumin, demethoxycurcumin, curcumin-BF2 complex and a monocarbonyl tetramethoxy curcumin derivative exhibited high larvicidal activity against *Culex pipiens* and *Aedes albopictus*	(32)
6	Synergistic effects of botanical curcumin-induced programmed cell death on the management of *Spodoptera litura* Fabricius with avermectin	10/1 μg/mL avermectin/curcumin mixed regent	*Spodoptera litura* Fabricius (Cutworm)	Curcumin has synergistic effects toward the pesticide avermectin by inducing programmed cell death	(129)
7	Environmentally safe photodynamic control of *aedes aegypti* using sunlight-activated synthetic curcumin: photodegradation, aquatic ecotoxicity, and field trial	4.2 mg/l	*Aedes aegypti* (Yellow fever mosquitoes)	Curcumin can act as an environment-friendly photosensitizer to control *A. aegypti* larval population by promoting oxidative storms *via* the photodynamic effect	(124)
8	Activation of the ROS/CncC and 20-hydroxyecdysone signaling pathways is associated with xanthotoxin-induced tolerance to λ-cyhalothrin in *Spodoptera litura*	0.2% in artificial diet	*Spodoptera litura* (Cutworm)	Curcumin is a CncC agonist and activated 20E signaling pathway	(130)

TABLE 4 Evaluation of curcumin's beneficial effects in the model insects and organisms.

S. No.	Study	Effective dosage	Insect	Main findings	References
1	Curcumin stimulates biochemical mechanisms of *Apis Mellifera* resistance and extends the apian life span	3 μg/ml	*Apis mellifera* (Western honey bee)	Curcumin increased apian life span, increased oxidation related protein levels and decreased DNA-methylation	(132)
2	Curcumin supplementation increases survival and lifespan in *Drosophila* under heat stress conditions	0.2 mg/g	*Drosophila melanogaster* (Fruit fly)	Curcumin fed drosophila were observed to have increased survival rates with enhance thermal tolerance	(133)
3	Developmental and behavioral toxicity induced by acrylamide exposure and amelioration using phytochemicals in *Drosophila melanogaster*	10 μM	*Drosophila melanogaster* (Fruit fly)	Curcumin along with another phytochemical have the potential in reducing acrylamide induced toxicity in *Drosophila melanogaster*	(134)
4	Regulation of heme oxygenase and its cross-talks with apoptosis and autophagy under different conditions in *Drosophila*	1 mg/mL (of the medium)	*Drosophila melanogaster* (Fruit fly)	Curcumin induced increased expression levels of heme oxygenase in drosophila brain	(135)
5	Screening bioactive food compounds in honey bees suggests curcumin blocks alcohol-induced damage to longevity and DNA methylation	100 μg/ml	*Apis mellifera* (Western honey bee)	Lifespan shortening caused by ethanol intake was restored when curcumin was co-administered through DNA methylation changes	(136)
6	Impacts of diverse natural products on honey bee viral loads and health	100 ppm	*Apis mellifera* (Western honey bee)	Curcumin fed bees showed low levels of *Gilliamella*, a honeybee bacterial symbiont	(137)

The recommended dosage of curcumin, while regulating intestinal microbiota or any other parasitic infection, was 200–400 mg/kg (47, 48). Meanwhile, 400–450 mg/kg of curcumin was found to be suitable for reducing mycotoxins like Aflatoxins (97, 106). Jin et al. (103) and Zhang et al. (94) suggested that 400–500 mg/kg of curcumin is best for improving meat quality in ducks, whereas 50–100 mg/kg of curcumin in broilers. In another study, 150 mg/kg of curcumin has a positive impact on increasing egg production (99). Curcumin's role in improving poultry and livestock production by enhancing their meat quality, egg-laying performance, and other productive traits has been demonstrated by various researchers. However, it should be noted that the beneficial dosage of curcumin still remains a debating issue. Since different studies have various experimental protocols and designs, it is difficult to conclude the beneficial and safe dose for practical usage. Standardizing the correct dosage regime of curcumin as feed additives for a particular function is the demand of the situation. Therefore, more research should be conducted in this direction. On the other hand, insect pest management studies conducted so far only examined curcumin's insecticidal activity; no studies investigated curcumin and their analogs side effects in plants, livestock, and poultry animals.

8. Conclusions and future prospects

Curcumin has been the subject of extensive investigation during the last few decades. Along with other biomedical applications, curcumin is an excellent nutraceutical candidate for animals and also a good insect repellent. Our aim with this review was to bring together the most recent available literature information and a thorough assessment based on the existing data. Livestock and poultry nutrition influence human health directly or indirectly since a significant part of the everyday meal comes from animals and their products. Insect vector-borne diseases and agricultural insect pests are getting increasingly worrisome, and the over use of chemical insecticides and unpredictable climatic changes are responsible for insecticide resistance in them. Therefore, it is necessary to control insect pests, especially using natural products such as curcumin, to avoid the hazardous effects of chemical insecticides. So, there will be a massive space of research opportunities in developing curcumin as a nutraceutical candidate and insect repellent.

