frontiers | Research Topics

Natural compounds/products and livestock productivity: enhancing antioxidant levels, gut health, mitigating greenhouse gas emissions, and disease control

Edited by
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Natural compounds/products and livestock productivity: enhancing antioxidant levels, gut health, mitigating greenhouse gas emissions, and disease control

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Editorial: Natural compounds/products and livestock productivity: enhancing antioxidant levels, gut health, mitigating greenhouse gas emissions, and disease control

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Editorial on the Research Topic
Natural compounds/products and livestock productivity: enhancing antioxidant levels, gut health, mitigating greenhouse gas emissions, and disease control

Growing concerns about antimicrobial resistance, drug residues in animal-derived foods, and the environmental consequences of conventional livestock production have prompted a global shift toward natural alternatives that are safe, multifunctional, and sustainable. Among these, plant-derived bio-actives, microbe-based products, and other natural compounds are emerging as valuable tools to enhance animal health, productivity, and resilience to disease. This shift is particularly relevant given the increasing need to phase out antibiotics as growth promoters and address the ecological pressures linked to intensive livestock systems. This Research Topic brings together 36 articles, comprising original articles, review and meta-analysis selected from 49 submissions, representing contributions from China (31 papers) and five other countries, Japan, Ethiopia, the United States, Korea, and Portugal. The papers span a wide range of livestock species, including ruminants (cattle, sheep, goats, yaks), monogastrics (broilers, layers, ducks, pigeons, geese), and pseudo-ruminants (donkeys), reflecting a global effort to explore natural solutions across diverse production systems.

A key strength of this Research Topic is its integrative perspective. Rather than focusing on single physiological outcomes, the studies address the multifunctional roles of natural compounds, from enhancing antioxidant defenses, modulating gut microbiota, and supporting disease resistance, to reducing methane emissions and improving product quality. This unified framework reflects a growing recognition that sustainable livestock production requires holistic interventions that promote animal health, environmental stewardship, and food safety. The studies can be broadly classified into five categories of natural compounds: including phytogenic compounds, including single-plant

extracts, multi-herbal formulations, and essential oils (e.g., *Artemisia annua*, theaflavins, *Pueraria* extract, *Broussonetia papyrifera*, *Ziziphus spina-christi*, *Gynura procumbens*, and oregano oil); phyto-microbial systems, which combine plant substrates with microbial fermentation to enhance bioactivity and digestibility (e.g., *Phragmites australis* silage improved with *Bacillus subtilis* and LAB); microbial-derived products, including functional bacteria, yeasts, and bacteriocins (e.g., *Heyndrickxia coagulans* SANK70258, *Rhodotorula mucilaginosa* ZTHY2, and Microcin J25); mineral and vitamin-based compounds, such as Fe-Gly, folic acid, taurine, and vitamin C, that improved metabolism, immunity, and tissue integrity; and oligosaccharides and polysaccharides, including chitosan oligosaccharides and *Lagenaria siceraria* polysaccharides, which play key roles in gut health and immune modulation.

The research themes explored in these papers span several functional domains, performance and antioxidant status (18 papers), immune modulation and gut health (16 papers), reproduction performance and embryo development (six papers), methane mitigation and environmental sustainability (three papers), carcass characteristics and animal product quality (10 papers), and feed ingredient innovation and nutritional strategies (11 papers). Together, these studies reported the potential of natural compounds as viable, regionally adaptable alternatives to synthetic drugs and additives. Many of these compounds are more accessible to smallholder farmers, who play a major role in livestock production across developing and transitional nations.

Key findings

Natural products or compounds as ingredients

One of the key future directions in animal nutrition is improving the utilization of unconventional feed ingredients, particularly low-cost, high-fiber resources, to reduce reliance on conventional feedstuff and reduce competition between livestock and human food systems. Integrating *Bacillus subtilis* (BNCC109047) with both homofermentative and heterofermentative lactic acid bacteria enhanced the fermentation quality and nutritional value of *Phragmites australis* (reed), a fibrous and affordable biomass (Liu, Zhang, et al.). This microbial synergy offers a promising strategy for improving the silage quality of lignocellulosic forages, especially in tropical regions where such resources are abundant during the dry season. In a complementary study, Zheng et al. showed that incorporating *Broussonetia papyrifera* silage into ruminant diets improved antioxidant capacity, enhanced mucosal immunity by increasing immunoglobulins and interleukins, and reduced the abundance of potentially pathogenic bacteria such as *Turicibacter* and *Romboutsia* in Kazakh sheep. Therefore, naturally fermented feeds may address feed shortages while promoting gut health and reducing antibiotic dependence.

Feed composition, including bioactive compounds can significantly influence milk quality. In a novel study, Zhou, Huang, et al. reported that fermented *Codonopsis pilosula* residue improved milk yield and metabolite profiles in lactating donkeys. The improvement was linked to immune activation, increased antioxidant capacity, and enhanced glucose and lipid metabolism. This suggest that bioactive residues from medicinal plants could offer dual benefits of improving animal performance while minimizing waste. Global demand for soybeans, largely produced in the Americas, has raised environmental concerns due to its intensive production and transport. While soybean remains an excellent protein source, more sustainable, low-cost alternatives are gaining attention. For example, a soybean-free, low-protein diet comprising rice, rice bran, rice co-product (RCC) meal, sweet potatoes, black tea powder, and tangerine peel powder reduced cost per kilogram of weight gain, increased lean meat yield, reduced cooking losses, and improved pork fatty acid profiles (notably increasing C22:6 and n−3 PUFAs) (Fu et al.). Additionally, this diet lowered the *Firmicutes/Bacteroidetes* ratio and increased the abundance of *Lactobacillus* spp in the colon. This illustrate the potential of unconventional feeds to improve meat quality, gut health and improvement in fiber degrading microbes. However, despite the appeal, long-term use of unconventional feed ingredients often raise questions about their safety, consistency, optimal dosage, and effects on microbiota, animal health, and product quality. Jack et al. (3) noted the nutritional benefits of microalgae like *Spirulina* in ruminants. Yet, the physiological differences between monogastric and ruminant require caution. Spínola et al. found that including *Spirulina* at up to 15% of the diet in broilers impaired weight gain, increased gut viscosity, enlarged digestive tract segments, and altered meat quality by reducing the n-6/n-3 ratio and α-tocopherol content. These effects may be due to their limited ability to digest microalgal cell walls, particularly at young age. Thus, while unconventional feed ingredients from fibrous forages and plant residues to microalgae offer great potential to reduce costs and improve animal health, careful consideration of species-specific responses, inclusion levels, and long-term effects remain essential. Continued research in microbial fermentation, bioactive compound profiling, and gut microbiome interactions will be key to unlocking their full value in modern animal production.

Natural products on stress and animal welfare

Stress is an unavoidable aspect of livestock production, triggered by physical exertion, transportation, extreme temperatures (especially heat), overcrowding, and intensive management. Many of these factors lead to oxidative stress at the cellular level, depleting antioxidant defenses, weakening immune function and increasing free radical production (1). Free radical levels can rise due to aging, infections, injuries, poor diet, or environmental disruptions, further amplifying oxidative stress. Transportation, whether from hatcheries to farms or farms to markets and slaughterhouses is a key contributor to physiological stress in animals. In yaks, for example, transport stress was linked to body weight loss and elevated plasma concentrations of lactate dehydrogenase, creatine kinase, malondialdehyde (MDA), cortisol, and lipopolysaccharides, all of which are biomarkers of oxidative and inflammatory responses. However, rumen-protected glucose (RPG) combined with taurine (TAU) supplementation minimized post-transport weight loss, improved antioxidant capacity, and

enhanced immune function, effectively alleviating stress responses in yaks (Wang, Zhao, et al.). In poultry, similar stress mitigation strategies are gaining attention. Qu et al. demonstrated that beyond classical antioxidants like selenium, zinc, and vitamins C and E, a compound herbal extract containing *Astragalus*, *Epimedium*, and *Fructus Ligustri Lucidi* (AEF) enhanced immune function and antioxidant status in goslings. AEF improved intestinal morphology (as seen in better villus height to crypt depth ratios), reduced the expression of inflammatory markers, and improved meat quality under peroxide-induced oxidative stress. This suggest broader applications of such compounds in other avian monogastric.

Beyond environmental stress, certain physiological stages, such as the perinatal period, place additional demand on high-yielding dairy cows. During this phase, bovine mammary epithelial cells (BMECs) experience accelerated metabolism and generate excess reactive oxygen species (ROS), contributing to oxidative damage and increasing susceptibility to mastitis, a prevalent and economically costly disease affecting milk yield and quality. Baicalin, a flavonoid derived from *Scutellaria baicalensis*, exhibited potent antioxidant, anti-inflammatory, and anti-apoptotic effects. It regulated oxidative stress-induced cell apoptosis in BMEC challenged with hydrogen peroxide (H_2O_2), offering a potential natural therapeutic alternative to manage periparturient oxidative stress (Kong et al.). In clinical mastitis, inflammation of the mammary gland is typically due to microbial infection or physical injury. Conventional management often involve minimizing pathogen exposure or injecting antibiotics directly into the udder, which carries the risk of residue accumulation (4). A promising alternative is the use of *Macleaya cordata* extract, which was reported to improve immune indices and reduce somatic cell count (SCC), serum amyloid A (SAA), and endotoxins in cows with mastitis, without negatively impacting milk production (Khattab et al.). Another critical stressor is weaning, particularly in neonatal piglets transitioning from milk to solid feed. This phase is often associated with diarrhea, inflammation, oxidative stress, and growth depression. Common interventions include zinc oxide and antibiotics, which face increasing regulatory restrictions due to environmental and resistance concerns. In this context, taurine, a naturally occurring amino sulfonic acid with antioxidant properties, has emerged as a promising alternative. It reduced diarrhea rates, enhanced intestinal barrier function, and alleviated oxidative and inflammatory damage in weaned piglets via activation of the Nrf2/HO-1 signaling pathway (Zhou, Wu et al.).

Natural products on Gut health and Immune functions

With the global ban on indiscriminate use of antibiotics as growth promoter in livestock production, producers and researchers are increasingly seeking safe, effective alternatives to maintain animal health and productivity. Gao Y. et al. emphasized that optimizing feed formulations and exploring novel, local, eco-friendly additives like *Atractylodes lancea* have become critical. These natural compounds show promise in improving performance, immunity, reducing heat stress, and supporting gut health. Gut-related issues in livestock are often linked to imbalances in the gut microbiota, weakened mucosal barriers, or inflammation. A key component of gut health is the function of Trefoil Factor Family (TFF) proteins, which help protect the gastrointestinal lining and regulate immune responses and it was recommended that feed additives promoting TFF protein synthesis could serve as natural alternatives to antibiotic growth promoters, helping to preserve gut integrity and resist infections (Fasina et al.). A recent study by Zhang Z. et al. demonstrated that polysaccharides extracted from *Lagenaria siceraria* (Molina) Standl. (LSP) helped regulate gut microbiota composition and increased the production of beneficial short-chain fatty acids. These changes contributed to improved intestinal immunity, better development of immune organs, and enhanced growth performance in broiler chickens. The small intestine, especially the jejunum, is the primary site for nutrient absorption in livestock, due to its high osmotic pressure (2). When its functions are compromised, animal health and growth can be severely affected. To prevent/manage this, Gang et al. found that water extracts of *Artemisia annua* improved immune function and antioxidant capacity in the small intestines of lambs, and also upregulated genes linked to mucosal health. One major challenge of indigenous pigs is their relatively slow growth, which often lead to increased reliance on antibiotics to prevent disease or as growth promoter. However, Wu H. et al. reported that *Pueraria* extracts offered a natural alternative by boosting beneficial microbes like *Bacillus acidi lactici* and *Saccharomycetes*, while reducing harmful *E. coli* and *Salmonella* in the jejunum. These shifts improved growth, feed efficiency, and immune responses, including higher immunoglobulin M and stronger intestinal barrier function.

While antibiotics have traditionally been added to livestock water or feed to support growth and health, increasing restrictions and concerns about resistance have prompted the exploration of natural alternatives. One promising example is fermented Shuanghuanglian, an herbal blend made from Honeysuckle flower, Baical skullcap root, and *Fructus forsythiae*. According to Xu Y. et al., adding 0.5% of this mixture to the drinking water of laying hens significantly improved their production performance, offering a natural way to support commercial poultry farming. Likewise, black tea-derived theaflavins (TF) enhanced laying performance, antioxidant capacity, yolk color, and lipid metabolism in hens (Zhou L. et al.), offering a natural way to improve egg quality. These improvements could reduce reliance on artificial yolk colorants and enhance overall egg quality in commercial operations. In broilers, ginseng stem-leaf extract has also shown promise. As reported by Zhang P. et al., supplementation improved growth performance and meat quality while enhancing antioxidant status, immune function, and cholesterol metabolism to varying degrees. Among ducks, low immunity remains a persistent challenge that contributes to high mortality rates. Addressing this, Ai et al. found that adding *Gynura procumbens* extract to duck feed significantly increased serum total antioxidant capacity, improved immune responses, and enhanced meat quality, offering hope for healthier, more productive duck farming.

Essential oils (EOs) are another natural tool with antimicrobial and performance-boosting benefits. Li et al. in their meta-analysis showed that EOs in calf diets improved milk production

and beta-hydroxybutyric acid levels without disrupting rumen function. Oregano essential oil (OEO), in particular, reduced excessive immune responses and intestinal damage in Holstein bulls (Xu M. et al.), suggesting a role in supporting gut health and immunity. For newborn calves with underdeveloped digestion, Zhang M. et al. found that a compound herbal extract (CHE) made from Honeysuckle, Astragalus, Magnolia bark, and Tangerine peel improved digestion, immunity, microbiota balance, and growth, without toxic effects.

Microbial

Heyndrickxia coagulans SANK70258, a Gram-positive bacterium, improved the growth performance of pigs, particularly during the lower-risk growing phase (Aida et al.). Microcin J25 (MccJ25), an antimicrobial peptide and a stable bacteriocin produced by *Escherichia coli* improved systemic metabolism, enhanced antioxidant defenses, strengthened intestinal barrier integrity and ultimately promotes pigeon health and survival (Cao et al.). This study support the application of MccJ25 as a functional feed additive in poultry production. Similarly, *Rhodotorula mucilaginosa* ZTHY2, a marine yeast strain, also improved the growth performance of ducks by enhancing immune function and antioxidant capacity. It upregulated serum components such as C3, C4, IgG, and cytokines (IFN-γ, IL-2, IL-4, IL-6, TNF-α), and modulated their gene expression in key immune organs (Wu J. et al.).

Natural products/compounds on sustainability

Methane reduction

Accurate methane emission data from ruminant production systems in Africa remain limited, primarily due to the high cost and limited availability of advanced measurement equipment such as Laser methane detector, GreenFeed, SF6, and respiratory chamber. Nevertheless, a consistent pattern emerging from reports is that ruminants in Africa tend to have high methane emission intensity, often attributed to the fibrous nature of native forages, widespread reliance on crop residues and agricultural by-products, and generally low product output (weight gain and milk yield). To address this challenge, Bature et al. reported on the potential of some locally available phytogenic feed additives, including trees, herbs, shrubs, and tannin-rich forages, as sustainable tools for reducing methane emissions in ruminants. In Ethiopia, Bekele et al. demonstrated that Menz sheep fed test diets containing *Acacia nilotica* and *Ziziphus spina-christi* leaves, either alone or in combination with brewery spent grains (BSG), exhibited a methane reduction of up to 67%. The methane-reducing effect was largely attributed to the condensed tannins present in these forages, which are known to modulate rumen fermentation and suppress methanogenesis. Similarly, in Korea, Bharanidharan et al. showed that when *Pharbitis nil* seeds were used in ruminant diets, there was up to 17.2% decrease in methane yield (g/kg OM), alongside a 7.6% reduction in urinary nitrogen excretion. In addition, the inclusion of *P. nil* increased metabolizable energy intake by 14.7% (as a percentage of gross energy intake) and reduced rumen protozoa populations, particularly *Entodinium caudatum*, by 40%, further supporting its anti-methanogenic and eco-friendly properties. These show the potential of locally available, plant-based feed additives as practical and cost-effective strategies for methane mitigation, particularly in low-input systems where conventional feed technologies may not be feasible. Apart from reduced emissions, this approach also offers benefits for nutrient utilization and productivity, contributing to both environmental sustainability and livestock development goals across diverse agro-ecological regions.

Nutrient excretion reduction

Excessive use of inorganic trace elements in livestock feed is common to meet nutritional needs, but it often leads to gastrointestinal stress and high levels of excretion, which can harm the environment. For piglets, iron injections are routinely given after birth, yet unused iron in the gut can be taken up by harmful microorganisms. Organic iron sources, such as iron glycinate (Fe-Gly), offer better digestibility and absorption. Supplementing piglet diets with 50 mg Fe-Gly has been shown to significantly improve feed intake, daily weight gain, and reduce diarrhea rates by 40%. It also enhanced iron status by increasing serum total iron-binding capacity, indicating more efficient iron transport. Fe-Gly helped protect against enterotoxigenic *Escherichia coli* (ETEC)-induced intestinal damage by improving jejunal morphology and regulating genes linked to gut health. This suggest that Fe-Gly not only meets the piglets' iron needs but also limit iron availability to pathogens like ETEC, supporting gut health under infectious stress (Gao Q. et al.). In finishing pigs, replacing all inorganic trace elements with up to 70% organic alternatives maintained performance, improved antioxidant capacity, reduced fecal excretion, enhanced gut health and microbiota diversity, and presented a more sustainable approach for modern pig production (Xu et al.).

Reproductive health

Reproduction remains one of the most sensitive and performance-defining processes in livestock systems. Recent studies have demonstrated that natural compounds and microbial derivatives can profoundly impact reproductive health and efficiency, particularly during critical windows such as late gestation, lactation, and early development. Maternal microbe-derived antioxidants (MA) supplementation increased litter size at weaning, improved milk quality (higher dry matter and fat), and reduced oxidative stress markers (Tang et al.). Furthermore, piglets born to these sows had better post-weaning growth and immunological resilience, emphasizing the maternal diet's programming effect. The antioxidant-rich MA compounds fermented mixtures involving *Rosa roxburghii*, sea buckthorn, and probiotics, enhanced redox balance and shifted gut microbial profiles in both dams and offspring. In a complementary approach, Zhao et al. evaluated a composite plant extract supplement,

ALAEm, during late gestation. Derived from nine traditional Chinese medicinal herbs, ALAEm improved live piglet births, placental vascular development, and reduced inflammatory markers, linked to upregulation of nutrient transport genes and antioxidant defenses in the placenta. These outcomes support the role of botanical extracts in reducing gestational stress and improving uteroplacental efficiency, a key bottleneck in sow productivity under antimicrobial-free systems. Similarly, folic acid improved folate status in eggs and offspring and modulated ovarian gene expression linked to reproductive competence in broiler breeders. These molecular effects, especially in Wnt signaling and steroid biosynthesis pathways, indicate folic acid regulatory role beyond basic nutrition. Du et al. explored *in ovo* vitamin C delivery in broilers, showing accelerated hatching, improved yolk absorption, and better hepatic metabolic indicators. Though studied in avian models, these results have relevance for prenatal antioxidant support in mammals under suboptimal conditions. Research evidence show that chitosan oligosaccharide (COS) can mitigate intrauterine growth restriction (IUGR), a persistent challenge in pig production. COS supplementation reduced stillbirth and mummified fetuses, and enhanced placental transporter expression and VEGFA signaling. These improvements translated into better gut function, redox status, and immune parameters in IUGR piglets, reinforcing the placenta-gut-health axis in neonatal survival and performance (Wang, Fang, et al.). The adoption of natural compounds such as MA, folic acid, COS, plant extracts, and vitamins is no longer an experimental novelty; they represent scalable, sustainable tools to enhance reproductive efficiency while reducing reliance on synthetic additives and antibiotics.

Others

Study by Liu, Liao, et al. on the physicochemical profiles of mixed rumen microbes (MRM), showed how surface tension (ST) and specific surface area (SSA) of rice straw-derived neutral detergent fiber (NDF) influence rumen microbial function and fermentation efficiency. The result showed those physical characteristics of the fermentation environment such as substrate structure and liquid surface tension, play a pivotal role in modulating microbial behavior, fermentation outcomes, and gut homeostasis. The study adds a novel dimension to our understanding of how physical feed properties and medium conditions can be manipulated to optimize rumen function, particularly in fiber-rich diets.

Conclusions

A promising future direction is the exploration of phyto-microbe synergy, harnessing the combined benefits of plant metabolites and microbial products to improve animal health and productivity while ensuring safety and sustainability. The botanical and microbial biodiversity found across tropical, arid, semi-arid, temperate, and subtropical zones offers an untapped reservoir of bioactives with localized benefits. However, further research is needed to identify active ingredients, determine effective dosages, and evaluate the long-term use of these compounds to ensure consistency and efficacy. This editorial collection marks a step forward in aligning livestock research with One Health and eco-efficient production goals, providing a scientific basis for the responsible use of natural compounds in livestock feeding systems.

Author contributions

MA: Conceptualization, Writing – original draft, Writing – review & editing. VP: Writing – review & editing. SS: Writing – review & editing. AS: Writing – review & editing.

Conflict of interest

The authors declare that the research was without any commercial or financial relationships that could be construed as a potential conflict of interest.

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Effect of oregano essential oil on intestinal immunoglobulin G in Holstein dairy bulls

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Introduction: Immunoglobulin G (IgG) is important in mediating humoral immunity and in the maintenance of immune homeostasis in the intestinal mucosa. Oregano essential oil (OEO) is a natural herbal extract that possesses antimicrobial, antioxidant, anti-inflammatory, and immunomodulatory properties. As the effects of OEO on intestinal mucosal immunity in Holstein dairy bulls remained unclear, we investigated the effect of dietary supplementation of OEO on IgG levels and IgG^+ cells residing in the intestinal tract in Holstein dairy bulls.

Methods: Twelve Holstein bulls in good health of approximately 10 months of age were selected for the experiment and randomly equally divided into two groups. The control (CK) group was fed a basal ration, and in the OEO group, the basal ration was supplemented with OEO (20 g/head/day). After 300 days of feeding, tissue samples of the jejunum, ileum, and colon of the bulls in each group were collected for histopathological analysis, immunohistochemistry, and enzyme-linked immunosorbent assays, respectively.

Results: The jejunum, ileum, and colon of bulls in the CK group had obvious pathological damage, whereas the structure of each intestinal segment was clear and intact. In the OEO group, pathological damage was significantly reduced. IgG^+ plasma cells were diffusely distributed in the lamina propria of the jejunum, ileum, and colon in the CK and OEO groups, with no significant difference between the groups. OEO supplementation significantly reduced the number of IgG+ plasma cells in each intestinal segment, with the highest decrease rate being noted for the ileum (22.87%), followed by the colon (19.45%) and jejunum (8.52%). ELISA test results and immunohistochemical results were mutually verified. The change in IgG content was consistent with the trend of change in the number of IgG^+ plasma cells.

Discussion: Our findings suggest that OEO supplementation does not alter the diffuse spatial distribution of IgG^+ plasma cells in the intestines of Holstein dairy bulls, but lowers immunoglobulin levels to normal levels, significantly reduces intestinal damage, and may enhance mucosal immune defence barrier function by inhibiting inflammatory reactions.

KEYWORDS

oregano essential oil, Holstein dairy bull, IgG, jejunum, ileum, colon, oregan

1 Introduction

The intestinal tract not only is a key site for digestion and nutrient absorption but also is considered as the largest immune organ. The intestinal mucosal immune system consists of mucosa associated lymphoid tissues (e.g., pai lymph nodes and mesenteric lymph nodes), effector lymphocytes dispersed between the lamina propria and intestinal epithelium, and corresponding effector molecules. The latter effectively monitor antigens passing through the intestinal tract, such as bacteria, viruses, and large proteins in food, to activate a mucosal immune response. Immunoglobulin IgA^+, IgG^+, and IgM^+ cells play an important role in maintaining intestinal mucosal immune system homeostasis by secreting immunoglobulins (1, 2).

Immunoglobulins are important effector molecules in the humoral immune response to pathogens. They kill pathogens and limit their proliferation through, for example, conditioning, antibody-dependent cell-mediated cytotoxicity, and complement activation (3). They are also involved in regulating and coordinating immune responses by binding to antigens and to Fc receptors on immune cells. Further, they initiate and regulate the inflammatory and cytotoxic effects of various immune cells (4). Immunoglobulins are classified as IgA, IgG, IgM, IgD, and IgE. IgG is the main class of immunoglobulins produced during the immune response to foreign antigens and effectively protects the body (5, 6). IgG has a longer half-life and is more abundant and more effective in protecting the immune system than the other classes (7). IgG plays an important role in disease prevention in animals (8, 9). IgG binds to antigens and to a wide range of cells, such as phagocytes, lymphocytes, and mast cells (10). In mammals, IgG is the only antibody capable of passing through the placenta, completing trans-mammalian intestinal epithelial and placental transport via the neonatal Fc receptor for effective neonatal protection (11). Bovine IgG prevents inflammatory reactions induced by gastrointestinal infections, upper respiratory infections, and lipopolysaccharides (12). In calves, IgG is not only very important for the prevention of diarrhoea (13), but also has been associated with hypertension (14), hypogammaglobulinaemia (15), and nephritis. In addition, IgG deposition is associated with the balance of helper T-cells (Th)1/2 cells (16). IgG levels are accurate markers of the extent of bacterial and viral infections, and high levels of IgG provide long-lasting immunity to the host. Therefore, studying IgG is an effective means for determining host immune defences.

Alternative feed additives can provide a nutritional strategy to effectively prevent metabolic disorders in ruminants by improving the metabolic and immune status of animals (17). Oregano essential oil (OEO) is a plant oil extracted from oregano. Its major components are carvacrol and thymol (18). In animals, OEO can promote growth, exerts antioxidant, antibacterial, and anti-inflammatory properties, and is a natural alternative to antibiotics. OEO can promote gastrointestinal tract development (19), and effectively reduces the incidence of mastitis and diarrhoea in Holstein cows and improves their health (20). Further, OEO can improve the growth and slaughter performance of Holstein bulls, improve their meat quality, and enhance their antioxidant capacity (21). Wang et al. (22, 23) showed that adding OEO to the feed increased IgG levels and promoted postpartum recovery in dairy cows. Jia et al. (24, 25) found that OEO supplementation reduced pro-inflammatory interleukin 6 (IL-6) serum levels in Mongolian goats and sheep, thus alleviating inflammatory responses. However, the effects of OEO on intestinal mucosal immunity in Holstein dairy bulls remain unclear. Calves, especially male calves, play an important role in the herd and ensuring the health and production performance of calves is vital for dairy farms (26). In intensive feeding, the immune system tends to be weakened, so the use of antibiotics to strengthen the immunity of cattle is very necessary (27). Therefore, we assessed the effects of OEO dietary supplementation on IgG expression and the number of IgG^+ plasma cells residing in the jejunum, ileum, and colon of Holstein bulls. Our aim was to provide evidence for further research on the mechanism of OEO, with the ultimate goal to improve the production performance of Holstein bulls.

2 Materials and methods

2.1 Test materials

Twelve healthy Holstein dairy bulls of approximately 10 months of age and with similar body weights (345.19 ± 3.89 kg) were obtained from Huarui Agricultural Company, Minle County, Zhangye City, Gansu Province, China. The animals were randomly equally divided into two groups. The control (CK) group was fed a basal ration [the nutrient composition of the basal diet is presented in Supplementary Table S1 (NRC, 2007)], and in the OEO group, OEO (Ralco Inc., Marshall, MN, United States) was added to the basal ration [20 g/head/day, based on previous research findings by our team (28)]. At the end of the 300-day feeding trial, the bulls were slaughtered after 24 h of fasting and 2 h of water fasting. Tissue samples were collected from the jejunum, ileum, and colon. Part of the samples was fixed by immersion in 4% formaldehyde solution, and the other part was stored at −80°C until analysis.

2.2 Haematoxylin-eosin staining

Paraffin sections (4 μm) were routinely prepared and deparaffinised to water. The sections were stained with haematoxylin for 6 min, rinsed with tap water for 30 min, subjected to hydrochloric acid alcohol differentiation for 10 s, and washed with tap water for 30 min for the colour to return to blue. Then, the sections were stained with eosin for 8 min and subjected to gradient alcohol dehydration and neutral gum sealing. Finally, the sections were scanned using a pathology slide scanner (Model No. DX1, Sruidi Medical Technology Co., Ltd., Shandong, China) and photographed to observe pathological changes in the jejunum, ileum, and colon.

2.3 Immunohistochemistry

The SABC immunohistochemical staining kit (Product No. SA1020) was used. Paraffin sections (4 μm) were routinely prepared and deparaffinised to water. They were subjected to antigen repair (citrate heat repair) for 10–15 min, cooled down to room temperature, and washed with distilled water. The sections were incubated in 3% H_2O_2 deionised water at room temperature to eliminate endogenous peroxidase activity and then washed three times with distilled water for 5 min each time. Drops of 5% normal goat serum blocking solution were added, and the sections were incubated at room temperature for 30 min to reduce non-specific binding. Primary antibodies IgG (Item No. ab6927, Abcam, United States) were added at a dilution of 1:600 (determined as the optimal working concentration among multiple dilutions tested), and the sections were incubated at 37°C for 1–2 h or at 4°C overnight (which had the best effect). As a negative blank control,

we used phosphate-buffered saline (PBS) instead of the primary antibodies. The sections were washed three times with PBS for 5 min each time. Then, we added biotin-labelled secondary antibody (goat anti-mouse/rabbit IgG) working solution (2 mg/1 mL), and the slides were incubated at 37°C for 30–40 min and then rinsed with PBS three times for 5 min each time. Horseradish peroxidase-labelled streptavidin working solution (2 mg/1 mL) was added dropwise, and freshly prepared diaminobenzidine chromogen solution was added for colour development. The degree of colour development was monitored under a microscope. Colour development was terminated by washing with water. The sections were washed with distilled water wash, stained with haematoxylin for 1 min, and washed with water again. After gradient alcohol dehydration, the sections were sealed with neutral gum. The immunohistochemically stained sections were scanned and photographed using a pathology slide scanner. For each section, IgG^+ cells in 10 random fields of view were counted, and densities were calculated using Image-ProPlus 6.0.

2.4 Enzyme-linked immunosorbent assay

Proteins were extracted using a whole protein extraction kit (BL521A, Solarbio technology Co., Ltd., Beijing, China). One gram of tissue sample (for samples, the fresh weight is required to be maintained, and the weight in the sample is not less than 50 mg, generally based on 1 g) was weighed and added to a lysis buffer (containing phosphatase inhibitor, protease inhibitor, and PMSF) on ice. The solution was mixed well and grounded with 2 mm magnetic beads until there were no obvious tissue pieces. The lysates were centrifuged at 4°C 12,000 × g for 30 min. The supernatants were aspirated into new centrifuge tubes and mixed again with 2 mm magnetic beads to ensure adequate grinding, and then centrifuged at 12,000 × g for 30 min at 4°C. The supernatants were aspirated into new centrifuge tubes. Protein contents were determined using a BCA protein concentration kit (BL521A). Finally, IgG^+ protein concentrations in the jejunum, ileum, and colon were determined in using a Bovine IgG Enzyme Immunoassay Kit (YJ330698; Shanghai Enzyme-linked Biotechnology Co., Ltd., Shanghai, China) in strict accordance with the manufacturer's instructions.

2.5 Statistical analysis

Statistical analyses were performed using IBM SPSS 26.0 software. The results are expressed as mean ± standard error. Means of two groups were compared using independent *t*-tests, and means of multiple groups were compared using one-way ANOVA. Differences were considered statistically significant at $p < 0.05$.

3 Results

3.1 Histological findings

Haematoxylin/eosin staining revealed inflammatory cell infiltration with varying degrees of intestinal villi detachment in some parts of the intestinal tract, as shown in Figures 1–3. The

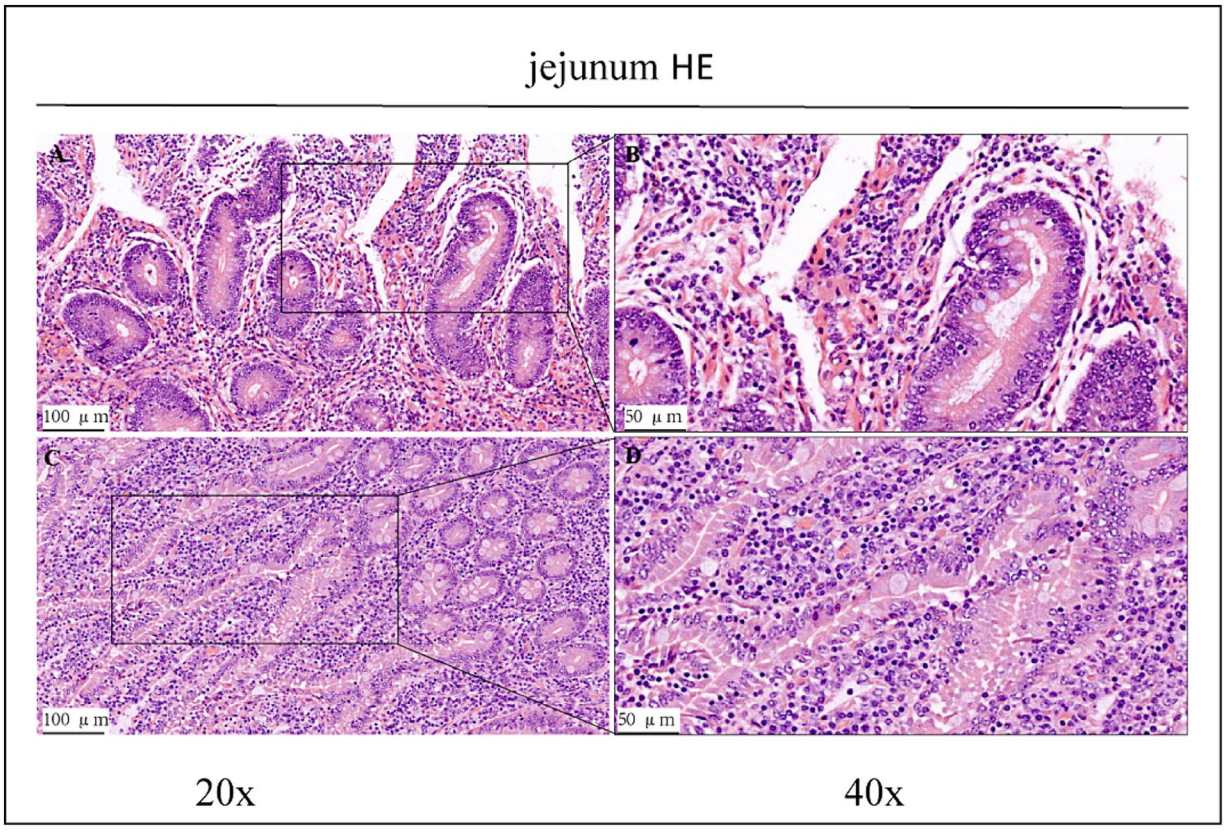

FIGURE 1
HE staining results of jejunum in CK group and OEO group (EO). **(A,B)** Are the CK group. **(C,D)** Are OEO group (EO). And the image on the right (scale, 50 μm) is a local magnification from the image on the left (scale, 100 μm).

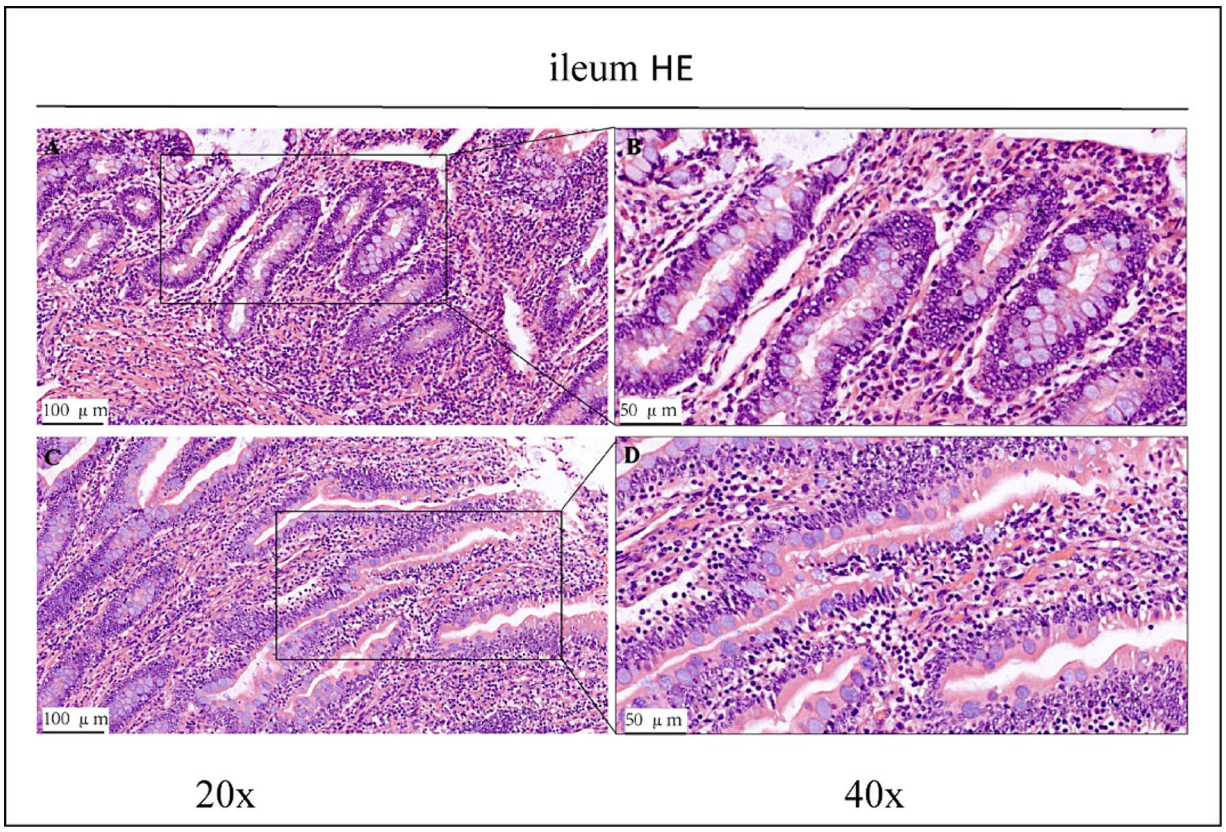

FIGURE 2
HE staining results of the ileum of control group (CK) and OEO group (EO). **(A,B)** Are the control group. **(C,D)** Are OEO group. And the image on the right (scale, 50 μm) is a local magnification from the image on the left (scale, 100 μm).

findings suggested that the experimental animals may have suffered from inflammation. Differences in inflammatory characteristics and the degree of inflammation between the CK and OEO groups could not be accurately determined, and further experimental verification is required.

3.2 Immunohistochemical findings

As shown in the microscopic images in Figure 2, secretory cells were clearly visible, and their shapes were largely oval. Their cytoplasm stained brownish-yellow, and the nuclei were located on one side, and wheel-shaped. IgG^+ cells were distributed in the jejunum, ileum, and colon, and their distribution was different in different intestinal segments. In the jejunum, they were mainly distributed in the lamina propria of the intestinal villi and of the basal part of the villi (Figure 4). In the ileum, IgG-secreting cells were mainly distributed in the lamina propria of the villi (Figure 5). In the colon, IgG-secreting cells were predominantly found in the lamina propria around the intestinal glands and a small amount was observed in the lamina propria of the intestinal villi (Figure 6). The cells were quite numerous and distributed diffusely throughout the intestine.

The patterns of change in IgG-secreting cells among the three intestinal segments were largely the same ($p > 0.05$) in the CK and OEO groups. The density of IgG^+ cells in all three intestinal segments was significantly reduced in the OEO group compared to the CK group (Figures 7A–C). As shown in the figure, there was no significant change in the cellular distribution of IgG^+ cells in the OEO group compared to the CK group. In the jejunum, the IgG^+ cell density was reduced by 8.52% in the OEO group compared to the CK group, in the ileum by 22.87%, and in the colon by 19.45% (Table 1). As shown in Figure 2, IgG secretory cells were significantly less distributed in the ileum than in the jejunum and colon in both the CK and EO groups, whereas the numbers of these secretory cells in the jejunum and colon were similar ($p > 0.05$) (Figure 7D). Thus, dietary supplementation of OEO may inhibit the inflammatory response in the intestine and lower the level of IgG^+ cells to normal.

3.3 Effect of OEO on IgG^+ expression levels in the jejunum, ileum, and colon

In enzyme-linked immunosorbent assays (ELISAs), serial dilutions of known standards are used to generate a standard curve, generally optical density versus concentration, from which the exact amount of the target in an unknown sample can be calculated. ELISA results showed that the expression level of

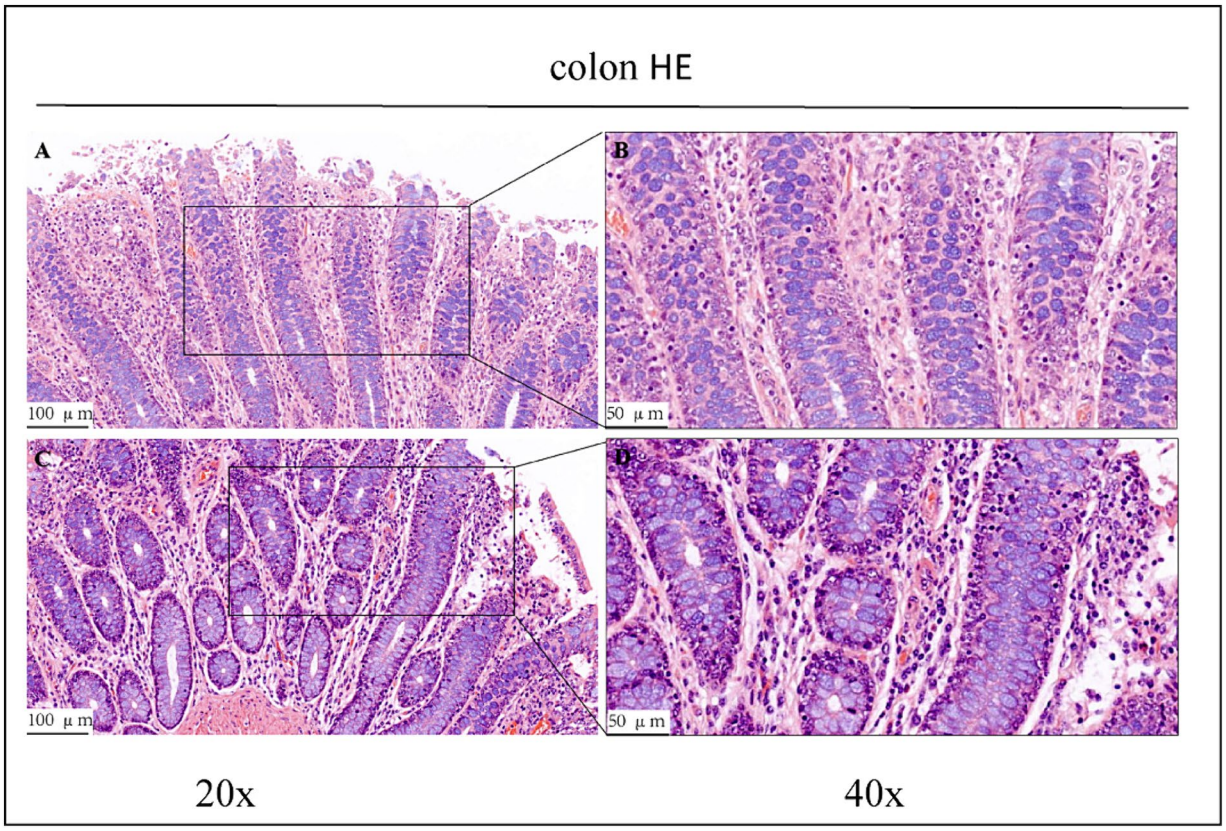

FIGURE 3
HE staining results of the colons of control group (CK) and OEO group (EO). **(A,B)** Are the control group. **(C,D)** Are oregano essential oil group. And the image on the right (scale, 50 μm) is a local magnification from the image on the left (scale, 100 μm).

IgG followed a similar trend as IgG^+ cell density (Table 2), and that OEO supplementation reduced IgG expression in the intestinal tract of Holstein dairy bulls (Figure 8). The differences in IgG^+ cells among the three intestinal segments did not correspond to the differences in expression, as the lowest expression was found in the jejunum.

4 Discussion

Histopathological analyses revealed varying degrees of damage to the jejunum, ileum, and colon of Holstein dairy bulls in the CK group (Figure 1). In intensive production, high-concentrate rations are often used to meet the nutritional needs of cattle for fattening and milk production and to achieve high productivity in the short term. In cows, high-concentrate diets are often associated with a large number of inflammatory cells infiltrating the mammary glands, suggesting that the feeding of high-concentrate diets induces inflammatory responses in the mammary glands of cows (29). Further, prolonged high-concentrate feeding can lead to acidosis and cause damage to the digestive tract epithelium of the host, thus affecting its digestive and immune capabilities and consequently, its performance (30–33). High-concentrate diets induce the activation and epigenetic modification of NF-κB and MAPK signalling pathways in mammary tissues of dairy cows, thus promoting pro-inflammatory cytokine gene expression, which ultimately leads to inflammatory responses in the mammary tissues (34). Therefore, we conclude that the Holstein dairy bulls in the CK group in this study had some intestinal damage due to long-term high-concentrate feeding.

IgG, as the most abundant immunoglobulin in serum, is the major anti-infection immune antibody. Therefore, it can be used as an important indicator for the diagnosis of inflammation. Immunohistochemical staining revealed the presence of a large number of IgG-secreting cells in the lamina propria of the intestinal mucosa of Holstein dairy bulls, suggesting that IgG plays an important role in protecting the intestinal mucosa. Both immunohistochemical staining and ELISA results showed that the addition of OEO significantly reduced IgG^+ plasma cell numbers and IgG expression levels in all intestinal segments. This may be because when inflammation occurs in the organism, the binding of antigens and antibodies triggers massive immunoglobulin production, leading to abnormally high immunoglobulin levels in the organism (35, 36). OEO is rich in phenolic substances that have anti-inflammatory properties and

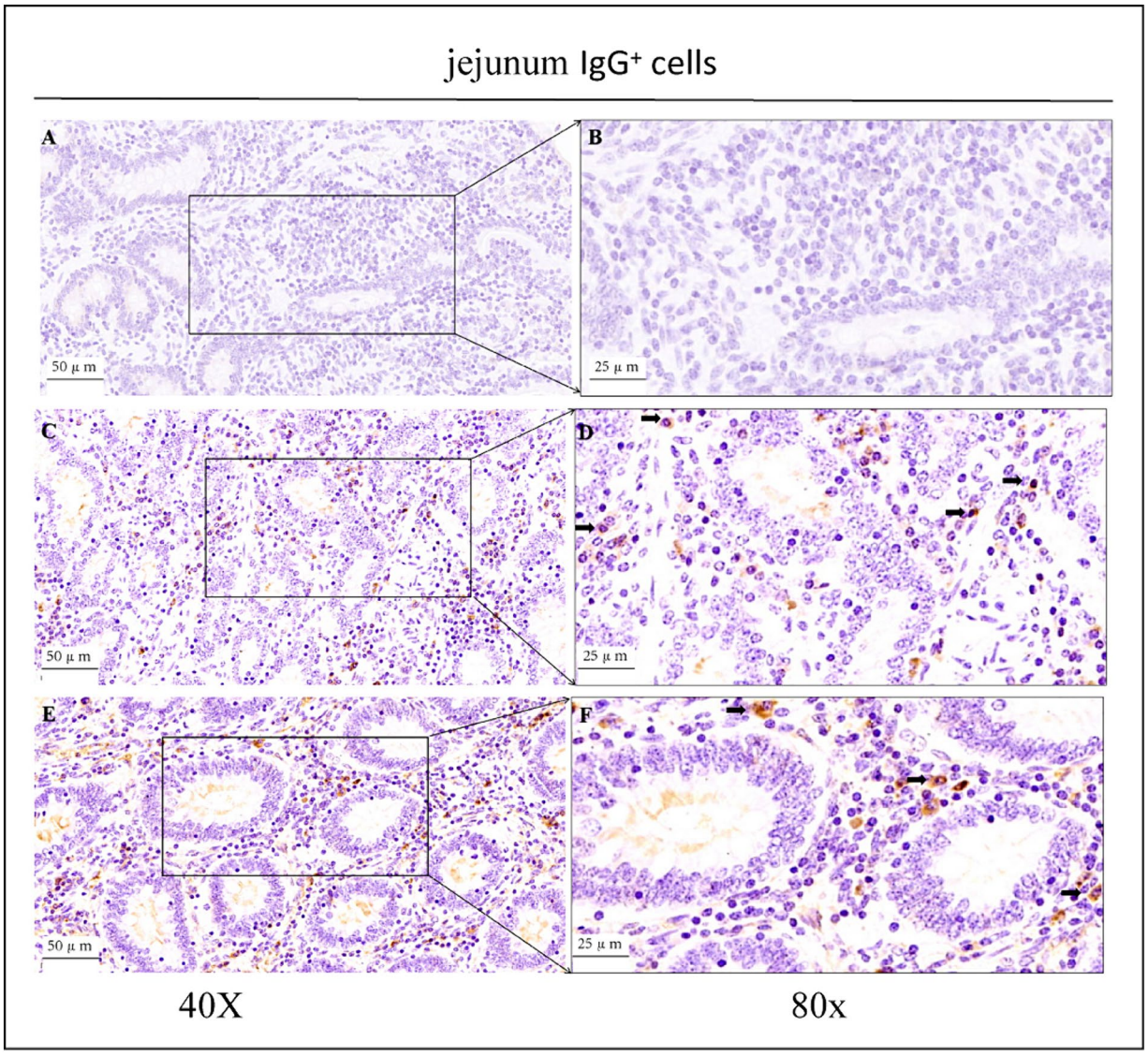

FIGURE 4
Distribution of IgG$^+$ cells in the jejunum of depopulated Holstein bulls. **(A,B)** Are negative controls. **(C,D)** Are controls. **(E,F)** Are OEO groups. Arrows indicate IgG$^+$ cells, and the right image (scale bar, 25 μm) is a magnification of the left image (scale bar, 50 μm).

reduce IgG secretion to normal levels by reducing IgG secretion to achieve an anti-inflammatory effect. These anti-inflammatory properties of OEO enable it to reduce the inflammatory response caused by damage to the intestinal mucosal barrier and to promote intestinal barrier function and mucosal barrier integrity. Essential oils have a protective effect on the intestinal mucus layer, reducing the exposure and adsorption of harmful substances. Plant essential oils can improve intestinal barrier function and reduce the degree of inflammation by regulating the expression levels of genes related to intestinal inflammation and other related gene (37). OEO exerts anti-inflammatory effects by regulating the expression of inflammatory factors such as IL-1β, IL-6, and tumour necrosis factor-α (38–40), which in turn reduce the risk of intestinal inflammation in Holstein dairy bulls. The level of intestinal IgG-secreting cells varies somewhat between species, providing a basis for different parts of the digestive tract to function to different degrees. The presence of numerous IgG-secreting cells in a particular site indicates a high capacity to prevent the invasion of toxic and harmful substances into the intestinal mucosal epithelium in that site (41).

In conclusion, dietary supplementation of OEO did not alter the spatial distribution of IgG$^+$ plasma cells in the jejunum, ileum, and colon of Holstein dairy bulls, but it did significantly reduce the number of IgG$^+$ plasma cells and IgG content in the three segments of the intestine. Our findings indicate that OEO can alleviate intestinal inflammation in Holstein dairy bulls and exerts anti-inflammatory action by reducing immunoglobulin

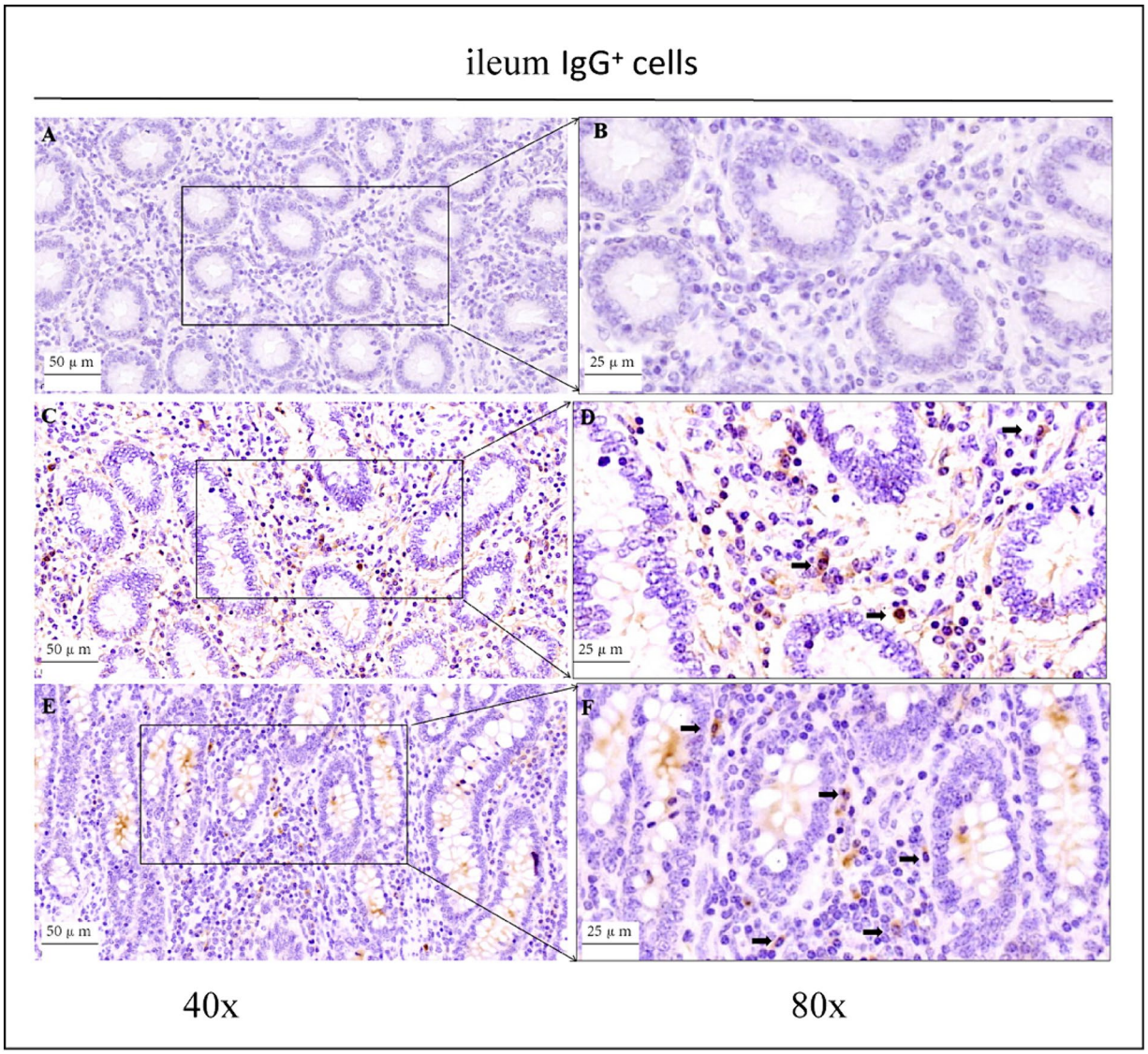

FIGURE 5
Distribution of IgG$^+$ cells in the ileum of depopulated Holstein bulls. **(A,B)** Are negative controls. **(C,D)** Are controls. **(E,F)** Are OEO groups. Arrows indicate IgG$^+$ cells, and the right image (scale bar, 25 μm) is a magnification of the left image (scale bar, 50 μm).

levels in the intestinal tissues to enhance body immunity. Therefore, OEO can be used as a feed additive in cattle breeding to promote host immunity, thus effectively enhancing production performance. The detailed mechanism underlying the regulation of IgG$^+$ plasma cell production by OEO requires further study.

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 approved by Animal Policy and Welfare Committee of Gansu Agricultural University (Ethic approval file No. GSAU-Eth-AST-2022-035). The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

MX: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Project administration, Software, Validation, Writing – original draft, Writing – review & editing.