Curcumin is proven to have many beneficial effects in maintaining livestock and poultry animal's health and performance. Also, it is evident that curcumin can be a viable alternative to antibiotics. However, we still do not fully understand which biochemical route or mechanism is targeted by curcumin. This uncertainty arises because there can be some other factors influencing the specific animal to show positive modifications regardless of the issue being studied. In this context, detailed studies needed to be employed on a molecular level after the administration of curcumin. Also, quantitative and qualitative analyses of curcumin in different tissues of animals are needed. Beneficial dose and toxicity in relation to practical applications are yet to be determined. Despite evidence that curcumin is a healthy feed additive to replace antibiotics for livestock and poultry, comparative research between curcumin antibiotics is still lacking. Taken together, we propose that large-scale evaluations are needed to suggest the precise and beneficial dose to livestock and poultry. Moreover, studying the impact of curcumin on digestion in livestock and poultry animals is an area worthy of future research. There are certain insect pests that disrupt the healthy wellbeing of livestock and poultry animals called ectoparasites. They cause major economic loss by interrupting the good productivity of animals, followed by many factors such as poor growth and reproductive performance. So, we put forward the idea of investigating more on these ectoparasites and whether curcumin and its modified formulations have specific effects on them. Regardless of the evidence that curcumin shows insecticidal activity in various insect vectors and agricultural pests through larvicidal, ovicidal, and other growth-inhibiting activities, the large-scale application of curcumin are still limited, largely because of a lack of a comprehensive understanding of their modes of action. No proper molecular authentication or conclusive structure-activity relationship on how curcumin causes these effects inside the insect's biological system. Hence, deciphering the genetic and molecular mechanism behind the interplay can open wide research opportunities in developing insecticides. As such, a better understanding of the effects of curcumin makeup on the host will enable us to fully utilize the curcumin substances for economically effective and sustainable insect pest management. Moreover, in agricultural pests, adequate toxicity evaluations of the crops are required.

Recent advances in genomics and bioinformatics have created several opportunities for investigating the physiological effect, toxicity, and protein composition of egg and poultry meat proteins and the safe use of curcumin as an animal feed additive. Also, researchers should prioritize expanding the application of genomics and bioinformatics tools to understand the curcumin interaction on the biological systems of livestock, poultry, and insects. It is a known fact that curcumin nanoformulations are more effective than free curcumin. However, almost no studies have been conducted using them to improve animal performance and control insects. To effectively promote curcumin nanoformulations as a feed additive or insect repellent, precise information regarding the beneficial dose and toxicity of the particular compounds against the host is required. Additionally, the comparison of information between the advantages of free curcumin and curcumin nanoformulations is also needed. To be approved for use in livestock and poultry, curcumin nanoformulations first undergo appropriate characterization and large-scale toxicity testing evaluation. Moreover, before proposing curcumin nanoformulations for practical applications, more detailed research should also concentrate on the long-term consequences of these formulations. Further, it is good to use it in curcumin alone or in combination with other synthetic or natural substances as a feed additive or insect repellent. So additional research should be conducted in this direction. The inclusion of curcumin supplements in livestock and poultry feed seems possible in the near future. Even though the use of curcumin to control insect pests is still in its infancy, encouraging results from recent studies are driving further investigations.

Author contributions

AS, TM, and AK: conceptualization. AS: literature collection and writing—original draft preparation. AS, ES, and AK: writing—review and editing. KN, GG, AK, TM, SK, and MM: suggestions and

comments. 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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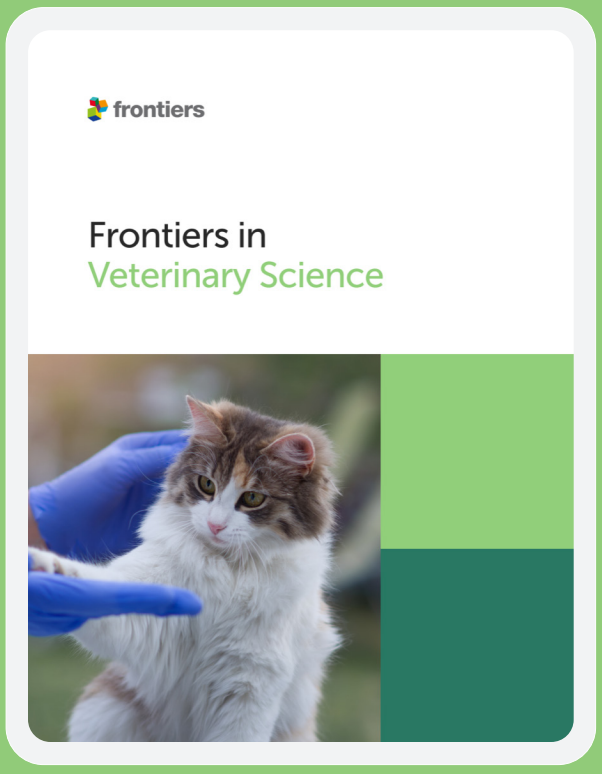

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