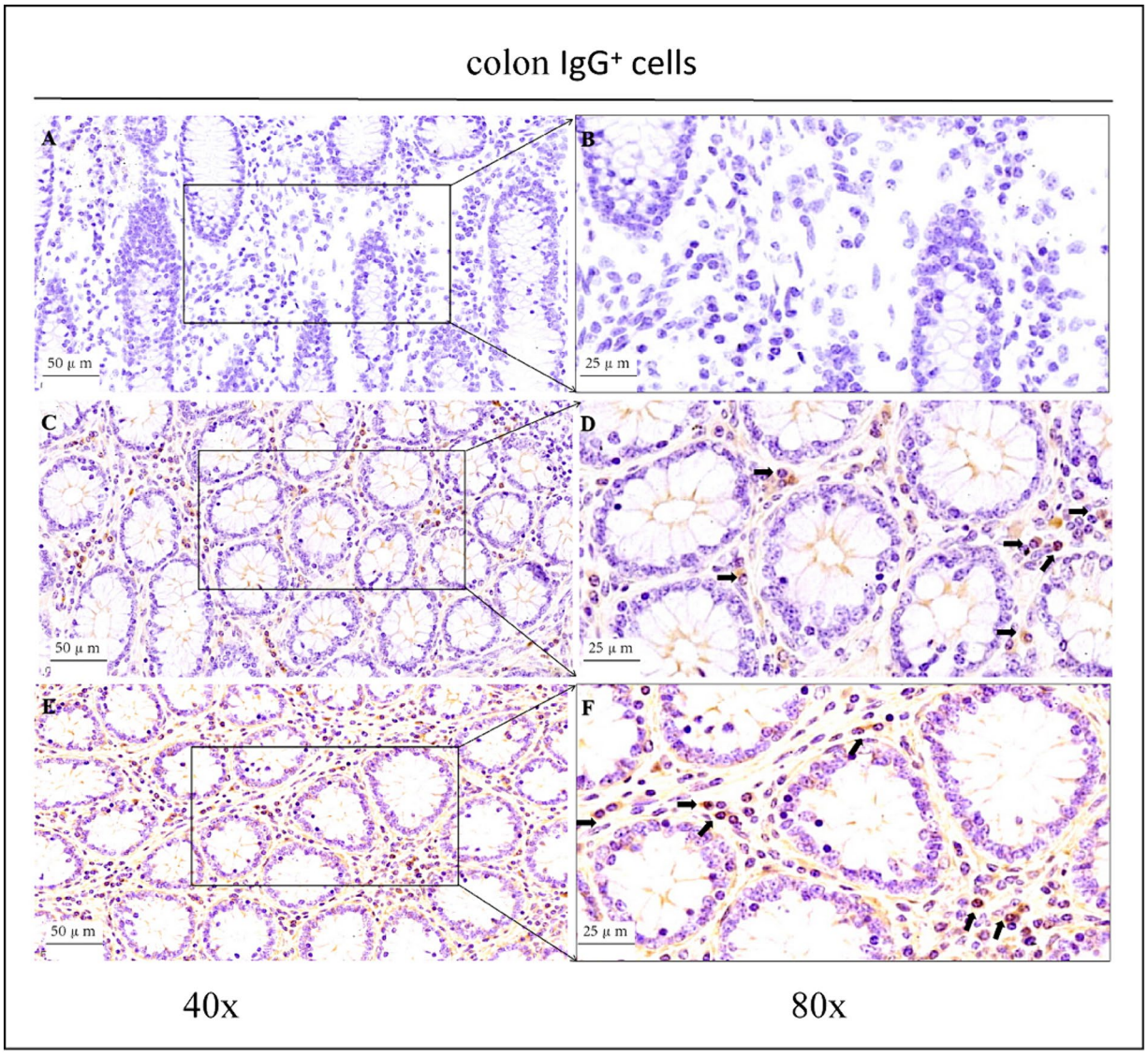

FIGURE 6
Distribution of IgG⁺ cells in the colon of depopulated Holstein bulls. **(A,B)** Are negative controls. **(C,D)** Are controls. **(E,F)** Are OEO groups. Arrows indicate IgG⁺ cells, and the right image (scale bar, 25μm) is a magnification of the left image (scale bar, 50μm).

WZ: Formal analysis, Methodology, Supervision, Validation, Writing – review & editing. FK: Conceptualization, Investigation, Supervision, Writing – review & editing. BW: Data curation, Resources, Writing – review & editing. JP: Data curation, Writing – review & editing. JS: Methodology, Software, Writing – review & editing. QL: Data curation, Methodology, Software, Writing – review & editing. PH: Methodology, Software, Writing – review & editing. YM: Methodology, Writing – review & editing. QC: Methodology, Writing – review & editing. ZZ: Methodology, Writing – review & editing. ZL: Conceptualization, Data curation, Formal analysis, Methodology, Project administration, Software, Writing – review & editing.

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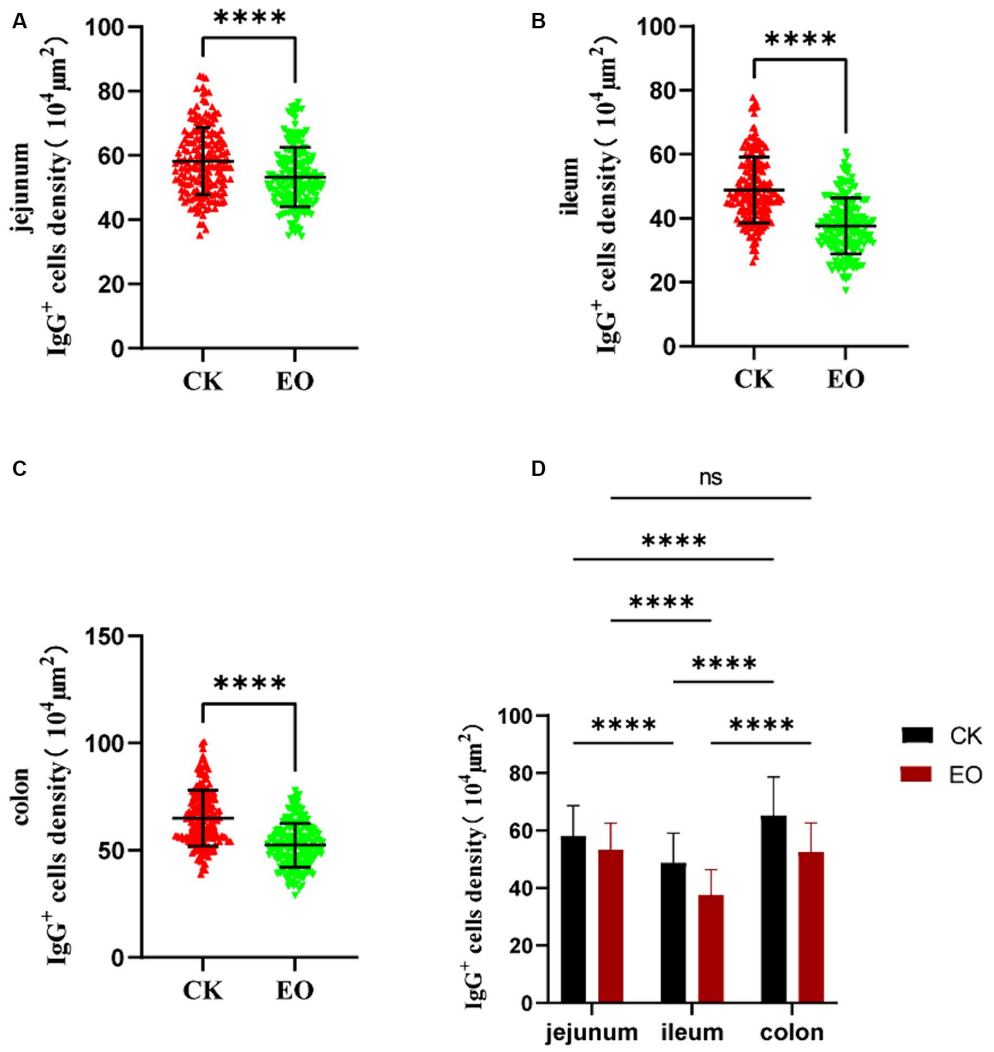

FIGURE 7
Effect of addition of oregano essential oil on IgG⁺ cell density in jejunum, ileum, and colon. **(A)** The effect of oregano essential oil on Holstein bull jejunum IgG⁺ cell density. **(B)** Effect of OEO on IgG⁺ cell density in the ileum of Holstein bulls. **(C)** Effects of oregano essential oil on colon IgG⁺ cell density in Holstein bulls. **(D)** Comparison of positive cells in three intestinal segments between OEO group (EO) and control group (CK). [OEO group (EO); control group (CK) "**" indicates statistically significant difference ($p < 0.01$)]. "****" indicates a significant difference.

TABLE 1 Distribution of IgG in different intestinal segments of Holstein bulls.

Items	CK group	OEO group	*p*-value	Decrease rate (%)
Jejunum/$10^4\mu m^2$	58.24 ± 0.78	53.28 ± 0.69	<0.0001	8.52
Ileum/$10^4\mu m^2$	48.79 ± 0.77	37.63 ± 0.66	<0.0001	22.87
Colon/$10^4\mu m^2$	65.03 ± 0.98	52.38 ± 0.76	<0.0001	19.45

Differences in IgG-secreting cells among different sites in the intestine within the same group were analysed by one-way ANOVA [different letters indicate significant differences ($p < 0.05$)], and differences in IgG-secreting cells at the same site between the CK and OEO groups were analysed using independent samples *t*-tests (**$p < 0.01$). Reduction rate = $(X - Y)/X * 100\%$ (X is the density of positive cells in the CK group; Y is the density of positive cells in the OEO group).

TABLE 2 IgG expression levels in jejunum, ileum, and colon.

Items	CK group	OEO group	*p*-value
Jejunum/(pg/mg)	767.17 ± 40.57	758.20 ± 6.06	0.85
Ileum/(pg/mg)	1546.82 ± 94.98	1425.80 ± 118.21	0.45
Colon/(pg/mg)	1396.83 ± 198.37	1384.78 ± 208.05	0.96

This result was obtained from an independent samples *t*-test, $p < 0.05$, indicating a significant difference.

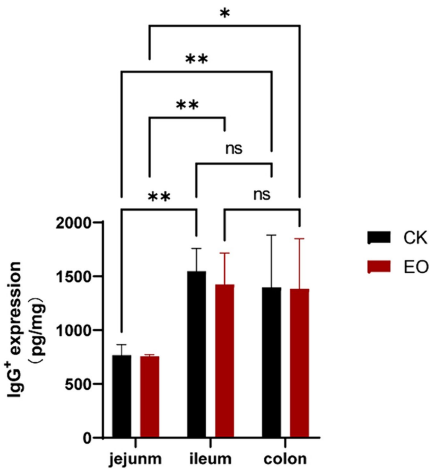

FIGURE 8
Effect of the OEO supplementation on IgG expression in the intestines of Holstein bulls OEO group (EO), control group (CK), [(**$p < 0.01$), (*$0.01 < p \leq 0.05$)].

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Conflict of interest

QC was employed by Jing Chuan Xu Kang Food Co., Ltd. ZZ was employed by Gansu Huarui Agriculture 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.

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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.2024.1382396/full#supplementary-material

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Effects of taurine on the growth performance, diarrhea, oxidative stress and intestinal barrier function of weanling piglets

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Oxidative damage resulting from weaning stress significantly impacts the growth performance and health status of piglets. Taurine, a dietary antioxidant with diverse functions, was investigated in this study for its protective role against weaning stress-induced oxidative damage and its underlying mechanism. Forty 28-day-old male castrated weaned piglets were randomly assigned to four groups. The control group received the basal diet, while the experimental groups were fed the basal diet supplemented with 0.1, 0.2%, or 0.3% taurine over a 28-day period. *In vitro*, H_2O_2 was utilized to induce oxidative damage to the jejunal mucosa of piglets via IPEC-J2 cells. The results demonstrated that taurine supplementation reduced the incidence of diarrhea in piglets compared to that in the control group ($p < 0.05$); the addition of 0.2 and 0.3% taurine led to increased average daily gain and improved feed conversion efficiency in weaned piglets, showing a linear dose-response correlation ($p < 0.05$). Taurine supplementation at 0.2 and 0.3% enhanced the activities of serum CAT and GSH-Px while decreasing the levels of serum NO, XOD, GSSG, and MDA ($p < 0.05$). Moreover, it significantly elevated the levels of GSS, Trx, POD, complex I, mt-nd5, and mt-nd6, enhancing superoxide anion scavenging capacity and the hydroxyl-free scavenging rate in the livers of weaned piglets while reducing NO levels in the liver ($p < 0.05$). Additionally, 0.2 and 0.3% taurine supplementation decreased serum IL-6 levels and elevated the concentrations of IgA, IgG, and IL-10 in weaned piglets ($p < 0.05$). The levels of occludin, claudin, and ZO-1 in the jejunum mucosa of weaned piglets increased with 0.2 and 0.3% taurine supplementation ($p < 0.05$). In IPEC-J2 cells, pretreatment with 25 mM taurine for 24 h enhanced the activities of SOD and CAT; reduced the MDA content; upregulated the mRNA expression of various genes, including *ZO-1*, occludin, claudin-1, *Nrf2*, and *HO-1*; and reversed the oxidative damage induced by H_2O_2 exposure ($p < 0.05$). Overall, the findings suggest that the inclusion of 2 and 3% taurine in the diet can enhance growth performance, reduce diarrhea rates, ameliorate oxidative stress and inflammation, and promote intestinal barrier function in weaned piglets.

KEYWORDS

weanling piglets, taurine, oxidative stress, intestinal health, Nrf2/HO-1 signaling pathway

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1 Introduction

Overall, piglet breeding plays a vital role in the process of pig breeding. The proper feeding and management of piglets directly impact key metrics such as slaughter time, rate, and weight, thus influencing the production efficiency of pig breeding (1, 2). Weaning is a critical phase in the pig growth cycle and is highly susceptible to environmental stimuli, feed quality, mycotoxins, and pathogenic microorganisms. These factors discussed may result in an overproduction of intestinal reactive oxygen species (ROS) in piglets, leading to oxidative stress. This oxidative stress can subsequently trigger inflammatory damage to the intestinal mucosa and compromise barrier function. As a consequence, piglets may experience reduced growth performance, diarrhea, and potentially fatal outcomes, thereby imposing substantial economic burdens on the swine industry (3, 4).

Oxidative stress occurs when animals release oxidative free radicals that surpass the body's antioxidant defenses, giving rise to various physiological or pathological manifestations (5, 6). Research indicates that the weaning process disrupts the redox balance in piglets, markedly reducing the activity of antioxidant enzymes while elevating free radicals and peroxide products, essentially inducing oxidative stress (7, 8). Cells respond to oxidative stress by activating various transcription factors, including nuclear factor E2-related factor 2 (Nrf2). Nrf2 plays a crucial role in regulating cellular redox balance, facilitating the expression of protective antioxidants, and initiating phase I detoxification reactions in mammals. It stands as a key target for the body in the management of oxidative stress (9). Dietary antioxidants such as anthocyanins and Vitamin E are potent natural antioxidants capable of efficiently scavenging free radicals, thereby safeguarding cells against oxidative damage and immune stress (10, 11). Consequently, enhancing the redox status of piglets is a crucial strategy for fostering healthy development.

Supplementing piglet diets with functional components has emerged as a feasible and effective approach for mitigating oxidative stress postweaning (7, 12). Taurine, a sulfur-containing nonprotein amino acid, plays pivotal roles in cellular processes such as volume regulation, osmotic pressure adjustment, protein phosphorylation, membrane stability, bile acid metabolism, neural modulation, calcium homeostasis, and detoxification and exhibits antioxidant and anti-inflammatory properties that are crucial for maintaining animal homeostasis (13, 14). Recent research underscores the ability of taurine to alleviate various oxidative damage-related ailments and its involvement in immune system modulation, offering significant benefits to animal production (15–17). Therefore, this study aimed to assess how taurine impacts growth performance, oxidative stress levels, immune function, and intestinal barrier integrity in weaned piglets.

2 Materials and methods

This animal study was reviewed and approved by the Hunan Agricultural University Institutional Animal Care and Use Committee (202105). Written informed consent was obtained from the owners for the participation of their animals in this study.

2.1 Materials

Taurine (purity ≥99%) and hydrogen peroxide (purity 30%) were purchased from Shanghai Aladdin Biochemical Technology Co., Ltd.

2.2 Experimental design and diets

Forty 28-day-old healthy castrated Duroc × Landrace × Large weaned piglets were randomly divided into 4 groups with 10 replicates in each group and 1 pig in each replicate. The plants were fed in a single pen and fed for 3 days. The control group was fed a basal diet, and the experimental group was fed a diet supplemented with 0.1, 0.2% or 0.3% taurine. The test period was 28 days. The basic diet was prepared according to the NRC 2012, and the feed composition and nutritional level are shown in Table 1. The piglet house was cleaned and disinfected before the experiment. During the experiment, the animals were fed twice a day, provided food freely and allowed to drink freely. The piglets were subjected to routine immunization procedures, and their health status was carefully observed and recorded every day.

2.3 Sample collection

After 12 h of fasting, the anterior vena cava of the piglets was collected on the morning of the 29th day of the experimental period. The whole blood was placed in an ordinary vacuum blood collection vessel. After standing at room temperature for 30 min, the mixture was centrifuged at 845 g and 4°C for 10 min, after which

TABLE 1 Diet composition and nutrient levels (as-fed basis).

Ingredients	Content	Nutrient levels[b]	Content
Corn, %	61.72	Digestible energy, MJ/kg	14.44
Soybean meal, %	11.88	Crude protein, %	18.38
Fermented soybean meal, %	6.00	Calcium, %	0.80
Fish meal, %	5.00	Total phosphorus, %	0.68
Whey powder, %	10.00	Available phosphorus, %	0.40
Fatty powder, %	1.59	Lysine, %	1.40
$CaHPO_4$, %	0.94	Methionine, %	0.50
Limestone, %	0.63	Methionine + cystine, %	0.76
NaCl, %	0.30	Threonine, %	0.90
L-Lysine HCl, %	0.55	Tryptophan, %	0.21
DL-Methionine, %	0.20		
L-Threonine, %	0.20		
Premix[a], %	1.00		
Total, %	100.00		

[a]The premix provided the following per kg of diet: V_A 10500 IU, V_{D3} 3,000 IU, V_E 22.5 IU, V_{K3} 3.0 mg, pantothenic 15 mg, riboflavin 7.5 mg, folic acid 1.5 mg, niacinamide 30.0 mg, thiamine 3.0 mg, VB_6 4.5 mg, biotin 0.12 mg, V_{B12} 0.03 mg, Zn 100 mg, Fe 104 mg, Mn 4.0 mg, Cu 6.0 mg, I 0.3 mg, and Se 0.3 mg.

[b]The digestible energy and amino acids were calculated according to the NRC (2012), and the analysis of crude protein, calcium, total phosphorus, and available phosphorus was conducted using the reference method provided by the AOAC (2006).

the supernatant was separated in a 1.5 mL EP tube. After quick freezing with liquid nitrogen, the samples were stored at −80°C. The patients were randomly anesthetized with pentobarbital sodium (40 mg/kg) and killed by bloodletting. The thoracic cavity and abdominal cavity were opened, and the liver and jejunum were separated. An appropriate amount of liver was removed from the frozen tube at the fixed position and stored at −80°C after quick freezing with liquid nitrogen. Approximately 20 cm of middle jejunum tissue was removed, and the tissue was rinsed with normal saline. The mucosal layer was scraped with a slide, collected in a cryopreservation tube for quick freezing with liquid nitrogen, and then stored at −80°C.

2.4 Growth performance

Piglets were weighed on an empty stomach on the mornings of day 1 and day 29 of the experiment. During the experiment, daily feed intake and residual feed weight were recorded to calculate the average daily gain (ADG), average daily feed intake (ADFI) and feed-to-gain ratio (F/G). The diarrhea of the piglets was observed and recorded at 5 PM every day, and the diarrhea rate (%) = number of piglets with diarrhea × 100/28.

2.5 Serum parameters

Serum catalase (CAT), glutathione peroxidase (GSH-Px), superoxide dismutase (SOD), total antioxidant capacity (T-AOC), superoxide anion scavenging capacity, hydroxyl radical scavenging rate, nitric oxide (NO), xanthine oxidase activity (XOD), glutathione (GSH), oxidized glutathione (GSSG), malondialdehyde (MDA) and peroxidase (POD) contents were detected by biochemical kits produced by Nanjing Jianchen Bioengineering Institute, China. Serum immunoglobulin A (IgA), immunoglobulin G (IgG), immunoglobulin M (IgM), interleukin-4 (IL-4), interleukin-10 (IL-10), interleukin-1β (IL-1β), interleukin-6 (IL-6), and tumor necrosis factor-α (TNF-α) were detected by ELISA kits, which were purchased from Jiangsu Enzyme Immunoassay Industry Co., Ltd., China. All procedures were performed in strict accordance with the kit instructions.

2.6 Analysis of hepatic oxidative status

The levels of glutathione synthase (GSS), thiredoxin (Trx), NO, superoxide anion scavenging capacity, hydroxyl radical scavenging rate, POD, complex I, mitochondrially encoded NADH dehydrogenase 5 (mt-ND5) and mitochondrially encoded NADH dehydrogenase 6 (mt-ND6) were measured. All operations were carried out in strict accordance with the instructions of the kit produced by Nanjing Jianchen Biotechnology Institute, China.

2.7 Analysis of jejunal mucosal barrier proteins

The levels of claudin-1, tight junction protein 1 (ZO-1) and Occludin in the jejunal mucosa were detected by ELISA kits purchased from Jiangsu Enzyme Immunoassay Industry Co., Ltd., China. All operations were carried out strictly according to the instructions of the kit.

2.8 Cell culture

Porcine jejunal epithelial cells (IPEC-J2) were cultured in complete DMEM/F12 supplemented with 10% FBS (OPCEL, Inner Mongolia Opcel Biotechnology Co., Ltd., BS1101) and 1% penicillin–streptomycin (Gibco, 15140-122) in a 5% CO_2 cell incubator at 37°C. When the cells reached 80% confluence, they were washed twice with sterile PBS and then subcultured or treated with drugs. The cell seeding ratios for culture vessels of various sizes were as follows: 5×10^6 cells/cm^2 for 100 mm cell culture dishes; 9×10^5 cells/cm^2 for 6-well cell culture plates; and 3×10^4 cells/cm^2 for 96-well cell culture plates.

2.9 Selection of the daidzein concentration

To determine the optimal taurine concentration, IPEC-J2 cells were seeded in 96-well cell culture plates with six replicates per treatment. After reaching approximately 80% confluency, the cells were subjected to two washes with sterile PBS. Subsequently, 100 μL of DMEM/F12 medium with various concentrations of taurosulfonate (0, 10, 25, 50, and 100 mM, supplemented with 100 μg/mL streptomycin and 100 U/mL penicillin) was added to each well for a 24 h incubation period. After another round of PBS washes, the model group received 100 μL of the treatment medium containing 0.6 mM H_2O_2 according to the methods of Li et al. (18), while the blank group was treated with the same volume of the treatment medium without H_2O_2. Cell viability assessment was conducted using the CCK8 assay after 1 h of incubation.

2.10 Measurements of T-AOC, SOD, and CAT activity and MDA content

The cells were cultured and treated in 6-well plates. Cells cultured to 80% confluence were preincubated with the treatment medium, either with or without 25 mM taurine, for 24 h. Subsequently, the cells were exposed to 0.6 mM H_2O_2 for 1 h, harvested and analyzed following the operational guidelines of the biochemical kits for T-AOC (G0115W), SOD (G0101W), CAT (G0105W), and MDA (G0109W) provided by Suzhou Grandis Biotechnology Co., Ltd., China.

2.11 RT-qPCR

Cells were categorized into four groups based on the presence or absence of 25 mM taurine and 0.6 mM H_2O_2 as per the culture method outlined in *section 2.10*. Upon completion of cell culture, the cells were harvested using a cell scraper, followed by total RNA extraction using the TRIzol method. Subsequently, the mRNA expression levels of the *Nrf2*, *HO-1*, *CAT*, *SOD*, *ZO-1*, occludin, and claudin-1 genes were analyzed through quantitative real-time PCR. The porcine-specific primers utilized in this study were custom-designed (Table 2). The

PCR cycles and relative expression assays adhered to the methodology outlined in our prior research study conducted by Yin et al. (19).

2.12 Statistical analysis

In animal experiments, a single-factor random design is utilized, with each pig serving as a designated statistical unit. Normally distributed data were subjected to single-factor ANOVA, along with pairwise group comparisons via the Duncan method. Polynomial contrast analysis is implemented to evaluate the linear and quadratic impacts of the additives. A nonparametric Kruskal–Wallis test was performed if the data deviated from a normal distribution, followed by a *post hoc* Dunn test for intergroup comparisons. The graphs were generated using GraphPad Prism 9.0.0 software. Statistical significance was determined at the $p < 0.05$ level. The results are presented as the means and standard errors of the means (SEMs).

3 Results

3.1 Growth performance

The impact of taurine on the growth performance of weanling piglets is detailed in Table 3. Compared with those in the control group, the inclusion of 0.2 and 0.3% taurine in the diet significantly enhanced the average final weight and ADG of the piglets while markedly reducing the F/G. The final weight and ADG of the piglets increased proportionally to the taurine concentration, while the F/G ratio decreased linearly. Moreover, supplementing the diets of weaned piglets with 0.1, 0.2%, or 0.3% taurine led to a significant reduction in diarrhea incidence, with both linear and quadratic effects.

TABLE 2 Primers used for gene expression analysis by real-time PCR.

Gene	Primer sequence (5′–3′)	Product length, bp
Nrf2	F: GACCTTGGAGTAAGTCGAGA	103
	R: GGAGTTGTTCTTGTCTTTCC	
HO-1	F: GAGAAGGCTTTAAGCTGGTG	74
	R: GTTGTGCTCAATCTCCTCCT	
SOD1	F: GAAGACAGTGTTAGTAACGG	93
	R: CAGCCTTGTGTATTATCTCC	
CAT	F: CCTGCAACGTTCTGTAAGGC	72
	R: GCTTCATCTGGTCACTGGCT	
ZO-1	F: TTGATAGTGGCGTTGACA	126
	R: CCTCATCTTCATCATCTTCTAC	
Claudin-1	F: GCATCATTTCCTCCCTGTT	97
	R: TCTTGGCTTTGGGTGGTT	
Occludin	F: CAGTGGTAACTTGGAGGCGTCTTC	103
	R: CGTCGTGTAGTCTGTCTCGTAATGG	
β-actin	F: CTGCGGCATCCACGAAACT	147
	R: AGGGCCGTGATCTCCTTCTG	

Nrf2, nuclear factor-erythroid2-related factor 2; *HO-1*, heme oxygenase-1; *SOD1*, superoxide dismutase 1; *CAT*, catalase; *ZO-1*, zonula occludens 1.

3.2 Serum antioxidant parameters

The analysis presented in Table 4 demonstrates that supplementing the diets of weaned piglets with 0.1, 0.2, and 0.3% taurine resulted in an increase in the serum GSH levels in comparison to those in the control group. The inclusion of 0.2 and 0.3% taurine led to elevated serum CAT activity and reduced levels of serum NO, XOD, GSSG, and MDA in the weaned piglets. Moreover, the introduction of 0.3% taurine enhanced the activities of GSH-Px and SOD in piglet serum and increased the T-AOC and POD, superoxide anion scavenging capacity, and hydroxyl radical scavenging rate. The study also revealed a linear increase in CAT, GSH-Px, SOD, T-AOC, superoxide anion scavenging capacity, the hydroxyl radical scavenging rate, GSH, and POD with increasing taurine concentration. Conversely, there were linear decreases in the levels of NO, XOD, GSSG, and MDA.

3.3 Liver antioxidant parameters

The findings presented in Table 5 illustrate the impact of taurine on the antioxidant capacity of the liver of weanling piglets. Compared with those in the control group, dietary supplementation with 0.1, 0.2, and 0.3% taurine resulted in increased levels of liver GSS, Trx, POD, and mt-ND5; enhanced superoxide anion clearance capacity; and an elevated hydroxyl-free clearance rate while reducing the liver NO levels in the weaned piglets. Moreover, administering 0.2 and 0.3% taurine led to increased levels of liver complex I and mt-ND6 in weanling piglets. The present study also revealed a linear increase in GSS, Trx, superoxide anion scavenging capacity, the hydroxyl radical scavenging rate, POD, complex I, mt-ND5, and mt-ND6 with increasing taurine concentrations and a linear decrease in NO levels.

3.4 Serum immune parameters

Table 6 shows that the supplementation of 0.1, 0.2, and 0.3% taurine in the diet of weaned piglets led to a significant reduction in the serum IL-6 levels and a notable increase in the IgA and IgG levels compared to those in the control group. Moreover, the addition of 0.2 and 0.3% taurine significantly elevated the serum IL-10 level, whereas the inclusion of 0.3% taurine notably increased the serum IL-4 and IgM levels. Notably, the levels of IL-4, IL-10, IgA, IgG, and IgM exhibited both linear and quadratic growth patterns in correlation with increases in the taurine concentration, while the level of IL-6 decreased linearly.

3.5 Jejunal mucosal barrier proteins

Table 7 shows that, in comparison to those in the control group, supplementation of the diets of weaned piglets with 0.1, 0.2, and 0.3% taurine led to a notable increase in occludin levels in the jejunal mucosa. Moreover, the inclusion of 0.2 and 0.3% taurine markedly elevated the levels of claudin and ZO-1. Concurrently, occludin, claudin, and ZO-1 levels exhibited both linear and quadratic increases proportional to the increase in taurine concentration.

TABLE 3 Effect of taurine on the growth performance of weanling piglets.

Item	Taurine inclusion level, %				SEM	*p*-value		
	0	0.1	0.2	0.3		Treatment	Linear	Quadratic
Initial body weight, kg	6.79	6.71	6.73	6.73	0.07	0.981	0.829	0.776
Final body weight, kg	12.65^{b}	13.00^{b}	14.02^{a}	14.48^{a}	0.21	0.002	0.000	0.871
ADG, kg/day	167.52^{b}	179.84^{b}	208.29^{a}	221.22^{a}	6.13	0.003	0.000	0.974
ADFI, kg/day	432.19	439.44	453.44	481.44	9.09	0.267	0.061	0.566
F/G	2.58^{a}	2.45^{a}	2.19^{b}	2.19^{b}	0.04	0.000	0.000	0.324
Diarrhea rate, %	11.43^{a}	1.34^{b}	0.00^{b}	3.93^{b}	1.07	0.000	0.001	0.000

ADG, average daily gain; ADFI, average daily feed intake; F/G, the ratio of feed intake to gain. The mean values of the same row of data without the same superscript letter were significantly different at the *p*-value ($n = 10$).

TABLE 4 Effect of taurine on serum antioxidant indices in weanling piglets.

Item	Taurine inclusion level, %				SEM	*p*-value		
	0	0.1	0.2	0.3		Treatment	Linear	Quadratic
CAT, nmol/min/mL	15.72^{b}	15.95^{b}	24.16^{a}	24.14^{a}	1.25	0.001	0.000	0.934
GSH-Px, nmol/min/mL	700.93^{b}	703.66^{b}	841.29^{a}	870.08^{a}	25.77	0.010	0.002	0.727
SOD, U/mL	197.46^{b}	209.04^{b}	248.75^{b}	388.57^{a}	22.23	0.000	0.000	0.017
T-AOC, μmol Trolox/mL	0.12^{b}	0.12^{b}	0.14^{b}	0.19^{a}	0.01	0.000	0.000	0.017
Superoxide anion scavenging capacity, %	16.81^{b}	16.96^{b}	17.35^{b}	18.64^{a}	0.24	0.010	0.002	0.120
Hydroxyl radical scavenging rate, %	3.46^{b}	3.55^{b}	3.78^{ab}	3.97^{a}	0.07	0.022	0.003	0.619
NO, μmoL/mL	0.36^{a}	0.33^{a}	0.25^{b}	0.24^{b}	0.02	0.006	0.001	0.670
XOD, U/mL	5.79^{a}	5.67^{a}	3.23^{b}	2.22^{b}	0.45	0.000	0.000	0.251
GSH, U/mL	0.08^{c}	0.14^{b}	0.17^{b}	0.24^{a}	0.02	0.000	0.000	0.604
GSSG, nmol/mL	3.61^{a}	3.18^{b}	3.14^{b}	2.71^{c}	0.11	0.001	0.000	0.955
MDA, nmol/mL	13.00^{a}	11.26^{a}	8.41^{b}	6.97^{b}	0.69	0.000	0.000	0.800
POD, U/mL	7.25^{b}	8.52^{b}	8.71^{b}	12.99^{a}	0.60	0.000	0.000	0.010

CAT, catalase; GSH-Px, glutathione peroxidase; SOD, superoxide dismutase; T-AOC, total antioxidant capacity; NO, nitric oxide; XOD, xanthine oxidase activity; GSH, glutathione; GSSG, oxidized glutathione; MDA, malondialdehyde; POD, peroxidase. The mean values of the same row of data without the same superscript letter were significantly different at the *p*-value ($n = 10$).

3.6 The concentration of taurine in IPEC-J2 cells

To determine the optimal treatment parameters for taurine. The cells were pretreated with various taurine concentrations for 24 h and subsequently either exposed or not exposed to 0.6 mM H_2O_2 for 1 h. Cell viability assessments revealed that the highest viability rate occurred in the 25 mM taurine treatment group (Figure 1). Consequently, we selected 25 mM as the optimal concentration for all subsequent experiments.

3.7 SOD activity, CAT activity, T-AOC and MDA content in H_2O_2-treated IPEC-J2 cells

This study focused on investigating the impact of taurine on antioxidant enzyme activity and MDA content in IPEC-J2 cells exposed to catalase. Biochemical kits (Figure 2) were utilized for analysis. Exposure to H_2O_2 significantly decreased the activities of CAT and SOD, as well as the T-AOC, while notably increasing the MDA content in the cells compared to those in the control group. As expected, 25 mM pretreatment for 24 h not only significantly boosted the antioxidant capacity of the cells but also ameliorated the oxidative stress injury caused by H_2O_2 exposure. This pretreatment led to a considerable increase in CAT and SOD activities and T-AOC, coupled with a reduction in MDA content.

3.8 Effect of taurine on the expression of genes involved in the Nrf2 signaling pathway in IPEC-J2 cells

The Nrf2 signaling pathway serves as a crucial regulatory mechanism in combating oxidative stress. Our study explored the impact of pretreatment with taurine on the expression levels of Nrf2 pathway genes in H_2O_2-exposed IPEC-J2 cells (Figure 3). Compared to those in the control group, the expression of the *Nrf2*, *HO-1* and *CAT* genes in the H_2O_2-treated group was notably lower. Conversely, pretreatment with 25 mM taurine for 24 h substantially upregulated the expression of *the Nrf2*, *HO-1* and *CAT* genes, leading to a significant amelioration of oxidative damage caused by H_2O_2 exposure.

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TABLE 5 Effect of taurine on antioxidant indices in the liver of weanling piglets.

Item	Taurine inclusion level, %				SEM	*p*-value		
	0	0.1	0.2	0.3		Treatment	Linear	Quadratic
GSS, U/g	0.39^{d}	0.43^{c}	0.52^{b}	0.58^{a}	0.02	0.000	0.000	0.297
Trx, ng/g	745.17^{d}	935.07^{c}	1052.05^{b}	1226.66^{a}	46.05	0.000	0.000	0.697
NO, μmoL/mgProt	0.19^{a}	0.18^{b}	0.16^{c}	0.10^{d}	0.01	0.000	0.000	0.000
Superoxide anion scavenging capacity, %	16.22^{d}	17.37^{c}	18.47^{b}	19.02^{a}	0.29	0.000	0.000	0.080
Hydroxyl radical scavenging rate, %	3.41^{c}	3.70^{b}	3.80^{b}	4.22^{a}	0.08	0.000	0.000	0.384
POD, U/mgProt	3.37^{d}	4.95^{c}	6.07^{b}	7.67^{a}	0.42	0.000	0.000	0.962
Complex I, pg/g	7.89^{c}	8.31^{c}	9.00^{b}	9.62^{a}	0.19	0.000	0.000	0.590
mt-ND5, pg/g	4.86^{d}	6.34^{c}	7.24^{b}	8.72^{a}	0.37	0.000	0.000	0.987
mt-ND6, pg/g	3.64^{c}	3.85^{c}	5.34^{b}	6.22^{a}	0.29	0.000	0.000	0.115

CAT, catalase; GSH-Px, glutathione peroxidase; SOD, superoxide dismutase; T-AOC, total antioxidant capacity; NO, nitric oxide; XOD, xanthine oxidase activity; GSH, glutathione; GSSG, oxidized glutathione; MDA, malondialdehyde; POD, peroxidase. GSS, glutathione synthase; Trx, thiredoxin; NO, nitric oxide; POD, peroxidase; Complex I, mt-ND5, mitochondrially encoded NADH dehydrogenase 5; mt-ND6, mitochondrially encoded NADH dehydrogenase 6. The mean values of the same row of data without the same superscript letter were significantly different at the *p*-value ($n = 10$).

TABLE 6 Effect of taurine on serum immune indices in weanling piglets.

Item	Taurine inclusion level, %				SEM	*p*-value		
	0	0.1	0.2	0.3		Treatment	Linear	Quadratic
IL-4, pg/mL	22.35^{b}	27.16^{b}	30.73^{b}	51.54^{a}	3.17	0.000	0.000	0.020
IL-10, pg/mL	35.86^{c}	49.92^{bc}	70.73^{b}	118.83^{a}	8.74	0.000	0.000	0.038
IL-1β, pg/mL	232.92	256.12	314.57	190.78	22.22	0.287	0.730	0.116
IL-6, pg/mL	346.53^{a}	284.70^{b}	202.42^{c}	92.72^{d}	25.57	0.000	0.000	0.160
TNF-α, pg/mL	80.40	71.28	78.28	80.97	4.16	0.864	0.833	0.525
IgA, μg/mL	263.28^{c}	345.53^{b}	293.92^{bc}	476.46^{a}	25.80	0.000	0.000	0.015
IgG, μg/mL	3.67^{c}	6.26^{b}	6.03^{bc}	13.55^{a}	1.08	0.000	0.000	0.010
IgM, mg/mL	7.27^{b}	8.10^{b}	8.04^{b}	15.44^{a}	0.97	0.000	0.000	0.000

IgA, immunoglobulin A; IgG, immunoglobulin G; IgM, immunoglobulin M; IL-4, interleukin-4; IL-10, interleukin-10; IL-1β, interleukin-1β; IL-6, interleukin-6; TNF-α, tumor necrosis factor-α. The mean values of the same row of data without the same superscript letter were significantly different at the *p*-value ($n = 10$).

3.9 Effect of taurine on barrier function-related gene expression in IPEC-J2 cells exposed to H_2O_2

The impact of taurine pretreatment on the expression levels of tight junction-related genes in IPEC-J2 cells exposed to H_2O_2 is illustrated in Figure 4. Compared to those in the control group, H_2O_2 exposure markedly decreased the expression of the *ZO-1*, occludin, and claudin-1 genes. Conversely, pretreatment with 25 mM taurine for 24 h significantly upregulated the expression of the *ZO-1*, occludin, and claudin-1 genes, effectively reversing the downregulation induced by H_2O_2.

4 Discussion

Weanling stress leads to diarrhea, reduced growth performance, compromised intestinal function, and other harmful effects, resulting in notable economic losses for the pig breeding industry (20, 21). This study aimed to investigate the potential benefits of taurine on the growth performance, oxidative stress, and intestinal barrier of weaned piglets. Currently, supplementing diets with antioxidants is an effective strategy for mitigating oxidative stress and enhancing the growth performance and health status of weaned piglets (7). Furthermore, past research has indicated that antioxidants can serve as alternatives to antibiotics in facilitating smooth weaning for piglets (22). Our findings revealed that the addition of taurine to the diets of weaned piglets, particularly at the 0.2 and 0.3% levels, significantly increased both the average final weight and daily gain of the piglets while reducing the feed-to-meat ratio. Consistent with the findings of a recent study by Wang et al. (23), taurine supplementation at 0.2 and 0.4% notably enhanced the growth performance of weaned piglets. Moreover, previous studies have suggested that taurine could mitigate the adverse impacts of LPS on the growth performance of piglets and broilers (16, 24). During the weaning process, piglets often experience diarrhea due to diverse external stressors and incomplete development. Each year, a considerable number of weaned piglets succumb to or are culled because of weaning stress-related diarrhea, resulting in

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TABLE 7 Effects of taurine on jejunal mucosal immune indices in weanling piglets.

Item	Taurine inclusion level, %				SEM	*p*-value		
	0	1	2	3		Treatment	Linear	Quadratic
Claudin-1, ng/g	636.88^{c}	682.24^{c}	1038.14^{b}	1469.29^{a}	88.18	0.000	0.000	0.000
ZO-1, ng/g	714.90^{c}	756.72^{c}	879.10^{b}	1258.34^{a}	55.84	0.000	0.000	0.000
Occludin, ng/g	33.49^{d}	47.28^{c}	66.02^{b}	133.75^{a}	10.01	0.000	0.000	0.000

ZO-1, zonula occludens 1. The mean values of the same row of data without the same superscript letter were significantly different at the *p*-value ($n = 10$).

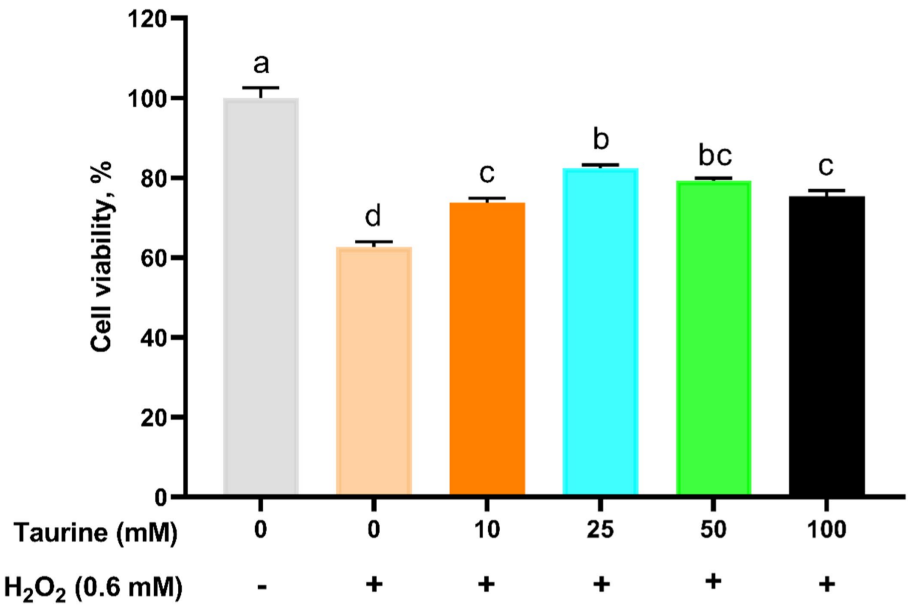

FIGURE 1
Effect of taurine on IPEC-J2 cell viability. IPEC-J2 cells were cultured in 96-well plates and preincubated with various concentrations of taurine (ranging from 0 to 100 mM) for 24 h, followed by exposure to 0.6 mM H_2O_2 for 1 h. The results, presented as the mean ± standard error of the mean (SEM), $n = 6$, indicate the values as a percentage relative to the untreated group with neither H_2O_2 nor taurine. Significant differences ($p < 0.05$) are denoted by the distinct letters a, b, c, and d. The following abbreviations were used: IPEC-J2, porcine intestinal epithelial cells; H_2O_2, hydrogen peroxide.

significant losses in the breeding sector (3, 25). Our research showed that taurine effectively decreased the rate of diarrhea in weaned piglets compared to that in the control group. Subsequent studies revealed that taurine improved the oxidative stress levels and intestinal barrier function of piglets, further corroborating the potential of taurine as an antioxidant substitute for feed antibiotics to ameliorate the growth performance and diarrhea of weaned piglets.

Weanling stress is not only closely related to potential changes in the immune system and intestinal barrier function but also results in severe oxidative stress (26). The body's redox reactions underlie various biochemical pathways and cellular functions, primarily reliant on the delicate equilibrium between the oxidative and antioxidant systems. An imbalance due to excessive ROS production or inadequate ROS scavenging by antioxidants induces oxidative stress, leading to cell apoptosis, tissue damage, metabolic disorders, inflammation, diarrhea, and reduced production performance in piglets (27, 28). Studies by Wen et al. (29) highlighted a notable increase in malondialdehyde (MDA) levels in the blood of weaned piglets, along with decreased SOD and GSH-Px activities. Taurine can scavenge reactive oxygen species and mitigate lipid peroxidation. The levels of SOD, GSH-Px, and MDA in blood and tissues are common biomarkers of oxidative stress. MDA is a metabolite of autolipid peroxidation, and an increase in GSH and SOD levels reflects an increase in host antioxidant capacity. Our research demonstrated that taurine supplementation enhances serum GSH-Px activity and GSH levels and improves superoxide anion and hydroxyl radical scavenging capacities while reducing NO, XOD, and GSSG serum levels. Moreover, oxidative stress is intricately linked to ferroptosis, where both GSH-Px and GSH play crucial roles as regulators by facilitating the reduction of ROS (30, 31). In conclusion, our findings align with previous research suggesting that taurine enhances the serum antioxidant capacity of weaned piglets.

The liver, an essential organ, performs various vital functions, including detoxification, metabolism, bile secretion, and immune defense (32). It exhibits high sensitivity to exogenous substances, rendering it susceptible to oxidative stress induced by drugs, viruses, or toxins. Weanling stress can trigger oxidative stress in the liver of piglets (33, 34). Previous research has demonstrated that weaning decreases the activities of SOD, CAT, and GSH-PX in the liver of piglets (35). Numerous studies support the protective role of taurine in liver injury. In our study, taurine increased the levels of GSS, Trx, POD, complex I, mt-nd5, and mt-nd6 in the livers of weaned piglets.

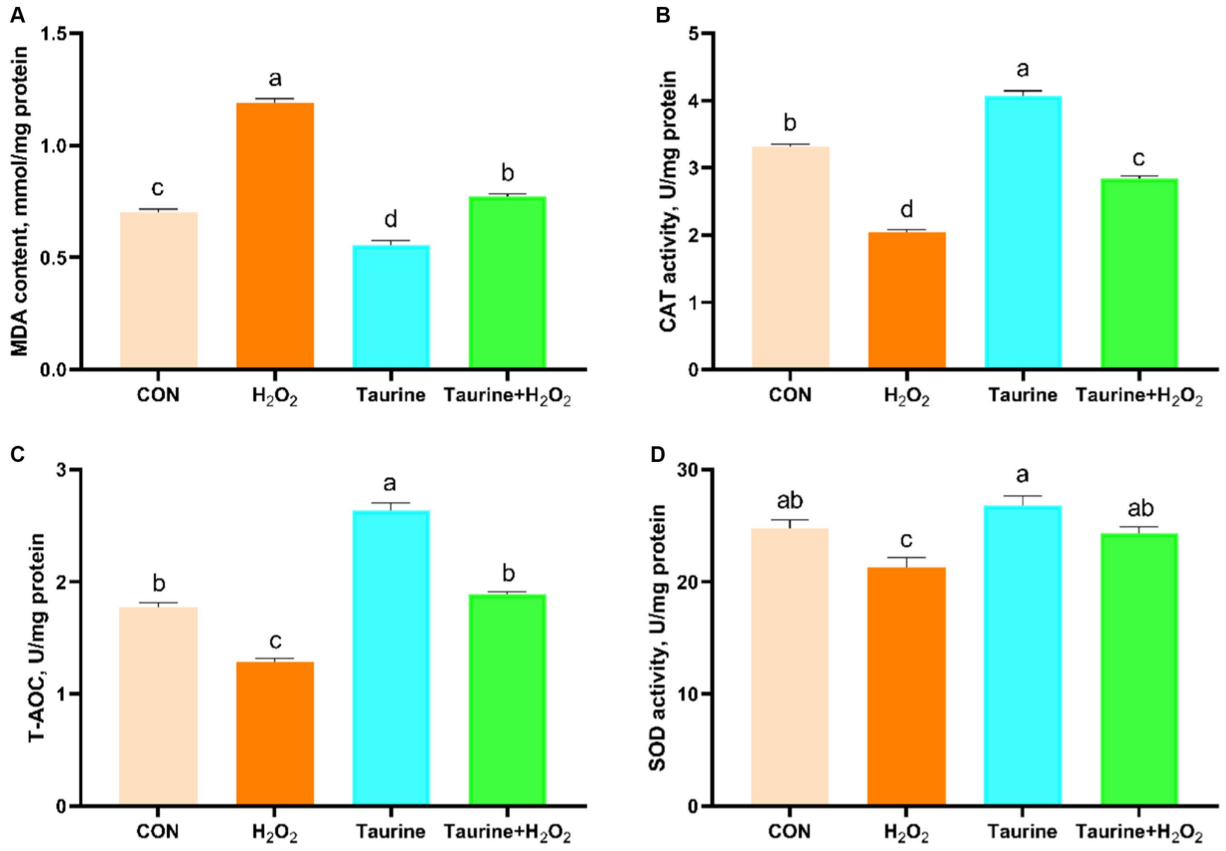

FIGURE 2
Effect of taurine on antioxidant enzymes and MDA in IPEC-J2 cells. **(A)** MDA content. **(B)** CAT activity. **(C)** Total antioxidant capacity. **(D)** SOD activity. IPEC-J2 cells were cultured in 6-well plates and pretreated with either 25 mM taurine or no taurine for 24 h, followed by exposure to 0.6 mM H_2O_2 or no H_2O_2 for 1 h. The results are presented as the mean ± standard error of the mean (SEM), $n = 6$. Statistical significance, denoted by $p < 0.05$, is indicated by values assigned unique alphabetical labels (a, b, c). IPEC-J2, porcine intestinal epithelial cells; H_2O_2, hydrogen peroxide; MDA, malondialdehyde; CAT, catalase; T-AOC, total antioxidant capacity; SOD, superoxide dismutase.

It enhanced superoxide anion scavenging capacity and the hydroxyl free radical scavenging rate and notably reduced NO levels in the liver. Wu et al. (36) reported that taurine inhibits the increase in MDA and decrease in antioxidant enzyme activity in rat livers induced by aflatoxin B1. Shi et al. (37) reported the efficacy of taurine in alleviating liver oxidative damage from oxidized fish oil in young catfish. Our findings align with these studies, suggesting the potential of taurine to enhance the antioxidant capacity of the livers of weaned piglets.

Weanling stress commonly results in compromised immune function and an inflammatory response (38). During weaning, piglets are exposed to bacteria, toxins, and antigens from the intestinal cavity, which can infiltrate tissues, organs, and bloodstream through the submucosa, leading to disruptions in the intestinal immune system and triggering inflammation (39, 40). Excessive proinflammatory cytokines can exacerbate damage to intestinal integrity and epithelial function. Researchers studied the gene expression of inflammatory cytokines in weanling piglets and noted a significant increase in IL-6 levels in the intestinal tract, as did the levels of IL-1β and TNF-α (41, 42). Taurine exhibits anti-inflammatory properties and contributes significantly to managing cardiovascular diseases and metabolic inflammatory conditions such as diabetes mellitus and nonalcoholic fatty liver disease (15, 43, 44). Neutrophils are the primary contributors to tissue damage in mastitis. Studies have shown that taurine can mitigate neutrophil aggregation, hinder cxcl2 expression, and alleviate mastitis caused by *Streptococcus uberis* in dairy cows (45). Additionally, research has demonstrated that taurine supplementation can ameliorate liver cell swelling and inflammatory infiltration in piglets afflicted with mycotoxins, reducing the serum levels of the proinflammatory factors IL-1β, IL-6, IL-8, and TNF-α (46). Our study corroborates these findings, showing that incorporating taurine into the diets of weaned piglets increases the serum levels of IgA, IgG, IgM, IL-4, and IL-10 while reducing IL-6 levels. This finding suggested that taurine plays a crucial role in immune regulation, counteracting immune function impairment in weaned piglets due to weanling stress.

The jejunum serves as a vital organ for nutrient absorption from the external environment and plays a crucial role in communication with both the internal and external environments (47, 48). Due to its sensitivity to internal and external factors, the jejunum can experience oxidative stress, leading to abnormalities in its morphology and structure, damage to barrier functions, and compromised digestion and absorption capabilities (49). Consequently, oxidative damage to the jejunal mucosa is closely linked to the development of conditions such as growth retardation and diarrhea in livestock and poultry (12, 50). In this study, the supplementation of weanling piglets with taurine significantly elevated the levels of claudin, ZO-1, and occludin in the jejunal mucosa. Previous research by Shi et al. (37) demonstrated that

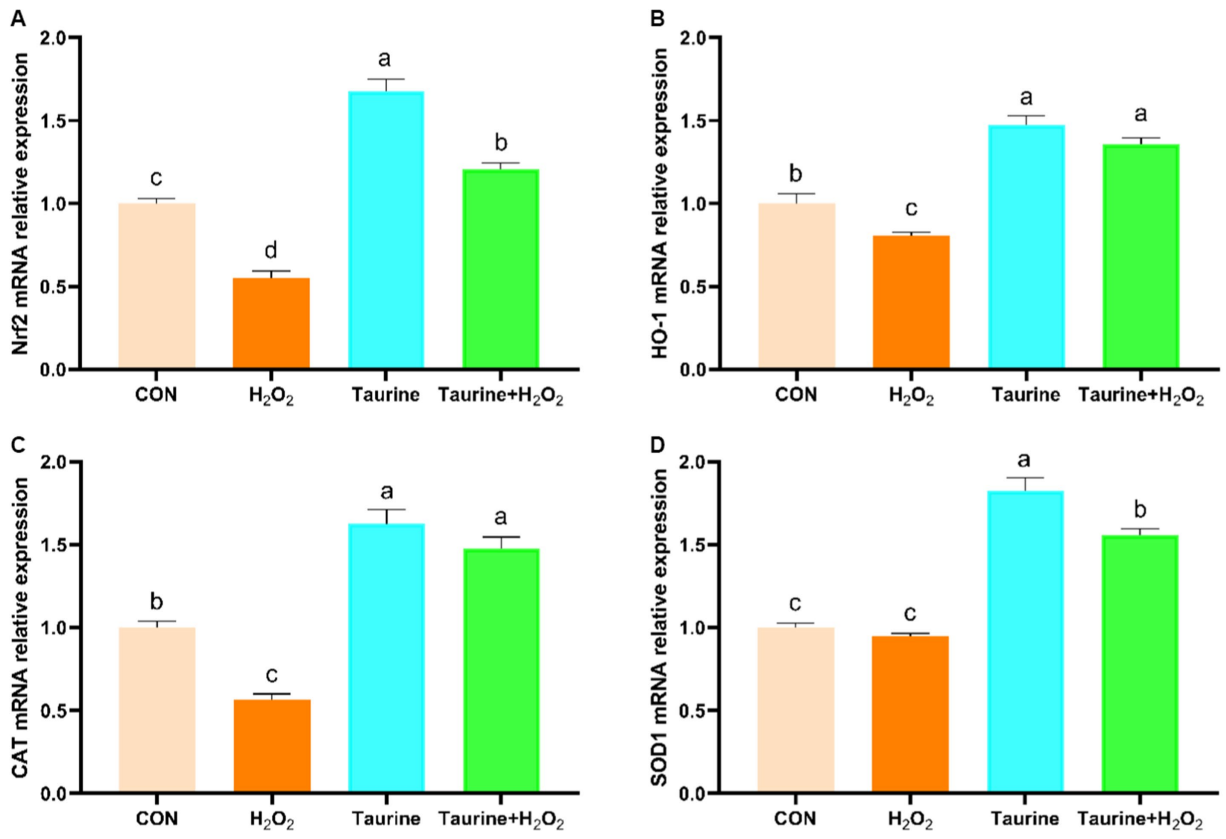

FIGURE 3
Effect of taurine on the expression of genes involved in the Nrf2 signaling pathway in IPEC-J2 cells. **(A)** *Nrf2* mRNA expression relative to that of β-actin. **(B)** *HO-1* mRNA expression relative to that of β-actin. **(C)** *CAT* mRNA expression relative to that of β-actin. **(D)** *SOD1* mRNA expression relative to that of β-actin. IPEC-J2 cells were cultured in 6-well plates and pretreated with either 25 mM taurine or no taurine for 24 h, followed by exposure to 0.6 mM H_2O_2 or no H_2O_2 for 1 h. The results are presented as the mean ± standard error of the mean (SEM), $n = 6$. Statistical significance, denoted by $p < 0.05$, is indicated by values assigned unique alphabetical labels (a, b, c). IPEC-J2, porcine intestinal epithelial cells; H_2O_2, hydrogen peroxide; MDA, malondialdehyde; CAT, catalase; T-AOC, total antioxidant capacity; SOD, superoxide dismutase.

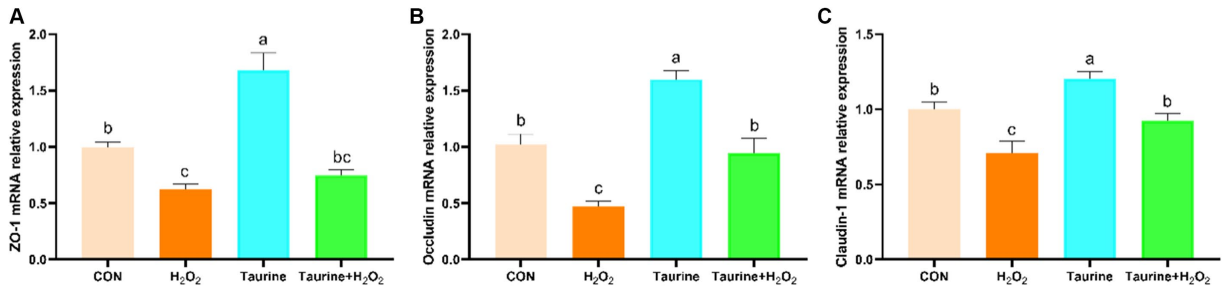

FIGURE 4
Effect of taurine on barrier function-related gene expression in IPEC-J2 cells. **(A)** *ZO-1* mRNA expression relative to that of β-actin. **(B)** Occludin mRNA expression relative to that of β-actin. **(C)** Claudin-1 mRNA expression relative to that of β-actin. IPEC-J2 cells were cultured in 6-well plates and pretreated with either 25 mM taurine or no taurine for 24 h, followed by exposure to 0.6 mM H_2O_2 or no H_2O_2 for 1 h. The results are presented as the mean ± standard error of the mean (SEM), $n = 6$. Statistical significance, denoted by $p < 0.05$, is indicated by values assigned unique alphabetical labels (a, b, c). IPEC-J2, porcine intestinal epithelial cells; H_2O_2, hydrogen peroxide; ZO-1, zonula occludens 1.

incorporating taurine into a diet containing oxidized fish oil markedly upregulated intestinal *ZO-1* and *ZO-2* mRNA expression in catfish seedlings. Additionally, Zhao et al. (24) reported that taurine could increase the protein expression of ZO-1, occludin, and claudin-1 in the colons of piglets challenged with LPS. These findings align with those of the present study, reinforcing the notion that taurine has the potential to alleviate Weanling stress-induced impairment of intestinal barrier function.

The intestinal structure is primarily composed of villi and crypts, which are enveloped by a single layer of columnar epithelial cells (51,

52). These absorptive cells, constituting 90 to 95% of epithelial cells, feature apical microvilli and house various enzymes responsible for the breakdown and absorption of sugars and proteins (53–55). Furthermore, intestinal epithelial cells act as a crucial interface between immune cells and environmental agents, assisting in the detection and response to food, symbiotic bacteria, and pathogens to protect against pathogen invasion (56, 57). Thus, intestinal epithelial cells play a pivotal role in intestinal function. Oxidative stress has the potential to harm the intestinal mucosa. In this investigation, IPEC-J2 were utilized to replicate the oxidative damage observed in the intestinal mucosa due to weanling stress in piglets through exposure to H_2O_2. The presence of H_2O_2 triggers the generation of ROS and results in lipid damage, leading to the production of lipid degradation biomarkers (MDA). The present study revealed that pretreatment of IPEC-J2 cells with 25 mM taurine for 24 h significantly enhanced the activities of antioxidant enzymes and reduced the MDA content. Additionally, taurine pretreatment for 24 h substantially mitigated the oxidative damage induced by H_2O_2 exposure in IPEC-J2 cells, reversing the decreases in SOD and CAT activities and the increase in MDA content caused by H_2O_2 exposure. SOD is widely distributed in the mitochondrial matrix of animal cells and plays a crucial role in scavenging oxygen free radicals to protect intestinal mucosal cells from oxidative damage, making it a vital antioxidant in the body (58). Furthermore, taurine pretreatment for 24 h reversed the downregulation of genes encoding barrier proteins induced by H_2O_2, underscoring the role of taurine in ameliorating H_2O_2-induced mucosal injury.

The Nrf2 signaling pathway, which is crucial for the oxidative stress response, plays a vital role in maintaining the body's redox homeostasis. Excessive ROS production can impede Nrf2 expression, leading to a redox imbalance (59, 60). Our study investigated the expression of Nrf2 pathway-related genes in IPEC-J2 cells using qPCR. We observed that taurine significantly enhanced *Nrf2* and *HO-1* gene expression, reversing the downregulation induced by H_2O_2 exposure. HO-1, a target of Nrf2, directly regulates HO-1 promoter activity through Nrf2, exhibiting potent antioxidant effects by scavenging ROS and defending against harmful substances such as peroxides and free radicals (61, 62). Taurine pretreatment also upregulated *SOD* and *CAT* gene expression in IPEC-J2 cells. Consistent with previous research, taurine shows promise for alleviating oxidative stress. Researchers have demonstrated that taurine, through *Nrf2* activation, protects pig mammary gland epithelial cells from oxidative stress induced by H_2O_2 (63). Furthermore, studies by Wang et al. (64) revealed that taurine could mitigate the downregulation of *Nrf2* and *HO-1* induced by 5-fluorouracil, ameliorating mucosal inflammation in mouse jejunum and colon tissues. Our findings support the potential of taurine as a dietary supplement for combating oxidative stress.

5 Conclusion

This study demonstrated that taurine enhances the growth performance of weaned piglets, reduces the incidence of diarrhea, alleviates oxidative stress, and mitigates inflammatory responses associated with weaning. Taurine supplementation can upregulate the expression of genes and proteins related to tight junctions in the jejunal intestinal epithelium. Additionally, taurine has the potential to ameliorate oxidative damage in intestinal epithelial cells through the Nrf2/HO-1 pathway. In conclusion, taurine has emerged as a promising dietary supplement for mitigating oxidative damage in weanling piglets.

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 studies were approved by Hunan Agricultural University Institutional Animal Care and Use Committee (202105). The studies were conducted in accordance with the local legislation and institutional requirements. Written informed consent was obtained from the owners for the participation of their animals in this study.

Author contributions

MZ: Data curation, Formal analysis, Investigation, Methodology, Software, Writing – original draft, Writing – review & editing. ZW: Formal analysis, Investigation, Methodology, Software, Writing – original draft, Writing – review & editing. DD: Formal analysis, Investigation, Software, Writing – original draft, Writing – review & editing. BW: Investigation, Software, Writing – original draft, Writing – review & editing. XZ: Investigation, Software, Writing – original draft, Writing – review & editing. BZ: Investigation, Software, Writing – original draft, Writing – review & editing. CW: Investigation, Software, Writing – original draft, Writing – review & editing. YZ: Conceptualization, Investigation, Project administration, Resources, Writing – original draft, Writing – review & editing.

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Conflict of interest

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Impact of long-term feeding a high level of Spirulina combined with enzymes on growth performance, carcass traits and meat quality in broiler chickens

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This study evaluates the effect of prolonged feeding with a high inclusion level of Spirulina, combined with peptidases, on broiler chicken's growth performance, digesta viscosity, carcass attributes and meat quality. The experiment involved 120 male broilers divided into 40 battery brooders, each housing 3 birds. Post 7-day acclimatisation with a corn and soybean-based diet, the birds were provided with one of four diets: a corn and soybean meal-based diet (CON), a mix incorporating 15% Spirulina (SP), a Spirulina-rich mix supplemented with 0.025% of commercial VemoZyme® P (SPV), or a Spirulina-rich mix supplemented with 0.10% of porcine pancreatin (SPP). The CON group had higher body weight and weight gain ($p < 0.001$) and a lower feed conversion ratio ($p < 0.001$) from day 7–21, compared to the Spirulina-fed groups. Spirulina-fed chickens significantly increased ileum viscosity ($p < 0.05$). Spirulina also elevated the weight ($p < 0.05$) of the duodenum and the length ($p < 0.001$) of the entire gastrointestinal tract compared to CON. Breast and thigh muscles from Spirulina-fed broilers displayed higher values of yellowness (b*) ($p < 0.001$), pigments ($p < 0.05$), and n-3 PUFA ($p < 0.01$), while n-6/n-3 ratio ($p < 0.001$) and α-tocopherol ($p < 0.001$) decreased relative to the CON. In conclusion, the introduction of a high level of Spirulina into broiler diets for an extended duration, has the potential to diminish birds' growth performance, possibly due to increased digesta viscosity. However, it does enhance the nutritional quality of the meat.

KEYWORDS

Spirulina, peptidase, broiler chicken, growth performance, meat quality

1 Introduction

The escalating demand for alternative protein sources as substitutes for conventional ingredients, like soybean (1), has stimulated interest in microalgae as a promising candidate. Notably, poultry has significant potential for integrating microalgae into their diets, indicating its potential for broader application in commercial animal feed (2). In particular, Arthrospira platensis, more commonly known as Spirulina, has gained recognition as an advantageous component of poultry nutrition (3). Spirulina's high protein content, fluctuating between 50 and 70% (4), positions it as a viable alternative or complement to traditional protein sources (5–7).

Spirulina, an edible microalga belonging to the blue-green algae group (*Cyanophyceae* or cyanobacteria), is recognized for its distinct filamentous multicellular structure and resilience in harsh environments (2, 8, 9). Its cell wall, akin to that of Gram-negative bacteria (10), is predominantly comprised of peptidoglycan and lipopolysaccharides, forming a robust multi-layered barrier (11). Aside from its high protein content and the presence of all essential amino acids (12), Spirulina serves as a bountiful reservoir of carbohydrates, a variety of vitamins including pro-vitamin A, vitamin C and vitamin E, as well as a host of minerals such as iron, calcium, chromium, copper, magnesium, manganese, phosphorus, potassium, sodium and zinc (13, 14). Further enriching its nutritional profile, Spirulina also provides essential fatty acids and an array of pigments including chlorophyll a, phycocyanin, carotenes and xanthophylls (4, 15–19).

Despite the nutrient-rich profile of Spirulina, the indigestible nature of its cell wall and the resilience of its protein-pigment complexes, known as phycocyanins, which are tied to microalgal thylakoid membranes, pose challenges to its protein's bioaccessibility and digestibility (20). To mitigate these issues, recent *in vitro* studies (21, 22) have investigated the potential of peptidases (EC 3.4), with or without prior treatments, in the hydrolysis of Spirulina proteins. Notably, Niccolai et al. (22) observed a remarkable increase of up to 81% in crude protein digestibility of Spirulina when applying a combination of pepsin and pancreatin. Furthermore, Costa et al. (21) confirmed the beneficial effect of pancreatin, especially when preceded by an extrusion pre-treatment, on the hydrolysis of protein potentially associated with phycocyanins. Peptidases catalyse the degradation of peptide bonds, are involved in the degradation of off-function proteins in cells and play an important role in cell homeostasis (23). However, proteins freed from Spirulina biomass could undergo a gelation process, potentially elevating the viscosity of the digesta and thereby entrapping other nutrients, which limits their absorption in monogastric animals (24, 25). This effect was demonstrated by Pestana et al. (25), who noticed a surge in digesta viscosity resulting in hindered growth performance in broilers fed a high level of Spirulina (15% of feed) supplemented with lysozyme (26). A similar observation was made when piglets were fed 10% of the microalga, where Spirulina proteins showed resistance to the activity of endogenous peptidases, consequently reducing protein digestibility (27). The application of 15% of Spirulina in broilers has been already studied, with no negative effect on production performance or meat quality (28).

Although the beneficial role of exogenous enzymes, such as carbohydrate-active enzymes (CAZymes), in enhancing the nutritional value of poultry diets is well-recognized (26, 29, 30), there remains a significant gap in our understanding of the *in vivo* impact of peptidases on augmenting the bioaccessibility of Spirulina proteins for monogastric animals. These enzymes are used extensively for their broad-spectrum activity against various substrates like barley, a key component of broiler diets (31, 32). CAZymes facilitate improved access to endogenous enzymes to their target substrates, by enhancing feed conversion and digestibility, mainly in young animals with immature digestive tracts (29). Similar to CAZymes, supplementing peptidases in diets that contain a high proportion of Spirulina could potentially boost broiler digestibility. One of the mixtures contains peptidase and α-amylase (VemoZyme® P) and the other encompasses pancreatic peptidase, lipase and amylase (porcine pancreatin). Despite limited information available on the function and applicability of VemoZyme® P in poultry nutrition, related enzymes from the same manufacturer, namely VemoZyme® F (a phytase) and VemoZyme® Plus (a complex enzyme preparation including carbohydrases), have been successfully utilized in laying hens (33) and broilers diets (34). Pancreatin, as a diverse enzyme mixture, is anticipated to aid broilers with an immature digestive tract in handling dietary changes, such as a transition from a corn and soybean meal-based diet to a Spirulina-enriched diet. Notably, previous studies employed pancreatin mixtures in broiler diets, demonstrating positive outcomes for fat digestibility (35). However, to our knowledge, the effects of this mixture on the degradation of algal proteins have not been explored yet.

Furthermore, while certain studies have scrutinized the influence of dietary Spirulina incorporation on broiler performance and meat quality (e.g., Pestana et al. (25), Abdelfatah et al. (36), Costa et al. (37), and Hassan et al. (38)), there exists a paucity of research probing into the combination of long-duration feeding periods with high inclusion levels of Spirulina.

Hence, the purpose of this study was to investigate the impact of incorporating 15% Spirulina in broiler diets, either alone or combined with commercial VemoZyme® P or porcine pancreatin extract, on broiler growth performance, gut viscosity, carcass traits and meat quality, spanning from day 7–35.

2 Materials and methods

2.1 Animal welfare statement

The experimental procedures conducted in this study were performed in compliance with ethical guidelines and regulations. The protocols followed were approved by the Ethics Commission of CIISA/FMV (Centre for Interdisciplinary Research in Animal Health, Faculty of Veterinary Medicine) and the Animal Care Committee of the National Veterinary Authority (Direção Geral de Alimentação e Veterinária, Lisboa, Portugal). Additionally, the study adhered to the principles and specific guidelines outlined in the European Union legislation (2010/63/EU Directive) concerning the use of animals in scientific research. The Animal Welfare Committee of the Higher Institute of Agronomy at the University of Lisbon (ORBEA/ISA) also approved the experimental procedures with animals, and the study was assigned a protocol code number 0421/000/000/2022.

2.2 Animals, management and diets

A total of 120 one-day-old male Ross 308 broiler chicks were housed in 40 wired-floor cages for 35 days, following the procedures

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described in Alfaia et al. (39), Pestana et al. (25), and Costa et al. (40). The birds were reared in a controlled environment room under standard brooding practices, with appropriate lighting conditions. At day 0, room temperature was maintained at 31°C, following 30°C on day 1, and from day 3 to 27, it decreased 1°C every 3 days to 20°C, after it remained constant until the end of the study. The temperature and ventilation in the room were continuously monitored throughout the study period from day 1–35. Also, the temperature at the cage level was monitored from day 1–35. Each cage measured 66 × 66 cm, with 2 drinking nipples and one feeder.

During the initial 7 days, the birds were provided with *ad libitum* access to a corn and soybean-based diet. From day 7–35, the birds were assigned to one of four dietary treatments: 1) a control diet based on corn and soybean meal (CON); 2) a diet containing 15% Spirulina powder (Allmicroalgae, Pataias, Portugal) (SP); 3) a diet containing 15% Spirulina powder supplemented with 0.025% of the commercial enzyme mix VemoZyme® P (VEMO, Sofia, Bulgaria) (SPV); and 4) a diet containing 15% Spirulina powder supplemented with 0.10% of porcine pancreatin extract (Merck, Darmstadt, Germany) (SPP). The VemoZyme® P enzyme had a proteolytic activity of 15,000 tyrosine units and an amylase activity of 400 units, as stated by the manufacturer. The porcine pancreatin extract contained 350 FIP-U/g protease, 6,000 FIP-U/g lipase and 7,500 FIP-U/g amylase. All diets were formulated to meet the nutrient requirements defined by the NRC (41), and the compositions and nutrient content analysis of the starter, and grower diets plus microalga powder are provided in Tables 1, 2, respectively.

The experimental design consisted of 10 replicate cages, with 3 birds per cage. Weekly measurements of broiler and feeder weights were recorded, and feed was provided daily to calculate body weight gain (difference between body weight of two consecutive weeks, divided by 7), average daily feed intake (weekly ingestion by each cage, divided by 7) and feed conversion ratio (ratio between weekly ingestion divided by 3 and weekly body weight gain). Diet samples were analysed for dry matter (DM) by drying a sample at 103°C until a constant weight was achieved. The nitrogen (N) content of the diets was determined using the Kjeldahl method according to AOAC method 954.01 (42), and crude protein content was calculated as 6.25 times the nitrogen content. Ash content was determined using AOAC method 942.05 (42). Crude fat was determined by extracting feed samples with petroleum ether using an automatic Soxhlet extractor (Gerhardt Analytical Systems, C. Gerhardt GmbH & Co. KG, Königswinter, Germany), following a prior hydrolysis with hydrochloric acid. The gross energy of the feed was determined using adiabatic bomb calorimetry (Parr 1,261, Parr Instrument Company, Moline, IL, United States).

On day 35, one bird from each cage, with the middle weight, was subjected to electrical stunning and manual exsanguination. Blood samples were collected in Sarstedt tubes (Numbrecht, Germany) and centrifuged to obtain serum. The gastrointestinal (GI) organs (crop, gizzard, duodenum, jejunum, ileum and cecum) were removed and emptied, and the weight of the crop, gizzard, liver, pancreas, duodenum, jejunum, ileum and cecum was recorded. The length of the duodenum, jejunum, ileum, and cecum was also measured. Duodenum in the proximal part of the intestinal tract, with a "U" shape around the pancreas. The jejunum is the distal part of the duodenum. Anterior to the junction of the cecum, is the ileum. The cecum is a paired tubular structure distal along the ileum from the ileo-caecal-colic junction (43). The viscosity of the contents of the small intestine was determined following the method described by Pestana et al. (25). Briefly, samples were collected from the duodenum plus jejunum and ileum, centrifuged for 10 min at 9.000 rpm, and the viscosity of the supernatant was measured using a viscometer (Model LVDVCP-II, Brookfield Engineering Laboratories, Middleboro, MA, United States) at a room temperature maintained at 24°C. Samples of breast and thigh muscles (deboned and skinless) were chopped and kept in aluminium foil, bagged in vacuum bags and stored at −20°C until analyses.

TABLE 1 Ingredient composition and nutrient content analysis of broiler experimental starter diets (day 7–14) (%, as-fed basis).

	CON	SP	SPV	SPP
Ingredients, %				
Corn	50.9	58.3	58.3	58.3
Soybean meal	41.8	23.2	23.2	23.2
Sunflower oil	3.40	0.0	0.0	0.0
Sodium chloride	0.400	0.400	0.400	0.400
Calcium carbonate	1.15	1.47	1.47	1.47
Dicalcium phosphate	1.80	1.10	1.10	1.10
DL-Methionine	0.150	0.050	0.050	0.050
L-Lysine	0.0	0.060	0.060	0.060
Vitamin-mineral premix[1]	0.400	0.400	0.400	0.400
Spirulina powder	0.0	15.0	15.0	15.0
VemoZyme® P	0.0	0.0	0.025	0.0
Porcine Pancreatin	0.0	0.0	0.0	0.100
Nutrient content[2]				
Gross energy, MJ/kg DM	19.1	18.5	18.3	18.2
Crude Protein, % DM	25.5	25.5	26.0	25.7
Crude fat, % DM	6.65	3.87	4.10	3.98
Ash, % DM	7.14	8.93	8.76	8.54

CON, Corn and soybean meal-based diet, in the control group; SP, 15% Spirulina; SPV, 15% Spirulina + 0.025% VemoZyme® P; SPP, 15% Spirulina + 0.10% Porcine pancreatin.
[1]Premix provided the following per kilogram of diet: pantothenic acid 10 mg, vitamin D_3 2,400 IU, cyanocobalamin 0.02 mg, folic acid 1 mg, vitamin K_3 2 mg, nicotinic acid 25 mg; vitamin B_6 2 mg, vitamin A 10000 UI, vitamin B_1 2 mg, vitamin E 30 mg, vitamin B_2 4 mg, Cu 8 mg, Fe 50 mg, I 0.7 mg, Mn 60 mg, Se 0.18 mg, Zn 40 mg.
[2]Nutrient content analysed.

2.3 Determination of carcass traits

The pH and colour analysis of the meat were conducted following the methods described in Pestana et al. (25) and Alfaia et al. (39). Briefly, the right breast (pectoralis major) and thigh (biceps femoris) muscles were deboned and skinned. Triplicate measurements were taken from three different spots on each muscle. The pH values were determined using a glass penetration pH electrode (HI9025, Hanna

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TABLE 2 Ingredient composition and nutrient content analysis of broiler experimental grower diets and microalga powder (day 14–35) (%, as-fed basis).

	Spirulina	CTR	SP	SPV	SPP
Ingredients, %					
Corn	–	55.6	62.2	62.2	62.2
Soybean meal	–	36.9	18.6	18.6	18.6
Sunflower oil	–	4.00	1.00	1.00	1.00
Sodium chloride	–	0.300	0.400	0.400	0.400
Calcium carbonate	–	1.08	1.39	1.39	1.39
Dicalcium phosphate	–	1.60	0.900	0.900	0.900
DL-Methionine	–	0.120	0.030	0.030	0.030
L-Lysine	–	0.0	0.050	0.050	0.050
Vitamin-mineral premix[1]	–	0.400	0.400	0.400	0.400
Spirulina powder	–	0.0	15.0	15.0	15.0
VemoZyme® P	–	0.0	0.0	0.025	0.0
Porcine Pancreatin	–	0.0	0.0	0.0	0.100
Nutrient content[2]					
Gross energy, MJ/kg DM	18.7	19.1	18.5	18.5	18.4
Crude Protein, % DM	52.5	23.6	23.5	23.1	23.3
Crude fat, % DM	6.56	7.88	5.19	5.30	5.14
Ash, % DM	21.6	6.41	8.21	8.27	8.19
Calcium, % DM	1.20	1.42	1.87	2.21	2.01
Available phosphorus, % DM	0.001	0.710	0.593	0.700	0.660
Sodium, % DM	4.51	0.299	0.860	1.03	0.94
Fatty acid profile, % total fatty acids					
14:0	0.432	0.072	0.078	0.119	0.113
16:0	39.7	9.90	16.1	16.2	15.9
16:1c9	4.95	0.133	0.796	0.797	0.776
17:0	0.410	0.065	0.105	0.122	0.123
17:1c9	1.88	0.035	0.215	0.222	0.221
18:0	1.75	3.05	3.19	2.56	2.58
18:1c9	6.34	31.8	27.1	26.7	27.4
18:2n-6	17.8	51.1	45.4	46.3	45.9
18:3n-3	15.8	1.27	3.36	3.43	3.46
20:0	0.189	0.379	0.415	0.426	0.431
20:1c11	0.9106	0.160	0.204	0.206	0.215
22:0	0.247	0.580	0.383	0.391	0.393
Diterpene profile, μg/g					
α-Tocopherol	29.5	58.1	57.3	51.1	47.5
α-Tocotrienol	–	4.37	4.36	3.77	4.29
β-Tocopherol		0.512	0.360	0.367	0.342
γ-Tocopherol + *β*-Tocotrienol	–	7.74	6.96	7.46	6.81
γ-Tocotrienol	–	10.9	10.1	10.6	10.2
δ-Tocopherol	–	0.904	0.752	0.687	0.751
Pigments, μg/g DM					
β-Carotene	294	1.34	115	121	107
Chlorophyll a[3]	1,600	13.3	504	517	538
Chlorophyll b[4]	1,404	26.8	74.0	67.0	70.9
Total chlorophylls[5]	3,004	40.0	578	584	608
Total carotenoids[6]	447	2.17	152	154	161
Total chlorophylls and carotenoids[7]	3,450	42.2	730	738	769
Minerals (mg/kg DM)					
Macrominerals					
Calcium (Ca)	12,010	14,202	18,735	22,143	20,110
Magnesium (Mg)	8,894	1955	1,526	1912	1869
Potassium (K)	3,230	11,961	10,587	11,501	11,161
Sulphur (S)	67.5	2,929	3,098	3,537	3,261
Total	69,405	41,135	48,476	56,403	52,458
Microminerals					
Copper (Cu)	946	13.2	15.3	16.7	18.4
Iron (Fe)	129,528	156	241	317	284
Manganese (Mn)	67.5	71.0	79.0	96.0	92.3
Zinc (Zn)	99.0	89.3	76.8	93.6	89.3
Total	131,697	329	412	523	484
Total macro and microminerals	201,101	41,464	48,888	56,926	52,942

CON, Corn and soybean meal-based diet, in the control group; SP, 15% Spirulina; SPV, 15% Spirulina +0.025% VemoZyme® P; SPP, 15% Spirulina +0.10% Porcine pancreatin.

[1]Premix provided the following per kilogram of diet: pantothenic acid 10 mg, vitamin D¬32,400 IU, cyanocobalamin 0.02 mg, folic acid 1 mg, vitamin K3 2 mg, nicotinic acid 25 mg; vitamin B6 2 mg, vitamin A 10000 UI, vitamin B1 2 mg, vitamin E 30 mg, vitamin B2 4 mg, Cu 8 mg, Fe 50 mg, I 0.7 mg, Mn 60 mg, Se 0.18 mg, Zn 40 mg.
[2]Nutrient content analysed.
[3]Ca: Chlorophyll a = 11.24 × A662 nm−2.04 × A645 nm.
[4]Cb: Chlorophyll b = 20.13 × A645 nm−4.19 × A662 nm.
[5]Ca + b: Total chlorophylls = 7.05 × A662 nm + 18.09 × A645 nm.
[6]Cx + c: Total carotenoids = (1,000 × A470 nm−1.90 × Ca−63.14 × Cb) / 214.
[7]Ccc: Total chlorophylls and carotenoids = (Ca + b) + (Cx + c).

Instruments, Woonsocket, RI, United States), while colour measurements [lightness (L*), redness (a*), and yellowness (b*)] were obtained using a Minolta CR-300 Chromameter (Minolta Camera Co. Ltd., Osaka, Japan) based on the CIELAB colour space. The data were recorded after a 24-h cooling post-mortem period at 4°C, followed by 1 h of air exposure.

2.4 Sensorial panel traits of breast meat

The sensory analysis was performed following the procedures outlined in Pestana et al. (25). In brief, the right skinless breast muscles were individually cooked in a water bath at 85°C using plastic bags until the internal temperature reached 78°C. For the sensory evaluation, the cooked muscle samples were trimmed of external connective tissue and cut into approximately 1 cm^3 cubes. These cubes were then kept at 60°C on pre-identified heated plaques. A panel of 10 trained assessors, selected and trained according to Cross (44) at the Faculty of Veterinary Medicine (University of Lisbon, Portugal), evaluated the samples. The sensory panel sessions consisted of five sessions, with eight random samples per session. The attributes assessed were tenderness, juiciness, off-flavours and overall acceptability. A structured 8-point scale was used for the sensory evaluation, with 1 representing extremely tough, dry, weak, and negative attributes, and 8 representing extremely tender, juicy, strong, and positive attributes for tenderness, juiciness, flavour and overall acceptability, respectively.

2.5 Determination of meat lipid oxidative stability

Approximately 1.5 g of minced meat from the left breast of each bird was divided into four portions and placed in plastic bags. These portions were then exposed to air and stored in a freezer at 4°C for 0 and 8 days. The concentration of thiobarbituric acid reactive substances (TBARS), an indicator of lipid oxidation, was measured on day 0 and day 8. The spectrophotometric method described by Mercier et al. (45) was followed to analyse the ability of malondialdehyde, a product of lipid oxidation, to form a pink-coloured chromogen that absorbs 532 nm light. The measurements were taken using a UV/visible spectrophotometer (Genesys 150, ThermoScientific, Madison, United States). For TBARS quantification, a standard calibration curve was prepared using 1,1,3,3-tetraethoxypropane (Fluka, Neu Ulm, Germany) as a precursor of malondialdehyde. The results are expressed as milligrams of malondialdehyde per kilogram of meat.

2.6 Determination of total cholesterol, diterpenes, pigments and minerals in meat and experimental diets

The determination of total cholesterol, β-carotene and tocopherols in both fresh meat (750 mg) and feed (100 mg) followed the protocol described by Prates et al. (46), with specific details provided in Pestana et al. (25), Alfaia et al. (39), Costa et al. (40). The measurements were performed in duplicate, and the concentrations were calculated based on the external standard technique using a standard curve of peak area versus concentration.

For the analysis of chlorophyll a, chlorophyll b and total carotenoids, the method outlined by Teimouri et al. (47) was employed, with slight modifications described in Pestana et al. (25), Alfaia et al. (39), and Costa et al. (40). All procedures related to pigment extraction and analysis were conducted under dim light conditions to minimize the photodegradation of pigments. The contents of pigments were determined using equations described by Hynstova et al. (16).

To determine the mineral profile, including calcium (Ca), potassium (K), magnesium (Mg), sodium (Na), phosphorus (P), sulphur (S), copper (Cu), iron (Fe), manganese (Mn) and zinc (Zn), the procedures outlined in Ribeiro et al. (48) and Costa et al. (40) were followed. Inductively coupled plasma optical emission spectrometry (ICP-OES) with an iCAP 7,200 duo instrument (Thermo Scientific, Waltham, MA, United States) was utilized for the analysis. Calibration curves using multi-element standards (PlasmaQual S22, SPC Science, Baie-D'Urfé, QC, Canada) were employed to quantify the different elements.

2.7 Determination of dry matter and total lipids in meat

The dry matter content of breast and thigh meats was determined following the method described by Rosenkranz (49). Freeze-dried samples were obtained by using a freeze-drier (Labogene, CoolSafe, Lillerod, Denmark) at −60°C and 2.0 hPa. The freeze-drying process was carried out until a constant weight was achieved. After freeze-drying, the samples were stored in desiccators at room temperature until further analysis.

To determine the total lipids in the meat samples, as well as in the feed, the freeze-dried breast and thigh muscles were subjected to lipid extraction according to the procedure outlined by Folch et al. (50). The extraction was performed using a mixture of dichloromethane and methanol in a ratio of 2:1 (v/v). The extracted lipids were then evaporated to dryness, and the fatty residue obtained was weighed gravimetrically. The measurements were performed in duplicate.

2.8 Determination of fatty acid composition in meat and experimental diets

The fatty acid composition of breast and thigh muscles was determined by converting the fatty acid residue into fatty acid methyl esters (FAME). This conversion was carried out using a combined basic and acidic transesterification procedure. Firstly, the fatty acids were transesterified using NaOH in anhydrous methanol (0.5 M) at 50°C for 30 min. Subsequently, HCl/methanol (1:1 v/v) was used for a second transesterification step at 50°C for 10 min (51). The same procedure was applied for the analysis of FAME in feed samples, except that direct transesterification was performed using HCl/methanol (1:1 v/v) at 70°C for 2 h.

The analysis of FAME was conducted following the method described by Pestana et al. (25). Gas chromatography (GC System, 7890A, Agilent Technologies, California, United States) comprising a Supelcowax® 10 capillary column (30 m × 0.20 mm internal diameter,

0.20 μm film thickness; Supelco, Bellefonte, PA, United States) and flame ionization detector, was employed for the analysis, using specific parameters. The injector temperature and detector temperature were set at 250°C and 280°C, respectively. Helium gas was used as the carrier gas at a flow rate of 1.0 mL/min, with a split ratio of 1:20. The gas chromatograph oven temperature was programmed as follows: an initial temperature of 50°C was maintained for 4 min, followed by a ramp of 13°C/min to 175°C (maintained for 20 min), and then a ramp of 4°C/min to 275°C (maintained for 44 min).

Identification of FAME was based on a comparison of retention times with a standard (FAME mix 37 components, Supelco Inc. Bellefonte, PA, United States) confirmed by gas chromatography coupled to mass spectrometry using a GC–MS QP2010-Plus (Shimadzu, Kyoto, Japan). Quantification of FAME was achieved using heneicosanoic acid (21:0) methyl ester as the internal standard. The resulting fatty acid composition was expressed as g/100 g of total fatty acids.

2.9 Statistical analysis

All the data were analysed using the Generalized Linear Mixed (GLM) model of the SAS program (SAS Institute Inc., Cary, NC, United States) for most variables, except for the TBARS values, the repeated measures in time (PROCMIXED) procedure of SAS were employed. The cage served as the experimental unit for body weight, body weight gain, average daily feed intake and feed conversion ratio, while the individual bird was considered as the experimental unit for gastrointestinal organs weight and length, and content viscosity and meat quality measurements. To determine statistical differences among dietary treatments, a significant multiple comparisons test was conducted using the PDIFF option, which was adjusted with the Tukey–Kramer method. A p-value less than 0.05 was considered statistically significant to indicate meaningful differences.

3 Results

3.1 Growth performance and gastrointestinal tract parameters

Table 3 compiles the data on birds' growth performance and gastrointestinal tract parameters in response to the different dietary conditions. Birds subjected to Spirulina diets manifested diminished body weights (BW) on day 35, in contrast to those sustained on the control diet (CON) ($p < 0.001$). The pattern persisted throughout the treatment span (days 14, 21, 28 and 35), wherein birds fed with Spirulina (SP, SPV and SPP) consistently showed lesser gains in body weight (BWG) and lower average daily feed intake (ADFI) relative to the control group (CON) ($p < 0.001$).

Similarly, the feed conversion ratio (FCR) displayed higher values for the Spirulina-fed groups (SP, SPV and SPP), as compared to the control group (CON) ($p < 0.001$). However, this trend was interrupted between days 21–28 and 28–35, wherein no significant FCR differences were noted. In addition, birds fed Spirulina, irrespective of enzyme supplementation, presented with increased viscosity in the ileum contents relative to their counterparts in the control diet (CON) ($p < 0.001$).

Despite these trends, most gastrointestinal (GI) tract organ weights remained largely unaffected by the dietary treatment ($p > 0.05$), as outlined in Table 4. Exceptions included the relative weights of the pancreas ($p < 0.001$) and duodenum ($p < 0.05$), which increased across all Spirulina-fed groups (SP, SPV and SPP). Moreover, the relative lengths of the duodenum, jejunum, ileum and caecum were greater in all Spirulina-fed groups (SP, SPV and SPP) ($p < 0.001$).

3.2 Carcass traits, meat quality and sensory evaluation

Table 5 presents a detailed analysis of the impact of dietary treatments on the carcass traits and meat quality of broiler birds. The study found no significant variance in the lightness (L*) and redness (a*) of the breast and thigh meats across the different dietary treatments ($p > 0.05$). However, significant differences were observed in terms of pH24h ($p < 0.05$) and yellowness (b*) ($p < 0.001$), both of which were markedly higher in birds fed Spirulina as compared to those subjected to CON.

As presented in Table 6, sensory evaluations, conducted by a trained panel for the breast meat, highlighted several significant differences between the diet groups. Birds that were fed Spirulina diets, irrespective of whether these were supplemented with enzymes, exhibited lower scores for meat tenderness, juiciness, and overall acceptability compared to those in the control group (CON) ($p < 0.001$). Nonetheless, the inclusion of both enzymes (SPV and SPP) appeared to counteract the decrease in meat flavour attributed to the Spirulina diets ($p < 0.05$). The addition of pancreatin seemed to particularly aid in reversing the decline in meat tenderness and overall acceptability. Of note, dietary treatments did not result in any significant changes in off-flavours ($p > 0.05$).

3.3 Oxidative stability, diterpenes and total pigments

Table 7 presents the findings on the oxidative stability of broiler breast meat across various dietary treatments over two time periods (day 0 and day 8). Oxidative stability is gauged by the measurement of TBA reactive substances, specifically MDA levels. It is worth noting that no significant interaction was found between dietary treatment and period ($p > 0.05$). On both day 0 and day 8, the MDA levels across all dietary treatments did not demonstrate significant differences ($p > 0.05$). However, a noteworthy exception was observed with the SPV treatment, which showed a marked increase in MDA levels between day 0 and day 8 ($p < 0.001$). This indicates that there might be an increase in the oxidation rate of broiler meat within this specific dietary group over time.

Table 8 depicts the impact of different dietary treatments on the diterpene profile and pigments in the meat of broilers. The data indicate a marked decrease in the levels of diterpenes, specifically α-tocopherol and γ-tocopherol+β-tocotrienol, in both breast and thigh meats of broilers fed with Spirulina-based diets when compared to the control group (CON) ($p < 0.001$). Moreover, the minor diterpenes, α-tocotrienol and β-tocopherol, were detected in breast meat but were absent in thigh meat. Conversely, the levels of pigments, including total chlorophylls, total carotenoids and their combined

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TABLE 3 Growth performance and gut content viscosity of broilers.

	CON	SP	SPV	SPP	SEM	*p*-value
Body weight, g						
Day 7	126	129	132	125	4.23	0.712
Day 14	339 [a]	268[b]	261[b]	255[b]	8.42	<0.001
Day 21	663[a]	447[b]	449 [b]	447[b]	18.0	<0.001
Day 28	1,041[a]	685[b]	698 [b]	696[b]	28.9	<0.001
Day 35	1,501[a]	998[b]	1,009[b]	1,035[b]	44.0	<0.001
Body weight gain, g						
Day 7–14	30.8[a]	19.8[b]	18.5[b]	18.6[b]	0.775	<0.001
Day 14–21	45.8[a]	25.6[b]	26.8 [b]	27.4[b]	1.60	<0.001
Day 21–28	54.0[a]	34.0[b]	35.6[b]	35.6[b]	2.02	<0.001
Day 28–35	65.9[a]	44.8[b]	44.5[b]	48.5[b]	2.73	<0.001
Day 7–35	50.9[a]	32.2[b]	32.5[b]	33.7[b]	1.55	<0.001
Average daily feed intake, g/pen						
Day 7–14	114[a]	93.7[b]	90.2[b]	89.7[b]	2.70	<0.001
Day 14–21	197[a]	132[b]	136[b]	140[b]	6.08	<0.001
Day 21–28	257[a]	173[b]	181[b]	184[b]	9.57	<0.001
Day 28–35	321[a]	234[b]	246[b]	256[b]	13.5	<0.001
Day 7–35	222[a]	158[b]	163[b]	167[b]	7.29	<0.001
Feed conversion ratio						
Day 7–14	1.30[b]	1.67[a]	1.75[a]	1.67[a]	0.0433	<0.001
Day 14–21	1.55[b]	1.84[a]	1.78[a]	1.8 [a]	0.0503	<0.001
Day 21–28	1.71	2.02	1.79	2.33	0.300	0.470
Day 28–35	1.76	1.90	2.17	1.89	0.127	0.159
Day 7–35	1.61[b]	1.78[a]	1.81[a]	1.83[a]	0.0364	<0.001
Content viscosity, cP						
Duodenum + jejunum	4.26	5.45	6.07	5.76	0.526	0.123
Ileum	6.23[b]	11.8[a]	12.8[a]	12.8[a]	0.903	<0.001

SEM, standard error of the mean.

[a,b] Different superscripts within a row indicate a significant difference ($p < 0.05$).

CON, Corn and soybean meal-based diet, in the control group; SP, 15% Spirulina; SPV, 15% Spirulina +0.025% VemoZyme® P; SPP, 15% Spirulina +0.10% Porcine pancreatin.

sum, were significantly elevated in the broiler breast and thigh meats from the Spirulina-fed groups (SP, SPV and SPP) compared to the control group (CON) (all $p < 0.001$, except $p = 0.003$ in total chlorophylls in breast meat). Among the Spirulina-fed groups, those combined with enzymes (SPV and SPP groups), exhibited higher levels of β-carotene in breast meat compared to the control group (CON) ($p = 0.008$), although no significant differences were found in the thigh muscle.

3.4 Meat lipids and fatty acid composition

Table 9 presents the total lipid content, total cholesterol content and fatty acid profile of both breast and thigh meats. The total lipid and total cholesterol contents of breast meat in Spirulina-fed birds were significantly lower ($p < 0.001$) than those fed the control diet (CON). However, the difference was not statistically significant in the thigh meat ($p = 0.061$ for total lipids, and $p = 0.683$ for total cholesterol).

For the fatty acid profile of breast meat, there was a significant increase ($p < 0.001$, in general) in the levels of several fatty acids (16:0, 17:0, 14:1c9, 16:1c9, 17:1c9, 18:1c11, 20:3n-6, 20:4n-6, 20:3n-3, 20:5n-3, 22:5n-3 and 22:6n-3), saturated fatty acids (SFA), and n-3 polyunsaturated fatty acids (n-3 PUFA) in Spirulina-fed birds. Additionally, Spirulina-fed groups (SP, SPV and SPP) led to decreased content of 18:2n-6 ($p < 0.001$), total PUFA ($p = 0.001$), n-6 PUFA ($p < 0.001$), n-6/n-3 ($p < 0.001$), and PUFA/SFA ($p < 0.001$) ratios. An interesting observation was a tendency for the content of 18:3n-6 to decrease with SP or SPP diets, and an increase with the SPV diet ($p = 0.046$). SPV and SPP diets also significantly increased 18:3n-3 ($p = 0.033$) and 20:2n-6 ($p = 0.002$) contents, respectively.

Similarly, for the thigh meat, Spirulina-fed birds exhibited increased contents of certain fatty acids (16:0, 17:0, 14:1c9, 16:1c9, 17:1c9, 18:1c11, 18:3n-3 and n-3 PUFA) compared to the CON group (majority $p < 0.001$). However, 18:2n-6 ($p = 0.003$), 18:3n-6 ($p = 0.001$) content, and n-6/n-3 ratio ($p = 0.022$) were significantly lower in

TABLE 4 Relative weight and length of the gastrointestinal tract of broilers.

	CTR	SP	SPV	SPP	SEM	*p*-value
Relative weight of GI[1] tract, g/kg BW						
Crop	3.51	5.00	4.92	5.37	0.573	0.127
Gizzard	17.2	18.6	18.5	16.3	1.26	0.510
Liver	23.8	21.5	21.7	22.2	1.07	0.439
Pancreas	2.58^{b}	3.13^{a}	3.36^{a}	3.08^{a}	0.119	<0.001
Duodenum	5.87^{b}	6.55^{ab}	7.29^{a}	7.46^{a}	0.346	0.009
Jejunum	11.4	10.2	11.8	10.6	0.644	0.289
Ileum	9.78	9.71	11.0	10.9	0.407	0.108
Caecum[2]	4.46	5.52	4.73	5.45	0.349	0.094
Relative length of GI tract, cm/kg BW						
Duodenum	20.2^{b}	28.2^{a}	29.1^{a}	28.1^{a}	1.53	<0.001
Jejunum	45.3^{b}	63.5^{a}	66.4^{a}	64.7^{a}	3.33	<0.001
Ileum	53.5^{b}	72.6^{a}	72.2^{a}	73.8^{a}	3.52	<0.001
Caecum	12.2^{b}	15.6^{a}	15.7^{a}	16.5^{a}	0.874	0.007

SEM, standard error of the mean.

^{a,b} Different superscripts within a row indicate a significant difference ($p < 0.05$).

CON, Corn and soybean meal-based diet, in the control group; SP, 15% Spirulina; SPV: 15% Spirulina +0.025% VemoZyme® P; SPP, 15% Spirulina +0.10% Porcine pancreatin.[1]GI, Gastrointestinal.

[2]Caecum, weight of 2 caecum.

TABLE 5 Carcass traits and meat quality of broilers' breast and thigh meats fed with Spirulina.

	CON	SP	SPV	SPP	SEM	*p*-value	CON	SP	SPV	SPP	SEM	*p*-value
	Breast meat						Thigh meat					
pH24h	5.81^{a}	5.57^{b}	5.56^{b}	5.55^{b}	0.034	<0.001	6.06^{a}	5.93^{ab}	5.96^{ab}	5.88^{b}	0.046	0.051
Lightness (L*)	59.81	57.71	58.99	58.15	0.983	0.453	54.45	54.90	56.20	55.10	0.757	0.419
Redness (a*)	4.40	4.99	5.03	4.86	0.360	0.592	7.13	8.15	8.68	8.92	0.542	0.110
Yellowness (b*)	9.20^{b}	20.93^{a}	22.87^{a}	21.36^{a}	0.730	<0.001	10.02^{b}	23.59^{a}	25.86^{a}	25.72^{a}	0.878	<0.001

SEM, standard error of the mean.

^{a,b} Different superscripts within a row indicate a significant difference ($p < 0.05$).

CON, Corn and soybean meal-based diet, in the control group; SP, 15% Spirulina; SPV, 15% Spirulina +0.025% VemoZyme® P; SPP, 15% Spirulina +0.10% Porcine pancreatin.

TABLE 6 Sensorial panel traits of broiler breast meat.

	CON	SP	SPV	SPP	SEM	*p*-value
Tenderness	6.04^{a}	5.00^{b}	5.31^{b}	5.63^{ab}	0.186	<0.001
Juiciness	5.45^{a}	4.56^{b}	4.44^{b}	4.61^{b}	0.157	<0.001
Flavour	6.01^{a}	5.48^{b}	5.59^{ab}	5.69^{ab}	0.121	0.013
Off-flavour	0.096	0.196	0.151	0.201	0.0706	0.684
Overall	5.85^{a}	4.92^{b}	5.06^{b}	5.31^{ab}	0.153	<0.001

SEM, standard error of the mean.

^{a,b} Different superscripts within a row indicate a significant difference ($p < 0.05$).

CON, Corn and soybean meal-based diet, in the control group; SP, 15% Spirulina; SPV, 15% Spirulina +0.025% VemoZyme® P; SPP, 15% Spirulina +0.10% Porcine pancreatin.

The different attributes evaluated in sensorial traits were quantified on a rating scale from 1 (low score) to 8 (high score), except flavour and off-flavour which were quantified from 0 (absence) to 8 (very intense).

Spirulina-fed groups (SP, SPV and SPP). There was a potential decrease in total PUFA content ($p = 0.049$) with the inclusion of Spirulina. The thigh meat of birds fed SP and SPV showed higher contents of 22:6n-3 ($p = 0.003$), SFA ($p = 0.008$), and a significantly lower PUFA/SFA ratio ($p = 0.005$) than the control (CON). The 20:5n-3 content was significantly increased by SP and SPP diets ($p = 0.005$), whereas SPV significantly reduced n-6 PUFA content ($p = 0.022$).

TABLE 7 Oxidative stability of broiler breast meat measured as TBA reactive substances.

Malondialdehyde, mg/kg	CON	SP	SPV	SPP	SEM	*p*-value		
						Diet	Period	Diet*Period
Day 0	0.17^{x}	0.18^{x}	0.14^{x}	0.15^{x}	0.021	0.137		
Day 8	0.31^{abx}	0.27^{bx}	0.56^{ay}	0.30^{abx}	0.087		<0.001	0.059

SEM, standard error of the mean.
^{a,b} Different superscripts within a row indicate a significant difference ($p < 0.05$).
^{x,y} Different superscripts between rows indicate a significant difference ($p < 0.05$).
CON, Corn and soybean meal-based diet, in the control group; SP, 15% Spirulina; SPV, 15% Spirulina +0.025% VemoZyme® P; SPP, 15% Spirulina +0.10% Porcine pancreatin.

TABLE 8 Diterpene profile and pigments of broiler meats.

	CON	SP	SPV	SPP	SEM	*p*-value	CON	SP	SPV	SPP	SEM	*p*-value
	Breast meat						Thigh meat					
Diterpene profile, μg/g												
α-Tocopherol	10.5^{a}	2.20^{b}	2.53^{b}	2.00^{b}	0.426	<0.001	9.63^{a}	3.80^{b}	4.41^{b}	3.61^{b}	0.428	<0.001
γ-Tocopherol+β-Tocotrienol	0.11^{a}	0.05^{b}	0.06^{b}	0.05^{b}	0.010	<0.001	0.114^{a}	0.060^{bc}	0.075^{b}	0.056^{c}	0.005	<0.001
α-Tocotrienol	0.13	0.24	0.16	0.18	0.063	0.663	nd	nd	nd	nd	nd	nd
β-Tocopherol	0.026^{a}	0.018^{b}	0.018^{b}	0.018^{b}	0.001	<0.001	nd	nd	nd	nd	nd	nd
β-Carotene	0.039^{b}	0.058^{ab}	0.062^{a}	0.061^{a}	0.005	0.008	nd	0.224	0.175	0.174	0.021	0.156
Pigments, μg/100g												
Chlorophyll a[1]	22.4^{b}	34.6^{ab}	40.2^{a}	32.2^{ab}	4.12	0.033	15.0^{b}	36.6^{a}	36.5^{a}	32.6^{a}	1.25	<0.001
Chlorophyll b[2]	27.3^{b}	56.4^{a}	63.8^{a}	53.1^{a}	6.17	0.001	25.3^{b}	60.8^{a}	63.1^{a}	56.4^{a}	2.37	<0.001
Total chlorophylls[3]	49.8^{b}	90.1^{a}	104^{a}	85.2^{ab}	9.80	0.003	40.3^{b}	97.4^{a}	99.6^{a}	89.0^{a}	3.54	<0.001
Total carotenoids[4]	29.4^{c}	242^{b}	288^{a}	253^{ab}	9.95	<0.001	38.1^{b}	230^{a}	251^{a}	223^{a}	12.17	<0.001
Total chlorophyll and carotenoid[5]	79.2^{b}	351^{a}	392^{a}	338^{a}	14.86	<0.001	78.4^{b}	327^{a}	351^{a}	312^{a}	11.10	<0.001

SEM, standard error of the mean; nd, not detected.
^{a,b,c} Different superscripts within a row indicate a significant difference ($p < 0.05$).
CON, Corn and soybean meal-based diet, in the control group; SP, 15% Spirulina; SPV, 15% Spirulina +0.025% VemoZyme® P; SPP, 15% Spirulina +0.10% Porcine pancreatin.[1]Ca: chlorophyll a = 11.24 × A662 nm−2.04 × A645 nm.
[2]Cb: chlorophyll b = 20.13 × A645 nm−4.19 × A662 nm.
[3]Ca + b: total chlorophylls = 7.05 × A662 nm + 18.09 × A645 nm.
[4]Cx + c: total carotenoids = (1,000 × A470 nm−1.90 × Ca−63.14 × Cb) / 214.
[5]Ccc: total chlorophylls and carotenoids = (Ca + b) + (Cx + c).

3.5 Mineral composition

The mineral composition of breast and thigh meats of broilers subjected to the different diets is presented in Table 10. The influence of Spirulina-enriched diets is seen to have differing effects on the mineral composition of breast and thigh meats. For the breast meat, both zinc content and overall micromineral concentration significantly decreased as a result of Spirulina integration ($p < 0.001$). Conversely, the copper content increased in the breast meat from broilers fed the SPP diet in comparison to the control group (CON) ($p = 0.030$). In the case of thigh meat, there was a significant decrease in the levels of phosphorus ($p = 0.014$), total macrominerals ($p = 0.016$), zinc ($p < 0.001$) and total minerals ($p = 0.014$) in groups fed Spirulina (SP, SPV and SPP), whereas the sodium and total micromineral content exhibited an increase ($p = 0.007$ and $p < 0.001$, respectively). A decrease in magnesium and potassium was also observed in the thigh meat of birds that were fed SPV and SPP diets ($p = 0.010$ and $p = 0.029$, respectively).

To put the above results into perspective, for instance, the zinc level in breast meat declined from 1.15 mg/100 g in the CON to 0.698 mg/100 g in the SPP group. Similarly, in the thigh meat, the phosphorus content was reduced from 246 mg/100 g in the CON to 234 mg/100 g in the SP and SPV groups. However, sodium content in the thigh meat increased from 102 mg/100 g in the CON to 111 mg/100 g in the SPP group.

4 Discussion

The presence of protein and pigment complexes attached to the thylakoid membrane (20, 52) in Spirulina, makes the hydrolysis of algal proteins difficult. This associated with the recalcitrance of

TABLE 9 Total lipid content, cholesterol content and fatty acid profile of broiler meats.

	CON	SP	SPV	SPP	SEM	*p*-value	CON	SP	SPV	SPP	SEM	*p*-value
	Breast meat						Thigh meat					
Total lipids, g/100 g	1.35^{a}	0.81^{b}	0.95^{b}	0.88^{b}	0.079	<0.001	2.90	2.32	2.39	2.45	0.162	0.061
Cholesterol, mg/g	0.79^{a}	0.61^{b}	0.67^{b}	0.62^{b}	0.025	<0.001	0.76	0.81	0.77	0.82	0.043	0.683
Fatty acid composition, g/100g fatty acids												
12:0	0.02	0.04	0.03	0.03	0.004	0.067	0.02	0.02	0.02	0.03	0.002	0.076
14:0	0.33	0.37	0.37	0.35	0.017	0.300	0.42^{b}	0.49^{ab}	0.50^{a}	0.46^{ab}	0.020	0.036
14:1c9	0.05^{b}	0.06^{a}	0.06^{a}	0.07^{a}	0.017	0.006	0.06^{b}	0.10^{a}	0.10^{a}	0.09^{a}	0.006	0.001
15:0	0.08^{b}	0.10^{ab}	0.10^{ab}	0.12^{a}	0.006	0.034	0.08^{b}	0.11^{a}	0.10^{ab}	0.12^{a}	0.007	0.018
16:0	16.6^{b}	20.7^{a}	21.0^{a}	20.8^{a}	0.286	<0.001	18.4^{b}	22.1^{a}	22.1^{a}	21.1^{a}	0.683	0.002
16:1c7	0.44	0.55	0.52	0.59	0.036	0.058	0.54^{b}	0.74^{a}	0.72^{a}	0.71^{a}	0.037	0.002
16:1c9	1.43^{b}	2.18^{a}	2.32^{a}	2.14^{a}	0.172	0.003	2.28^{b}	3.90^{a}	4.05^{a}	3.77^{a}	0.244	<0.001
17:0	0.16^{b}	0.25^{a}	0.25^{a}	0.25^{a}	0.011	<0.001	0.17^{b}	0.27^{a}	0.29^{a}	0.26^{a}	0.018	0.000
17:1c9	0.05^{b}	0.09^{a}	0.09^{a}	0.09^{a}	0.004	<0.001	0.06^{b}	0.11^{a}	0.10^{a}	0.08^{b}	0.007	<0.001
18:0	9.10	9.76	9.35	9.72	0.240	0.183	8.05	8.11	7.84	7.72	0.271	0.722
18:1c9	31.4^{a}	26.7^{b}	27.5^{b}	26.0^{b}	0.828	0.000	35.8	34.4	35.1	34.7	0.914	0.714
18:1c11	1.45^{b}	2.51^{a}	2.53^{a}	2.62^{a}	0.077	<0.001	1.39^{b}	2.02^{a}	2.09^{a}	1.99^{a}	0.074	<0.001
18:2n-6	29.1^{a}	21.5^{b}	21.7^{b}	21.9^{b}	0.484	<0.001	27.3^{a}	20.8^{b}	20.2^{b}	21.5^{b}	1.37	0.003
18:3n-6	0.09	0.07	0.10	0.07	0.009	0.046	0.07^{a}	0.06^{b}	0.05^{b}	0.06^{b}	0.004	0.001
18:3n-3	0.45^{b}	0.56^{ab}	0.58^{a}	0.56^{ab}	0.031	0.033	0.46^{b}	0.75^{a}	0.72^{a}	0.75^{a}	0.049	0.000
20:0	0.10	0.11	0.11	0.11	0.006	0.359	0.10	0.09	0.09	0.09	0.005	0.636
20:1c11	0.26^{b}	0.33^{ab}	0.35^{ab}	0.38^{a}	0.027	0.019	0.27	0.28	0.30	0.29	0.013	0.381
20:2n-6	0.43^{b}	0.50^{b}	0.50^{b}	0.65^{a}	0.038	0.002	0.23	0.19	0.18	0.21	0.019	0.357
20:3n-6	0.68^{b}	1.28^{a}	1.24^{a}	1.38^{a}	0.063	<0.001	0.38	0.52	0.50	0.51	0.040	0.069
20:4n-6	4.48^{b}	6.69^{a}	6.05^{a}	7.00^{a}	0.364	<0.001	1.86	2.36	2.16	2.24	0.260	0.572
20:3n--3	0.02^{c}	0.05^{b}	0.05^{b}	0.06^{a}	0.004	<0.001	0.02	0.03	0.02	0.03	0.003	0.133
20:5n-3	0.04^{b}	0.14^{a}	0.13^{a}	0.16^{a}	0.012	<0.001	0.02^{b}	0.05^{a}	0.04^{ab}	0.04^{a}	0.006	0.005
22:0	0.07^{b}	0.10^{a}	0.09^{ab}	0.10^{ab}	0.006	0.029	0.05	0.04	0.03	0.04	0.003	0.058
22:1n-9	0.03	0.04	0.05	0.04	0.005	0.060	0.04	0.06	0.07	0.04	0.014	0.194
22:5n-3	0.22^{b}	0.60^{a}	0.54^{a}	0.63^{a}	0.037	<0.001	0.04	0.06	0.05	0.06	0.005	0.208
22:6n-3	0.15^{b}	0.46^{a}	0.42^{a}	0.46^{a}	0.029	<0.001	0.09^{b}	0.25^{a}	0.20^{a}	0.20^{ab}	0.029	0.003
Others	2.63	4.02	3.73	3.52	0.409	0.111	0.06^{b}	0.16^{a}	0.16^{a}	0.15^{a}	0.021	0.004
Partial sums of fatty acids												
SFA	26.5^{b}	31.7^{a}	31.5^{a}	31.6^{a}	0.379	<0.001	27.4^{b}	31.3^{a}	31.0^{a}	29.9^{ab}	0.831	0.008
MUFA	35.2	32.4	33.5	32.0	0.992	0.130	40.5	41.6	42.5	41.7	1.19	0.682
PUFA	35.7^{a}	31.9^{b}	31.3^{b}	32.9^{b}	0.738	0.001	30.5	25.1	24.3	25.7	1.64	0.049
n-6 PUFA	34.8^{a}	30.1^{b}	29.6^{b}	31.0^{b}	0.704	<0.001	29.8^{a}	23.9^{ab}	23.1^{b}	24.5^{ab}	1.60	0.022
n-3 PUFA	0.88^{b}	1.81^{a}	1.72^{a}	1.87^{a}	0.053	<0.001	0.66^{b}	1.24^{a}	1.15^{a}	1.17^{a}	0.079	<0.001
Nutritional ratios												
n-6/n-3	39.9^{a}	16.8^{b}	17.3^{b}	16.6^{b}	0.611	<0.001	44.4^{a}	19.7^{b}	20.5^{b}	22.1^{b}	1.71	<0.001
PUFA/SFA	1.35^{a}	1.01^{b}	1.00^{b}	1.04^{b}	0.029	<0.001	1.16^{b}	0.81^{a}	0.79^{a}	0.87^{ab}	0.077	0.005

SEM, standard error of the mean; nd, not detected.

^{a,b,c} Different superscripts within a row indicate a significant difference ($p < 0.05$).

CON, Corn and soybean meal-based diet, in the control group; SP, 15% Spirulina; SPV, 15% Spirulina +0.025% VemoZyme® P; SPP, 15% Spirulina +0.10% Porcine pancreatin.

SFA = 12:0 + 14:0 + 15:0 + 16:0 + 17:0 + 18:0 + 20:0 + 22:0.

MUFA = 14:1c9+ 16:1c7 + 16:1c9 + 17:1c9 + 18:1c9 + 18:1c11 + 20:1c11 + 22:1n−9.

PUFA = 18:2n-6 + 18:3n-6 + 18:3n-3 + 20:2n-6 + 20:3n-6 + 20:4n-6 + 20:3n-3 + 20:5n-3 + 22:5n-3 + 22:6n-3.

n-3 PUFA = 18:3n-3 + 20:3n-3 + 20:5n-3 + 22:5n-3 + 22:6n-3.

n-6 PUFA = 18:2n-6 + 18:3n-6 + 20:2n-6 + 20:3n-6 + 20:4n-6.

TABLE 10 Mineral composition of broiler meats fed with Spirulina.

	CON	SP	SPV	SPP	SEM	*p*-value	CON	SP	SPV	SPP	SEM	*p*-value
	Breast meat						Thigh meat					
Macrominerals (mg/100g fresh weight)												
Calcium (Ca)	17.8	18.3	17.8	18.7	0.51	0.60	18.3	18.7	18.4	18.8	0.37	0.73
Magnesium (Mg)	25.8	26.9	26.2	26.1	0.45	0.35	20.4^{a}	19.1^{ab}	18.8^{b}	18.7^{b}	0.38	0.01
Phosphorous (P)	254	261	263	261	4.1	0.35	246^{a}	234^{b}	234^{b}	235^{b}	2.9	0.01
Potassium (K)	408	420	422	419	5.0	0.18	392^{a}	375^{ab}	376^{ab}	375^{b}	4.6	0.03
Sodium (Na)	101	94.5	92.6	96.1	2.80	0.15	102^{b}	109^{a}	109^{a}	111^{a}	1.9	0.01
Sulphur (S)	209	201	201	201	3.0	0.12	217	208	207	206	3.0	0.06
Microminerals (mg/100g fresh weight)												
Copper (Cu)	0.12^{b}	0.16^{ab}	0.17^{ab}	0.17^{a}	0.01	0.03	0.21	0.21	0.23	0.22	0.006	0.09
Iron (Fe)	1.26	1.30	1.30	1.35	0.03	0.30	1.46	1.45	1.38	1.44	0.028	0.18
Manganese (Mn)	0.06	0.06	0.06	0.06	0.00	0.95	0.06	0.06	0.06	0.06	0.003	0.41
Zinc (Zn)	1.15^{a}	0.70^{b}	0.71^{b}	0.70^{b}	0.03	<0.001	1.99^{a}	1.64^{b}	1.59^{b}	1.56^{b}	0.046	<0.001
Total	2.59^{a}	2.21^{b}	2.23^{b}	2.27^{b}	0.04	<0.001	3.71^{a}	3.36^{b}	3.26^{b}	3.29^{b}	0.060	<0.001
Total macro and microminerals	1,019	1,025	1,026	1,024	10.0	0.96	999^{a}	968^{b}	968^{b}	967^{b}	7.9	0.01

SEM, standard error of the mean; nd, not detected.

^{a,b} Different superscripts within a row indicate a significant difference ($p < 0.05$).

CON, Corn and soybean meal-based diet, in the control group; SP, 15% Spirulina; SPV, 15% Spirulina +0.025% VemoZyme® P; SPP, 15% Spirulina +0.10% Porcine pancreatin.

microalga cell walls, compromises nutrients' bioaccessibility and bioavailability (12). To improve the bioaccessibility of microalga nutrients in broilers, this study incorporated two commercial enzyme mixtures into the diets.

Spirulina is frequently used as a supplement or ingredient in poultry diets, with incorporation levels ranging between 0.25 and 20% (12). Notably, Valente et al. (53) suggested that microalga incorporation below 15% can augment meat quality without compromising performance. Conversely, higher incorporation levels may promote biomass gelation and escalate digesta viscosity, thereby potentially hindering nutrient digestibility and bioaccessibility (25, 54). In our study, broilers fed with Spirulina showed significantly lower BW and BWG, coupled with an increased FCR compared to the control groups. These findings echo the results of Pestana et al. (25) in a trial involving the same level of inclusion but over half the period (days 21–35). The present results can be attributed to the increased digesta viscosity in the digestive tract, particularly in the ileum, as suggested by Pestana et al. (25). This may have been due to an increase in digesta viscosity, compromising nutrient digestibility. However, interestingly, the FCR remained unaffected by the inclusion of the microalga in the diet from day 21–35. Moreover, an increase in the weight of the duodenum and the length of the duodenum, jejunum and ileum, as observed in Spirulina-fed groups (SP, SPV and SPP), could be a result of the increase in digesta viscosity. The enhanced viscosity can significantly influence nutrient digestibility, bioavailability and accessibility, leading to compromised animal growth and performance (55). In addition, it was reported that higher intakes of Spirulina (above 1.5%) may impact metabolic functions and slow growth rates in broilers (56), while smaller percentages can either improve or have no effect on poultry growth (57, 58). Further, high incorporation levels of Spirulina (up to 21%) can lead to protein gelation and increased digesta viscosity (24, 25), which in turn affects protein and amino acid digestibility and stalls animal growth (59). The discrepancies among various published studies may stem from factors like diverse Spirulina growing conditions, variable inclusion levels in diets and nutritional composition (57). Interestingly, in our study, the enzymatic supplementation in the diet did not reverse the decreased growth performance associated with microalga incorporation. However, the addition of pancreatin slightly mitigated the negative impact on body weight gain from day 28–35. The increase in digesta viscosity caused by high levels of Spirulina proteins in the diet may have trapped the enzymes, thereby inhibiting their action on feed nutrients, a phenomenon that warrants further investigation. An investigation by our group with Spirulina and combination of two types of pre-treatments, such as extrusion method or enzymatic (pancreatin and lysozyme) have shown good results in combining pre-treatments in growth performance, with similar FBW and BWG to control (37).

In the poultry industry, meat colour holds significant importance from the consumer's perspective. The colour preferences vary widely depending on factors like geographical location, dietary preferences and environmental conditions (60). The supplementation of Spirulina, particularly in concentrations ranging from 4.0 to 15%, has been noted to enhance the redness and yellowness of poultry meat while reducing its lightness. The elevated redness may be attributed to the high iron and mineral content, and the increased yellowness could be due to the greater proportion of carotenoids, such as β–carotene and zeaxanthin present in the flesh (60–62). Our study further reinforced these findings, with Spirulina incorporation deepening the yellowness of both breast and

thigh meats. This aligns with previous studies by Toyomizu et al. (60), Venkataraman et al. (63), and Altmann et al. (64). Notably, the latter demonstrated that diets supplemented with Spirulina led to more pronounced colour pigmentation of the skin, breast, and thigh meat in broilers. In a study by Moujahed et al. (65) it was observed that incorporating 2.5 and 5% Spirulina into the diet enhanced the yellowness of the breast and skin colour in Arbor Acres broilers. However, a lower level of Spirulina supplementation (0.25, 0.5, 0.75 and 1%) in the diet of Ross 308 male broilers did not significantly affect the colour of the breast meat (58). These differences are not only perceptible in instrumental analysis but also noticeable to the naked eye (64). An orange hue in poultry meat may be perceived as advantageous by consumers when Spirulina is incorporated into broilers' diets. Meat colour is a significant quality indicator in several countries, particularly in Japan (66), the United States, and Mexico (67). Interestingly, when the breast muscles were cooked for the sensory panel, no discernible differences between samples were found. For the sensory panel, the overall acceptance of all diets was positive (scores between 5 and 6), and the absence of any significant difference in off-flavours between treatment groups, positions Spirulina-fed chicken favourably when compared to meats from birds fed on fish oil diets (68) or those rich in docosahexaenoic acid from algal products, which may affect off-flavours in meat.

Substituting corn-based diets with Spirulina necessitates careful analysis. Spirulina is a novel ingredient incorporated in diets, so it is important to do randomized, double-blind and placebo clinical trials to test the microalga's functionality (13). The latter's higher PUFA content could contribute to lipid oxidation, thereby negatively impacting meat quality (69, 70). The PUFA content directly influences lipid oxidation (71), rendering thigh meat. This portion contains more lipids than breast meat, more prone to lipid oxidation (72, 73). Despite the greater lipid and PUFA content in thigh meat, we focused our analysis on TBARS in breast meat due to its prominence in consumers' diets. Levels of TBARS exceeding 0.8 mg/kg can indicate rancidity and adversely affect meat acceptability because of the resultant oxidized flavour, which is not acceptable to consumers (57, 74). In our study, TBARS levels in breast meat ranged from 0.14 to 0.56 mg/kg, significantly below this threshold.

Spirulina is a microalga renowned for its high content of antioxidants such as carotenoids, β-carotene, and tocopherols (75), which can protect lipids from oxidation (76). Our research indicates that the high levels of total carotenoids in both types of poultry meat may reflect Spirulina's antioxidant capabilities and its ability to enhance the oxidative stability of broiler meat. The high carotenoid content in chickens fed Spirulina could explain the increase in meat yellowness, as a greater proportion of carotenoids in the meat can result in heightened yellowness. In the thigh meat of Spirulina-fed groups (SP, SPV, and SPP), the α-tocopherol and γ-tocopherol+β-tocotrienol content was higher than in the breast meat, likely due to the thigh's higher fat content (77). Nevertheless, the decreased value of α-tocopherol in Spirulina-fed groups compared to the control might be attributed to several factors. Firstly, the oxidative stress induced by the higher polyunsaturated fatty acids (PUFAs) in Spirulina could increase the consumption of α-tocopherol as an antioxidant to counteract lipid peroxidation. Secondly, Spirulina's high phycocyanin content may provide an additional antioxidant effect, thereby reducing the need for α-tocopherol and causing its levels to decrease as it is utilized more rapidly in oxidative processes. Despite the reduction, the α-tocopherol levels in Spirulina-fed groups remain within the typical range found in chickens (77, 78). Of the vitamin E diterpenes, α-tocopherol was found in the highest concentrations, while other homologues were present at lower levels, consistent with typical values in broilers (77, 78). Therefore, our results indicate that incorporating 15% Spirulina into the diet can enhance the antioxidant function of broiler meat through its high carotenoid content and possibly through synergistic effects with its other antioxidant compounds.

Breast and thigh meats are recognized as lean meats due to their low-fat content, which is generally less than 5% (79). In this study, these criteria were met as the total lipid content ranged from 0.82 to 1.35% in the breast and 2.32 to 2.90% in the thigh. The thigh meat exhibited a higher cholesterol content than the breast, surpassing values reported by Pestana et al. (25). However, the introduction of Spirulina led to a decrease in the cholesterol values of breast meat.

The fatty acid profile was found to be affected by Spirulina in both types of meat, corroborating previous findings by Cortinas et al. (80) and Botsoglou et al. (81). The most abundant fatty acids across all diets were SFA, such as palmitic (16:0) and stearic (18:0) acids, monounsaturated fatty acids (MUFA), like palmitoleic (16:1c9) and oleic acid (18:1c9), n-3 PUFA α-linolenic acid (18:3n-3), and n-6 PUFA, such as linoleic acid (18:2n-6) and arachidonic acid (20:4n-6). These fatty acids are also the most prevalent in Spirulina. The incorporation of Spirulina in the diet increased the SFA content in both types of meat, with palmitic (16:0) and stearic (18:0) acids being predominant. SFA are more resistant to oxidation and are more readily deposited in meats (76). The arachidonic acid content was higher in breast meat that included Spirulina, which could be due to the conversion of linoleic acid (82). Furthermore, the linoleic acid content was reduced in the breast and thigh of broilers fed diets containing Spirulina, and arachidonic acid was not detected in the microalga powder or diets. Both cereal-based feed and Spirulina are weak sources of α-linolenic acid, with the latter containing less than 1% (83). However, the SPV for the breast and SP, SPV and SPP for the thigh, led to an increase in α-linolenic acid, thereby improving the n-3 PUFA content. Due to the competition for desaturase enzymes, which are the same for both n-6 and n-3 pathways, the conversion of essential fatty acids, mainly α-linolenic acid, along the entire n-3 pathway is a limited mechanism (84, 85). Thus, it is crucial to ensure an appropriate dietary linoleic acid/α-linolenic acid ratio to obtain an efficient conversion of α-linolenic acid into beneficial eicosapentaenoic acid (20:5n-3) and docosahexaenoic acid (22:6n-3) (86). In this study, an increase in eicosapentaenoic and docosahexaenoic acids was observed in the breast and thigh meat of broilers fed Spirulina. This suggests a possible conversion of α-linolenic acid into these long-chain PUFA (LC-PUFA), as they were not detected in the microalga. Additionally, the decrease in total PUFA in breast meat, and a trend towards a decrease in the thigh, may suggest an increase in the *de novo* synthesis of fatty acids, facilitated by Δ6 and Δ5 desaturases and elongases (87). Aside from the LC-PUFA content, the fatty acid profile from the breast and thigh meats was consistent with the diets, as reported by other studies (77, 88). The addition of VemoZyme® P and porcine pancreatin did not alter these fatty acid profiles.

The changes in meat fatty acid profile, particularly the increase in n-3 PUFA and the decrease in n-6/n-3 ratio, can be attributed to the composition of Spirulina. Spirulina is a rich source of n-3 fatty acids, including alpha-linolenic acid, which can be converted into longer-chain n-3 PUFA such as eicosapentaenoic acid and docosahexaenoic acid in broilers. The dietary inclusion of Spirulina likely enhanced the deposition of these fatty acids in the meat (4, 89). PUFA play essential roles in functions such as the production and metabolism of prostaglandins, thromboxanes and leukotrienes (89, 90). Dietary guidelines recommend a PUFA/SFA ratio in human diets above 0.45 and an n-6/n-3 ratio not

exceeding 4.0 (91). In this study, the guideline for the PUFA/SFA ratio was met, but the n-6/n-3 ratio in breast and thigh meats across dietary treatments significantly exceeded the recommended levels.

Minerals play a significant role in various physiological processes, including maintaining normal body fluids, regulating absorption, secretion and excretion, as well as contributing to skeletal structure and soft tissues (92). Although the integration of Spirulina into the diet did not influence the macro-mineral composition in the breast meat, in the thigh meat, there was a noticeable decrease in magnesium, potassium and total content among groups fed Spirulina (SP, SPV and SPP). Magnesium is a critical player in cellular functions, acting as a cofactor in all major metabolic pathways (93, 94). In contrast, sodium content was higher in groups fed Spirulina (SP, SPV and SPP) compared to the control diet (CON). Sodium and potassium play a crucial role in maintaining water balance, regulating muscle and heart activities, as well as hydrogen ion regulation, and gastric and kidney secretions. They are also integral constituents of blood and body fluids (92).

Regarding micro-minerals, zinc and total content were found to be lower in both breast and thigh meats of the Spirulina-fed groups (SP, SPV and SPP). Zinc, a vital micromineral, is important for the growth and development of broilers, as it influences DNA synthesis and the metabolism of lipids and carbohydrates (95–97). The decrease in zinc levels in the treatment groups may be explained by the high phytic acid content in Spirulina, which is known to bind zinc and reduce its bioavailability. Furthermore, the increased gastrointestinal viscosity due to Spirulina could impair the absorption of minerals, including zinc. These factors likely contributed to the reduced zinc levels observed in the Spirulina-fed groups. Nevertheless, this decreased should be considered if it is higher, because can me associated with severe physical and pathological changes, that influences growth, immune response and reproductive failure (97). Therefore, it is essential to consider the balance of minerals in the diets when incorporating Spirulina to ensure the optimal growth and development of broilers.

5 Conclusion

This study demonstrates that incorporating a high level of Spirulina (15%) into broiler diets for an extended period (from day 7–35), with or without enzyme supplementation, negatively impacts broiler growth performance, particularly body weight and body weight gain. The FCR remained unaffected by the inclusion of Spirulina from day 21–35. The increased digesta viscosity observed in Spirulina-fed groups appears to hinder nutrient digestibility, which could explain the compromised growth performance.

In addition, Spirulina integration enhances meat quality by reducing cholesterol content in breast meat and improving the antioxidant profile and n-3 PUFA levels in both breast and thigh meats. These changes potentially make the meat more nutritious and desirable for health-conscious consumers. The addition of commercial enzyme mixtures in this study was not effective in mitigating the increased viscosity in the digestive tract, suggesting that a combination of enzymatic and mechanical or physical pre-treatments may be necessary.

Future research should focus on exploring alternative strategies to improve the digestibility of Spirulina in broiler diets. This could include the use of different enzymatic formulations with specific activity against Spirulina proteins or combined mechanical and enzymatic pre-treatments. Additionally, long-term studies should investigate the potential health benefits of consuming meat enriched with n-3 PUFAs and antioxidants from Spirulina-fed broilers, providing a comprehensive understanding of its implications for both poultry production and human health.

Data availability statement

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

Ethics statement

The animal study was approved by ORBEA/ISA (approval code: 0421/000/000/2022), Instituto Superior de Agronomia, Universidade de Lisboa, Portugal. The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

MS: Formal analysis, Investigation, Writing – original draft, Writing – review & editing. MC: Formal analysis, Investigation, Methodology, Validation, Writing – original draft, Writing – review & editing. BT: Formal Analysis, Investigation, Methodology, Writing – original draft, Writing – review & editing. JPe: Formal analysis, Investigation, Methodology, Writing – original draft, Writing – review & editing. JT: Formal analysis, Investigation, Methodology, Writing – original draft, Writing – review & editing. CM: Formal analysis, Investigation, Methodology, Writing – original draft, Writing – review & editing. CA: Formal analysis, Investigation, Methodology, Writing – original draft, Writing – review & editing. VM: Formal analysis, Investigation, Methodology, Writing – original draft, Writing – review & editing. DC: Formal analysis, Investigation, Methodology, Writing – original draft, Writing – review & editing. MM: Formal analysis, Writing – original draft, Writing – review & editing. ML: Resources, Writing – original draft, Writing – review & editing. JPr: Conceptualization, Funding acquisition, Methodology, Project administration, Resources, Supervision, Validation, Writing – original draft, Writing – review & editing.

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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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Effect of ginseng stem leaf extract on the production performance, meat quality, antioxidant status, immune function, and lipid metabolism of broilers

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Introduction: The present study explores the effect of ginseng stem leaf (GSL) extract on the production performance, meat quality, antioxidant status, immune function, and lipid metabolism of white feathered broilers.

Methods: There were 6 replicates in each group, with 10 broilers in each replicate. In the 42 day trial, 300 AA broilers were randomly divided into five groups: control group (CON), 1.25% GSL extract group (GSL-L), 2.5% GSL group (GSL-M), 5% GSL group (GSL-H), and 45 mg/kg chlortetracycline group (CTC).

Results: The results showed that different doses of GSL extract could improve the body weight, feed to gain ratio (F/G), average daily feed intake (ADFI), average daily gain (ADG), and meat quality of broilers. Compared with the control group, the addition of different doses of GSL improved the antioxidant and immune abilities of broilers to varying degrees, and the effect of GSL extract was significant in the GSL-H group ($p < 0.05$). In addition, medium and high doses of GSL extract significantly reduced the blood triglyceride (TG) and total cholesterol (TC) contents of broilers ($p < 0.05$).

Discussion: Adding GSL extract to the feed has a positive impact on the body weight, meat quality, antioxidant capacity, immunity, and blood lipids of broilers.

KEYWORDS

broilers, ginseng stem leaf extract, production performance, meat quality, antioxidant, immunity, blood lipids

Introduction

Panax L. (the ginseng genus) is a group of medicinal plants within the family Araliaceae and all of its species possess broad application prospects. Among them, ginseng (*Panax ginseng* C. A. Mey.) is known as the "King of Herbs" in traditional Chinese medicine by virtue of its high medicinal and nourishing value. Ginsenoside, as one of the major bioactive components in *Panax ginseng*, has attracted extensive research interest. In recent years, chemical, pharmacological, and clinical studies have proven that ginseng stem leaf (GSL) saponins have similar pharmacological effects to ginsenosides. Ginseng stems and leaves contain abundant saponins, polysaccharides, volatile oils, organic acids, proteins, flavonoids, steroids, and other

active ingredients (1), which can significantly the enhance immune function. By comparing the effect of different parts of five-year-old ginseng (root/stem/leaf/flower/seed) on the immune system of immunosuppressed mice, some scholars have found that ginseng roots, leaves, and flowers can improve the viability of natural killer (NK) cells, enhance the immune organ index, improve cell mediated immune response, increase the content of CD4+ and the ratio of CD4+/CD8+, and restore the function of macrophages, while ginseng stems and seeds can enhance the thymus index, carbon clearance, splenocyte proliferation, NK cell viability, and IL-4 level of immunosuppressed mice (2). In addition, scholars have designed the compatibility of different compounds with ginsenosides to promote mouse immune response (3, 4), enhance intestinal mucosal immunity (5), and elevate vaccine efficacy (6). Total saponins of GSL can also improve the composition of intestinal flora, increase the abundance of probiotics, and thus enhance the secretion of intestinal IgA response (7). In terms of antiinfection, ginseng can inhibit the formation of bacterial biofilms and induce the dispersion and dissolution of mature biofilms (8). In addition, *in vitro* and *in vivo* studies have revealed the anti-influenza properties of ginseng and its derivatives. Ginsenoside compounds, especially Rb1, interact with hemagglutinin (HA) on the surface of influenza A virus and interfere with the binding of the virus to cell surface receptors, thereby inhibiting the invasion of the virus into host cells (9). In addition to Rb1, scholars have also demonstrated the anti-inflammatory activity of Rg1 (10), the hypoglycemic effect of Rb3 (11), the improving effect of Rb (Rb1, Rb2, Rb3) on the structure and diversity of human intestinal flora (12), as well as the functionality of Rg3 and Rh2 in anti-inflammation, immune regulation, and antioxidation (13), and Rd. is also reported to be a natural neuroprotective agent (14). All these studies indicate that GSL and its effective component saponins contribute to exerting anti-inflammatory response, improving intestinal flora, and regulating immune function. The main bioactive saponins of ginseng exist in the roots, stems, leaves, flowers, and berries, but the stems and leaves of ginseng are not fully utilized. In recent years, due to intensive feeding and environmental biological pollution, the potential application value of GSL in poultry production has been gradually highlighted. Yu et al. (15) found that total saponins from GSL significantly inhibited cyclophosphamide-induced oxidative stress in broilers and induced immune response of Newcastle disease virus (ND) and avian influenza (AI) bivalent inactivated vaccines (16). In addition, Yuan found that after injection of ND virus vaccine into broilers, the absorption rate of GSL saponin (E515-D)-adjuvanted vaccine was faster than that of Marcol 52 and #10 white oil-adjuvanted vaccine (17). Ginseng extract can reduce the negative physiological effects caused by heat stress during broiler breeding (18). Adding 3% red ginseng residue in the diet can significantly decline the mortality and serum cholesterol of chicks and also improve the meat quality of broilers (19). These results indicate that ginseng and its effective components have high application value in broiler feeding.

The residue and drug resistance of antibiotics in meat products have increasingly attracted public attention. As a kind of "safe, efficient, stable, and controllable" feed additive, plant extract has become the first choice of alternative anti-bacterial products in the breeding industry due to its advantages of less drug resistance, small toxic and side effects, low residue and anti-oxidation, which has also been widely promoted in the animal food production and feed industry in developed countries such as Europe and the United States. Numerous data have confirmed the high bactericidal and anti-bacterial properties of plant extracts (20), which can effectively eliminate poultry parasites (21) and other harmful pathogens and even exert excellent killing effect on some drug-resistant strains (22), thereby effectively protecting the intestinal health of animals, enhancing the digestive and immune systems, improving daily weight gain, and achieving antibiotic free and green breeding. Against the background of "total ban on antibiotics," taking GSL and its extracts as efficient feed additives has great practical significance for expanding and deepening the utilization of traditional Chinese medicine resources and promoting the green development of animal husbandry.

Therefore, the aim of this study was to verify that dietary GSL supplementation can replace feeding antibiotics and improve growth performance, meat quality, antioxidant status, immune function, and lipid metabolism of broilers.

Materials and methods

Experimental design and diet

This study was carried out in the animal experiment center of Shenyang Institute of Technology and got approval of the Animal Ethics Committee of College of Life Engineering, Shenyang Institute of Technology (Fushun, China, SITLLBA2024011). GSL extract (80.6% saponin, 0.02% crude fiber, 1.1% ash content, and 2.2% water) was purchased from SKY ENERGY Biotechnology Co., Ltd. A total of 300 1-day-old AA broilers (average weight 0.04 kg) were randomly divided into five treatment groups, with six replicates in each treatment group and 10 broilers (5 males and 5 females) in each replicate. The poultry house was fully rinsed and fumigated before the experiment. The experimental broilers were caged in upper, middle, and lower layers (1.75 long × 1.55 wide × 0.5 high, m). The temperature of the poultry house was raised to 32 ~ 35°C 24 h in advance, and then, the temperature was reduced by 2 ~ 3°C every week until it was maintained at 21 ~ 24°C, with continuous light and natural ventilation. The animals had free access to food and drinking water throughout the experimental period and were immunized according to the standardized procedures. The dietary treatments were divided into CON (i.e., basal diet), GSL-L (1.25% of GSL extract in the basal diet), GSL-M (2.5% of GSL extract in the basal diet), GSL-H (5% of GSL extract in the basal diet), and CTC (basic diet supplemented with 45 mg/kg chlortetracycline). The experiment lasted 42 days in total, with 1–21 days as the early stage and 22–42 days as the later stage. The relative humidity of the chicken house was controlled at 50 ~ 70% during the entire experiment. The basal diet for the experiment was corn-soybean meal. The formula was based on NRC (1994) and Chicken Feeding Standard (NY/T33-2004). Table 1 shows the composition and nutritional level of basal diet.

Sample collection

The blood of broilers was collected in the morning of the 42nd day, and one broiler was randomly selected from each replicate. Approximately 10 mL of blood was extracted from the vein under the chicken wing using a 5 mL syringe and centrifuged at 4000 × g for 10 min to isolate the serum, and then stored at-20°C for subsequent analysis. After blood collection, the broilers were sacrificed by cervical dislocation. The whole thigh (biceps femoris) and chest (pectoralis

TABLE 1 Composition and nutritional level of basal diet (air-dried).

Item	1-21d	22-42d
Diet composition %		
Corn	63.0	64.00
Wheat bran	1.68	1.95
Soybean meal	27.00	28.40
Fish meal	3.00	0
Soybean oil	1.40	1.60
Limestone	1.42	1.45
Dicalcium phosphate	0.60	0.7
Salt	0.30	0.3
Choline	0.10	0.1
Premix[1]	1.50	1.50
Total	100	100
Nutrient levels[2]		
ME (MJ/kg)	12.99	12.36
CP (%)	18.45	17.11
Lys (%)	0.77	0.88
Met (%)	0.34	0.33
Ca (%)	0.80	0.80
TP (%)	0.53	0.41

[1]Premix provided per kilogram of diet: VA 12000 IU, VD3 2,500 IU, VE 21.0 mg, VK3 3.0 mg, VB1 5.0 mg, VB2 9.0 mg, VB6 8.0 mg, VB12 2.03 mg, pantothenic acid 20.0 mg, niacin 50.0 mg, biotin 0.1 mg, folic acid 1.5 mg, Fe 94 mg, Cu 23 mg, I 0.7 mg, Zn 92 mg, Mn 101.4 mg, Se 0.04 mg. [2]Calculated value.

major) musculature were removed from the same broiler. The meat quality traits of thigh and chest stored at 4°C were analyzed. The abdominal cavity of broilers was opened after death from bloodletting, and the liver tissues were taken and stored in liquid nitrogen in a cryopreservation tube for future use.

Growth performance and organ index

Broilers in each replicate were weighed on days 21 and 42, and the feed consumption of each replicate was recorded on days 1–21 and 22–42. Average daily gain (ADG), average daily feed intake (ADFI), and feed to gain ratio (F/G) were calculated for each replicate. On the 42nd day, the weight of one broiler from each replicate was close to the average weight, followed by cervical dislocation. Immune organs (liver, spleen, thymus, and bursa of Fabricius) were collected to calculate the percentage of organs.

Meat quality

The samples of pectoral and thigh muscles on both sides of each broiler were collected. Within 12 h after death, the pH values of biceps femoris and pectoralis major muscles were measured using a penetrating electrode (Mettler Toledo, Changzhou, China) connected to a portable pH meter (FG2, Shanghai, China). The pH probe was calibrated with pH = 4 and pH = 7 standard buffer solutions before testing. Meat color (L* = relative lightness, a* = relative redness, and b* = relative yellowness) was measured with a handheld color reader (CR10, Konica Minolta INC, Osaka, Japan).

The reading from the inner surface of the sample represented the entire surface of the muscle, with white tiles (L*92.30, a*0.32, and b*0.33) as standards. Approximately 20 g of samples from thigh and chest muscles were used to determine the drip loss. Muscle samples were weighed and fixed in plastic bags filled with air to avoid evaporation, suspended in a 4°C cooler for 24 h, and then weighed again. The drip loss was calculated according to the weight loss and expressed as a percentage. After pH and drip loss measurements, the pectoral muscle samples were loaded into plastic bags and placed at room temperature before cooking. Then, the muscle samples were heated to an internal temperature of 70°C in a boiling water bath. The cooked muscle was cooled to room temperature and cut into pieces of $3 \times 1 \times 1$ cm3, parallel to the direction of the muscle fibers. The Warner-Bratzler shear force was assessed using a texture measurement system (Food Technology Corporation, Stirling, VA, USA).

Antioxidant capacity

About 0.3 g of liver samples stored in liquid nitrogen were put into a centrifuge tube, added with 0.9% normal saline nine times the weight of the sample, and homogenized at 4000 rpm/min for 10 min under the condition of ice water bath, and the supernatant was sucked with a pipette gun. The total antioxidant capacity (T-AOC) (A015-2-1), superoxide dismutase (T-SOD) (A001-3-2), glutathione peroxidase (GSH-PX) (A005-1-2), catalase (CAT) (A007-1-1), and malondialdehyde (MDA) (A003-1-2) were detected by corresponding kits. The absorbance at 425 nm and 520 nm was determined by a microplate reader (Bio-Tek ELx808). The above kits were purchased from Nanjing Jiancheng Bioengineering Co., Ltd. (Nanjing, China).

Detection of serum immune indexes

The sample serum was used for the detection of IgA (m1002792V), IgG (m1042771V), and IgM (m1027781V) according to the kit instructions. The kits were purchased from Shanghai Meilian Biotechnology Co., Ltd. (Shanghai, China), and the absorbance at 450 nm was detected with a microplate reader.

Analysis of biochemical and lipid indicators

The concentrations of total cholesterol (TC) (A111-1-1), triglycerides (TG) (A110-1-1), high-density lipoprotein cholesterol (HDL) (A112-1-1), and low-density lipoprotein cholesterol (LDL) (A113-1-1) in serum samples were detected using commercial kits (Nanjing Jiancheng Bioengineering Co., Ltd., Nanjing, China). The absorbance at 500 nm and 600 nm was detected with a microplate reader.

Statistics analysis

The experimental data were collated with Excel 2019, and SPSS 26.0 statistical software was used for one-way analysis of variance (ANOVA). Duncan's multiple comparisons were performed on the

data with significant differences. $p < 0.05$ and $p < 0.01$ are regarded as significant differences and extremely significant differences, respectively, while $0.05 < p < 0.1$ is indicative of a trend. Data are expressed as mean ± standard deviation.

Results

Effect of GSL extract on body weight of white feathered broilers

The effect of GSL extract on the body weight of white feathered broilers is shown in Table 2. At 21 and 42 days, there was no significant difference in the body weight of broilers in each group ($p > 0.05$), but with the increase of the dose of GSL extract, the body weight also showed a linear growth trend. In addition, the F/G of the high-dose group was significantly higher than that of the control group and CTC group ($p < 0.05$). Although there was no difference in ADFI and ADG among the groups ($p > 0.05$), the ADFI and ADG of GSL extract groups had an upward trend with the increase of dose.

Effect of GSL extract on meat quality of white feather broilers

The effect of GSL extract on the meat quality of white feather broilers is shown in Table 3. Compared with the control group, the medium-dose and high-dose groups showed significantly reduced chicken shearing force ($p < 0.05$), and the pH value of chicken in the high-dose group was significantly decreased ($p < 0$ 0.05), but there was no significant difference among other groups. The drip loss of the high-dose group was significantly lower than that of other groups ($p < 0.05$). The cooking loss of the high-dose group and CTC group was significantly lower than that of the control group and low-dose group ($p < 0.05$). Compared with the control group, the a* of the medium-dose group and high-dose group were significantly decreased ($p < 0.05$), but there was no significant difference among other groups ($p > 0.05$). L* and b* showed a linear correlation with the concentration of GSL extract, and there was no significant difference in L* and b* among the groups ($p > 0.05$).

TABLE 2 Effect of GSL extract on body weight of white feathered broilers.

Item	Group				
	CON	GSLL	GSLM	GSLH	CTC
21d (kg)	0.83 ± 0.07	0.86 ± 0.09	0.87 ± 0.06	0.88 ± 0.04	0.82 ± 0.09
42d (kg)	3.32 ± 0.14	3.31 ± 0.15	3.33 ± 0.17	3.34 ± 0.11	3.31 ± 0.21
ADFI (g)	85.3 ± 3.2	84.3 ± 3.7	85.2 ± 5.0	85.4 ± 4.1	85.2 ± 3.9
ADG (g)	35.7 ± 2.2	36.3 ± 1.4	36.5 ± 2.0	36.9 ± 2.1	35.8 ± 1.1
F/G	2.63 ± 0.11^{b}	2.69 ± 0.09^{ab}	2.74 ± 0.12^{ab}	2.77 ± 0.07^{a}	2.66 ± 0.07^{b}

In the same row of data, different small letters indicate significant differences ($p < 0.05$); the same small letters or no letters indicate no significant differences ($p > 0.05$). CON, basal diet; GSL-L, CON + 1.25% GSL extract; GSL-M, CON + 2.5% GSL extract; GSL-H, CON + 5% GSL extract; CTC, CON + 45 mg/kg chlortetracycline.

Effects of GSL extract on antioxidant indexes of white feather broilers

The effect of GSL extract on antioxidant indexes of white feather broilers is shown in Table 4. Compared with the control group, the CAT activity in the high-dose group was significantly increased ($p < 0.05$), and the CAT activity increased linearly with the increase of the dose of GSL extract. Compared with the control group, the activities of T-SOD and T-AOC were significantly increased in the high-dose group ($p < 0.05$). The activities of T-SOD and T-AOC in other experimental groups were enhanced with the increasing dose of GSL extract, and the activity of T-AOC in the CTC group was significantly lower than that in the high-dose group ($p < 0.05$). Compared with the control group, the content of MDA in the medium-dose group and high-dose group was significantly decreased ($p < 0.05$), and the differences in the content of MDA among the other groups were not significant, but the content of MDA showed a decreasing trend ($p > 0.05$). Although there was no significant difference in the content of GSH-PX among the groups ($p > 0.05$), it also increased with the increasing dose of GSL extract.

Effect of GSL extract on immune function of white feather broiler

Table 5 shows the effect of GSL extract on immune organ indexes of white feather broilers. Compared with the control group, the bursa of Fabricius index in the high-dose group and CTC group was increased significantly ($p < 0.05$). Compared with the high-dose group, the thymus index of the medium-dose group and low-dose group was decreased significantly ($p < 0.05$), and there was no significant difference among other groups ($p > 0.05$). There was no significant difference in spleen index among the groups ($p > 0.05$).

The effect of GSL extract on immunoglobulin indexes of white feathered broilers is shown in Table 6. Compared with the control group, the IgA content and IgG content in the medium-dose group and high-dose group were significantly increased ($p < 0.05$). In addition, compared with the CTC group, the IgG content in the high-dose group and the medium-dose group was increased significantly ($p < 0.05$). There was no significant difference in the content of IgM among the groups ($p > 0.05$), but with the increase of the dose of GSL extract, the content of IgM also showed an upward trend.

Effect of GSL extract on blood lipid metabolism of white feather broilers

Table 7 shows the effect of GSL extract on blood lipid indexes of white feather broilers. Compared with the control group, the contents of TC and TG in the medium-dose group, high-dose group, and CTC group were significantly decreased ($p < 0.01$). The HDL-C and LDL-C in the CTC group were significantly lower than those in the control group ($p < 0.05$), and there was no significant difference among other groups.

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TABLE 3 Effect of GSL extract on meat quality of white feather broilers.

Item	Group				
	CON	GSLL	GSLM	GSLH	CTC
shear force *N*	9.45 ± 0.29^{a}	8.53 ± 0.23^{ab}	6.76 ± 0.25^{b}	6.11 ± 0.25^{b}	8.55 ± 0.38^{ab}
pH(24 h)	5.64 ± 0.23^{a}	5.62 ± 0.30^{ab}	5.56 ± 0.31^{ab}	5.52 ± 0.22^{b}	5.74 ± 0.25^{ab}
drip loss %	5.30 ± 0.59^{a}	5.06 ± 0.50^{a}	4.84 ± 0.27^{a}	4.40 ± 0.44^{b}	4.96 ± 0.54^{a}
pressure loss %	21.73 ± 1.37^{a}	20.44 ± 1.67^{a}	17.54 ± 1.55^{ab}	14.46 ± 1.07^{b}	15.03 ± 1.06^{b}
L*	57.44 ± 2.60	57.81 ± 2.12	56.91 ± 2.25	57.75 ± 1.75	57.05 ± 3.63
a*	4.36 ± 1.06^{a}	3.98 ± 1.77^{ab}	3.10 ± 1.08^{b}	3.00 ± 0.74^{b}	3.43 ± 0.82^{ab}
b*	15.33 ± 3.49	13.65 ± 2.24	14.57 ± 1.50	14.30 ± 2.40	14.07 ± 2.98

In the same row of data, different small letters indicate significant differences ($p < 0.05$); the same small letters or no letters indicate no significant differences ($p > 0.05$). CON, basal diet; GSL-L, CON + 1.25% GSL extract; GSL-M, CON + 2.5% GSL extract; GSL-H, CON + 5% GSL extract; CTC, CON + 45 mg/kg chlortetracycline.

TABLE 4 Effects of GSL extract on antioxidant indexes of white feather broilers.

Item	Group				
	CON	GSLL	GSLM	GSLH	CTC
GSH-PX (U/mg)	33.51 ± 3.10	34.78 ± 3.68	35.18 ± 3.66	37.38 ± 3.63	37.75 ± 3.87
CAT (U/mg)	4.86 ± 0.58^{b}	5.05 ± 0.77^{ab}	5.28 ± 0.60^{ab}	5.31 ± 0.67^{a}	5.16 ± 0.62^{ab}
T-SOD (U/mg)	198.40 ± 8.46^{b}	226.84 ± 8.78^{ab}	241.72 ± 9.06^{ab}	285.87 ± 8.70^{a}	217.70 ± 9.13^{ab}
T-AOC (mmol/g)	0.11 ± 0.02^{b}	0.20 ± 0.02^{ab}	0.21 ± 0.02^{ab}	0.22 ± 0.02^{a}	0.12 ± 0.03^{b}
MDA (nmol/mg)	2.35 ± 0.32^{a}	1.97 ± 0.36^{ab}	1.67 ± 0.34^{b}	1.57 ± 0.35^{b}	1.76 ± 0.33^{ab}

Data are represented as the mean ± SD ($n = 6$). ^{a,b}Values within a row with different superscript letters indicate significant differences ($p < 0.05$) among groups, and the same superscript letters or no superscript letters indicate non-significant differences ($p > 0.05$). T-AOC: total antioxidant capacity; CAT: catalase; MDA: malondialdehyde; GSH-Px: glutathione peroxidase; T-SOD: superoxide dismutase.

Discussion

The synergistic treatment of feed and plant extract can reduce the anti-nutritional effect of anti-nutritional factors in feed, thus promoting the digestibility and utilization of energy and nutrients in broilers, elevating feed remuneration, and improving animal production performance (23, 24). GSL extract is rich in saponins and flavonoids, which can improve the production performance, immune function, and meat quality of quails (25). However, the effect of GSL extract on the production performance of white feathered broilers is not yet clear. Our results showed that the addition of GSL extract to the diet had no significant effect on the body weight of white feathered broilers, but the body weight increased with the increasing dose of GSL extract. After adding GSL extract, both ADG and F/G showed an upward trend, and the F/G of the group added with 5% GSL extract was significantly higher than that of the control group ($p < 0.05$), which was similar to the research results of Liu et al. (26) that adding ginseng extract increased the ADG and F/G of broilers. Saponins in American ginseng and ginseng can inhibit pancreatic lipase activity and reduce body weight, adipose tissue weight, and blood lipids of high-fat diet mice, thereby controlling obesity caused by high-fat diet (27, 28). The blood lipid indexes can comprehensively reflect the ab-sorption and metabolism of starch, lipids, and other nutrients in the feed of broilers (29). In this study, by detecting the cholesterol metabolism indexes in the blood of broilers, it was found that the TC and TG in the blood of broilers fed GSL extract were significantly reduced ($p < 0.05$). Kim M H (30) et al. found that adding 1% red ginseng to the diet could reduce TC and LDL-C, but not HDL-C in hyperlipidemic mice, which was in consistence with our experimental results. Collectively, these findings suggest that GSL extract can significantly improve the growth performance of white feathered broilers, especially in lowering lipid and increasing F/G.

pH value, drip loss, and shear force are the main physical indicators to evaluate meat quality (31). It is reported that the saponin component in ginseng extract pro-motes angiogenesis, which can rapidly regenerate the blood vessels of limbs and relieve vascular injury or local hypoxia (32). The development of blood vessels improves the efficiency of nutrient transport, accelerates the metabolism of limbs, and promotes the normal development of pectoral and thigh muscles (33). Therefore, we speculate that GSL extract can prevent the lignification of chicken breast meat and the growth restriction of acral tissues caused by long-term high-density breeding conditions. Our results demonstrated that GSL extract reduced the pH value, drip loss, and muscle shear force of chicken, thereby effectively improving meat quality. Similar results were also found by Morsy et al. (34) that adding 300 mg/kg ginseng extract to the diet improved the shear force of chicken during the growth period and reduced the pH value and drip loss.

Intensive feeding can lead to the production of excessive reactive oxygen free radicals in broilers. Therefore, it is of practical significance to develop feed additives that can reduce lipid peroxide, eliminate oxygen free radicals, and enhance disease resistance of broilers (35). SOD, CAT, and GSH-PX are essential for reducing excessive reactive oxygen radicals (36). GSL extract exerts its antioxidant effect mainly by regulating the production of SOD and GSH-PX, thereby indirectly affecting the antioxidant capacity of the body (37). In our experiment, adding 5% GSL extract to the basal diet could significantly increase

TABLE 5 Effect of GSL extract on immune organ index of white feathered broilers.

Item	Group				
	CON	GSLL	GSLM	GSLH	CTC
Thymus organ index (g/kg)	2.20 ± 0.07^{ab}	1.88 ± 0.09^{bc}	1.95 ± 0.07^{bc}	2.42 ± 0.11^{a}	2.19 ± 0.08^{ab}
Bursa organ index (g/kg)	1.16 ± 0.04^{c}	1.19 ± 0.06^{bc}	1.43 ± 0.06^{abc}	1.58 ± 0.05^{a}	1.52 ± 0.05^{ab}
Spleen organ index (g/kg)	0.10 ± 0.02	0.11 ± 0.03	0.11 ± 0.03	0.10 ± 0.02	0.16 ± 0.05

Data are represented as the mean ± SD ($n = 6$). ^{a,b}Values within a row with different superscript letters indicate significant differences ($p < 0.05$) among groups, and the same superscript letters or no superscript letters indicate non-significant differences ($p > 0.05$).

TABLE 6 Effect of GSL extract on immunoglobulin of white feather broilers.

Item	Group				
	CON	GSLL	GSLM	GSLH	CTC
IgA (μg/mL)	15.99 ± 2.17^{b}	25.67 ± 2.78^{ab}	34.42 ± 2.11^{a}	36.42 ± 2.21^{a}	25.75 ± 2.40^{ab}
IgM (μg/mL)	141.96 ± 9.18	148.21 ± 8.56	161.66 ± 8.64	206.06 ± 8.79	191.56 ± 8.43
IgG (μg/mL)	361.28 ± 18.39^{b}	457.53 ± 18.04^{b}	497.69 ± 16.81^{a}	534.41 ± 19.90^{a}	406.28 ± 19.46^{b}

Data are represented as the mean ± SD ($n = 6$). ^{a,b}Values within a row with different superscript letters indicate significant differences ($p < 0.05$) among groups, and the same superscript letters or no superscript letters indicate non-significant differences ($p > 0.05$). IgA, Immunoglobulin A; IgG, Immunoglobulin G; IgM, Immunoglobulin M.

TABLE 7 Effect of GSL extract on blood lipid indexes of white feathered broilers.

Item	Group				
	CON	GSLL	GSLM	GSLH	CTC
TC (mmol/L)	0.29 ± 0.04^{A}	0.25 ± 0.10^{AB}	0.17 ± 0.07^{BC}	0.18 ± 0.07^{BC}	0.12 ± 0.06^{C}
HDL-C (mmol/L)	4.65 ± 0.11^{a}	4.27 ± 0.15^{ab}	4.24 ± 0.13^{ab}	3.91 ± 0.14^{ab}	3.51 ± 0.11^{b}
LDL-C (mmol/L)	1.26 ± 0.07^{a}	0.93 ± 0.07^{ab}	1.11 ± 0.06^{ab}	1.03 ± 0.07^{ab}	0.82 ± 0.07^{b}
TG (mmol/L)	3.73 ± 0.04^{A}	3.51 ± 0.03^{A}	2.66 ± 0.02^{B}	2.70 ± 0.03^{B}	2.53 ± 0.01^{B}

Data are represented as the mean ± SD ($n = 6$). ^{a,b}Values within a row with different superscript letters indicate significant differences ($p < 0.05$) among groups, and the same superscript letters or no superscript letters indicate non-significant differences ($p > 0.05$). ^{A,B}Values within a row with different superscript letters indicate significant differences ($p < 0.01$) among groups, and the same superscript letters or no superscript letters indicate non-significant differences ($p > 0.01$). HDL: high-density lipoprotein; LDL: low-density lipoprotein; TG: triacylglycerol; TC: total cholestero.

the activities of T-SOD, T-AOC, and CAT in the liver of white feathered broilers and reduce the content of MDA in the liver ($p < 0.05$). Jiao et al. (38) also reported that ginseng extract enhanced SOD and T-AOC activities in the se-rum and liver of D-galactose-induced aging mice, while reducing MDA content. *In vitro* and *in vivo* tests have revealed that plant extracts achieve antioxidant effects by reducing the generation of oxygen free radicals and enhancing the activities of antioxidant enzymes such as SOD, GSH-PX, and CAT (39–41). The above results confirm that adding GSL extract to the diet can improve the serum antioxidant capacity of white feathered broilers and reduce oxidative stress damage.

The development of immune organs underlies the realization of immune function, and the immune organ index is an important indicator to measure the immune status of poultry (42). Spleen, thymus, and bursa of Fabricius are important sites for the formation and differentiation of T and B lymphocytes. Therefore, the organ indexes of spleen, thymus, and bursa of Fabricius are commonly used to evaluate the immune status of poultry. The higher the index, the stronger the immune system function (43). Song et al. (44) found that adding 300 mg/kg ginsenoside Rg1 could significantly in-crease the organ indexes of bursa of Fabricius, spleen, and thymus and enhance the immune function of broilers. Our results also showed that adding an appropriate dose of GSL extract to the diet of white feather broilers promoted the development of bursa of Fabricius and improved the immunity, which may be due to that the saponins contained in GSL extract can promote the proliferation of lymphocytes in bursa of Fabricius. Serum IgA, IgG, and IgM, as the most important signs reflecting the humoral immune function, are produced by the proliferation and differentiation of B cells into plasma cells after receiving antigen stimulation. The higher the titer of serum antibody, the stronger the anti-infection and disease resistance ability (45). Our experimental results showed that the addition of GSL extract to the diet improved the bursa of Fabricius index and increased the serum IgG content of white feather broilers. Similar results were revealed by Ma et al. [45] that GSL saponins combined with selenium effectively increased the content of serum IgA after broilers were vaccinated with Newcastle disease vaccine, indicating the potential of GSL extract strengthening immunity of broilers.

Incorporating varying doses of ginseng stem and leaf extracts into the diet can enhance the production performance and meat quality of broilers, while also improving their antioxidant status, cholesterol metabolism, and immune function. These changes indicate the potential application value of ginseng stem and leaf extracts in broiler production. However, to facilitate their widespread use in animal husbandry, further research is required to determine the optimal dosage, action targets, and mechanisms of action of the extracts. Such

studies will not only deepen our understanding of how ginseng stem and leaf extracts influence host health but also provide a theoretical basis for the development of novel green 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 in the article/supplementary material.

Ethics statement

The animal study was approved by Ethics Committee of Shenyang Institute of Technology. The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

PZ: Conceptualization, Funding acquisition, Project administration, Writing – original draft, Writing – review & editing. HZ: Software, Writing – original draft. CM: Data curation, Writing – original draft. QL: Validation, Writing – original draft. HY: Methodology, Writing – original draft. QZ: Writing – original draft, Methodology.

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Conflict of interest

HY and QZ were employed by Liaoning Zhongqing Xinze Biotechnology 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.

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Methane emission, nitrogen excretion, and energy partitioning in Hanwoo steers fed a typical TMR diet supplemented with *Pharbitis nil* seeds

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Two *in vivo* experiments were conducted to evaluate the potential of *Pharbitis nil* seeds (PA) as an anti-methanogenic additive to ruminant feed. In experiment 1, six Hanwoo steers (459.0 ± 25.8 kg) were fed either a total mixed ration (TMR; 32-d period) or TMR supplemented with PA at 5% dry matter (DM) intake (TMR-PA; 45-d period) for two consecutive periods. Fecal and urine outputs were measured in an apparent digestibility trial in both periods. Methane (CH_4) yield and heat energy (HE) were measured using respiratory chambers equipped with gas analyzers. In experiment 2, five rumen cannulated Holstein steers (744 ± 35 kg) were fed the same TMR or TMR-PA diets for 40 days; rumen samples were collected at 0, 1.5, and 3 h after feeding on the last day of the feeding period. In experiment 1, although there were no differences ($p > 0.05$) in nutrients or gross energy intake (GEI) between the groups, an increase ($p < 0.05$) in the apparent digestibility of DM (9.1%) and neutral detergent fiber (22.9%) was observed in the TMR-PA fed Hanwoo steers. Pronounced decreases ($p < 0.05$) in CH_4 (g/Kg DM; 17.1%) and urinary N excretion (% N intake; 7.6%) were observed in the TMR-PA group, leading to a 14.7% increase in metabolizable energy intake (% GEI). However, only a numerical increase ($p > 0.05$) in retained energy was observed due to the increase in HE loss. In experiment 2, a drastic decrease ($p < 0.05$) in rumen ammonia concentration (56.3%) associated with an increased ($p = 0.091$) rumen short-chain fatty acid concentration 1.5 h after feeding were observed in TMR-PA fed Holstein steers. A 26.6% increase ($p < 0.05$) in the propionate proportion during the treatment period clearly reflected a shift in the ruminal H_2 sink after 3 h of feeding. A 40% reduction ($p = 0.067$) in the relative abundance of rumen protozoa *Entodinium caudatum* was also observed. It was concluded that PA could be a natural feed additive for CH_4 and N emission abatement.

KEYWORDS

energy partitioning, Hanwoo, methane, nitrogen utilization, *Pharbitis nil*

1 Introduction

In addition to their environmental footprints, methane (CH_4) and nitrogen (N) emissions represent losses in livestock dietary energy and N utilization (1, 2). The total farm-gate greenhouse gas emissions from Korea were 19.3 $MtCO_2$ eq/year in 2021; CH_4 and nitrous oxide (N_2O) from livestock farming (enteric fermentation and manure management) contributed 6.59 and 1.05 $MtCO_2$ eq/year, respectively (3). The recent update of the nationally determined contribution of Korea includes a target to reduce the total absolute greenhouse gas emissions by 24.4% from their 2017 level (709.1 $MtCO_{2eq}$; i.e. reducing national greenhouse gas emissions to 536 $MtCO_{2eq}$) by the year 2030 (4). Furthermore, Korea has pledged to become carbon neutral by 2050 with a focus on low-carbon agricultural technologies targeting the development of low-methane feeds that improve livestock enteric fermentation and reduce CH_4 emissions (5). However, the substantial increase in the total cattle population, with a record of 3.35 million heads in the first quarter of 2024 (6), has raised concerns regarding their associated CH_4 and N_2O emissions.

Several dietary interventions have been proposed to mitigate the emissions of CH_4 and noxious N-containing compounds from ruminant husbandry (7, 8). The implementation of these interventions while avoiding changes to nutrient and energy utilization is a major goal in animal nutrition. Large-scale identification of plant materials rich in plant secondary metabolites, such as tannins and saponins, for CH_4 and N abatement in the livestock industry has been achieved in recent years (9, 10). Plant materials rich in bioactive lipid compounds and other unsaturated fatty acids (FAs) have been extensively studied to elucidate these effects (7, 8). However, most relevant results have been obtained by *in vitro* trials; only few plant materials such as hazel leaves (11, 12), tannin rich legumes (13, 14), and conventional or unconventional oil seeds (15–17) have been studied *in vivo* to determine their nutritional value and effects on CH_4 production and N utilization. Moreover, inconsistent results (18, 19) and adverse effects on nutrient digestibility (20) have encouraged ruminant nutritionists to explore novel feed additives with high nutritional value.

Pharbitis nil (Convolvulaceae) is an annual climbing herb widely distributed throughout Korea, Japan, and China, and are reported to be enriched in resin glycosides (21), chlorogenic acid derivatives (22), and other phenolic substances (23). Seeds of *Pharbitis nil* (PA) were also used as a purgative agent and in treating digestive disorders (24). Our previous *in vitro* and *in sacco* studies identified and evaluated the potential of PA for mitigating ruminal CH_4 and NH_3 production with improved nutrient digestibility at a range of 4.5%−9% dry matter (DM) supplementation (10, 25). We also found that PA is rich in nutrients such as neutral detergent fiber (NDF), crude protein (CP), and fat (25). Our previous studies demonstrated that the abundance of flavonoids and unsaturated FAs in PA probably had detrimental effects on ciliate protozoa (25), which are generally regarded as H_2 producers and are detrimental for ruminal N metabolism (8, 26). Therefore, the inclusion of PA in ruminant diets would be an effective strategy to prevent inefficient N utilization and CH_4 emission. However, *in vivo* experiments may be more appropriate for evaluating the nutritive value of PA because a drastic change in the Bacteroides: Firmicutes ratio was observed after PA incubation in rumen due to a probable defaunation effect (25).

Therefore, the present study investigated the effects of supplementing PA in the diet of Hanwoo steers at 5% DM intake on digestibility, CH_4 emissions, energy and N balance, and rumen protozoal diversity via reverse transcription quantitative polymerase chain reaction (RT-qPCR) analysis. We hypothesized that PA supplementation to ruminants would enhance nutrient digestibility and decrease protozoal abundance, followed by reductions in N and CH_4 emissions, thereby increasing dietary metabolizability and allowing spare energy to be used for productivity gains.

2 Materials and methods

2.1 Animal ethics statement

All experimental animals were obtained from the animal farm of Seoul National University. The methods and protocols for all experiments involving animals were approved by the Committee for the Institutional Animal Care and Use of Seoul National University (SNU-210615-3), and all experiments were performed in accordance with relevant guidelines and regulations.

2.2 Experiment 1: effects of PA on CH_4 production, energy balance, and productivity of Hanwoo steers

In experiment 1, six Hanwoo steers with an initial body weight (BW) of 459.0 ± 25.8 kg were randomly allocated to pens equipped with automatic Calan doors. All steers were offered a commercial total mixed ration (TMR; Table 1) as a basal diet for 21 days and allowed to adapt to the Calan doors to consume their individual feeds prior to commencement of experimental work. The experiment included two successive periods where the animals were first fed a basal diet for 32 days (TMR), followed by 45 days of PA supplementation at 5% DM (TMR-PA) of the basal diet. The TMR period included 21 days for diet adaptation, 3 days for respiratory chamber adaptation, 3 days for gas sampling, and 4 days for total feces and urine collection. The TMR-PA period included 35 days for diet adaptation, 3 days for respiratory chamber adaptation, 3 days for gas sampling, and 4 days for total feces and urine collection. Because only three steers could be housed in the respiratory chambers at a given time, steers began the experiment in groups of three, staggered by 1 week. The TMR diet was prepared every 14 days during the experimental period by a local feed mill. The prepared TMR diet was then packed in vinyl bags (20 kg per bag) and stored for feeding to the animals. The diet was provided at 2% initial BW twice daily (at 09:00 and 18:00), and the animals had unrestricted access to water. PA was purchased from the local market in Dongdaemun-gu, Seoul, Republic of Korea, ground to pass through a 1-mm screen (Model 4, Thomas Scientific, Chadds Ford Township, PA, USA), and mixed with the diet during feeding. The BWs of the animals were measured both at the beginning and end of the period to calculate the average daily gain (ADG). Feed

TABLE 1 Ingredients and chemical compositions of the dietary treatments.

	TMR	TMR-PA
Ingredient composition, %DM		
Corn DDGS	17.5	16.6
Corn gluten feed	11.0	10.4
Rye straw	10.7	10.2
Steam-flaked corn	9.7	9.2
Broken corn	9.4	8.9
Wheat flour	8.8	8.5
Fermented mixture[a]	7.3	7.0
Palm Kernel meal	7.0	6.6
Whole cottonseed	5.0	4.7
Rice bran	3.0	2.8
Tall fescue straw	2.6	2.5
Wheat bran	2.0	1.9
Pineapple pulp meal	0.6	0.5
Sodium bicarbonate	0.3	0.3
Limestone	0.1	0.1
Pharbitis nil seeds	–	5.0
Chemical composition, %DM		
Organic matter (OM)	93.3	93.3
Crude protein (CP)	16.4	16.7
Ether extract (EE)	4.8	5.2
Neutral detergent fiber (NDFom)[b]	38.7	38.9
Acid detergent fiber (ADFom)[c]	18.5	18.4
Gross energy (GE, MCal/kg)	4.3	4.4

TMR, total mixed ration; TMR-PA, TMR supplemented with Pharbitis nil seeds at 5% DM.
[a]Rice bran 50% and wheat bran 50% (w/w) with 0.1% Lactobacillus acidophilus KCTC3140 (1.2×10^7 CFU/ml) and 0.1% Saccharomyces cerevisiae KACC30068 (2.1×10^6 CFU/ml) were mixed and maintained at the optimal temperature for fermentation.
[b]Neutral detergent fiber assayed with heat-stable amylase and expressed exclusive of residual ash.
[c]Acid detergent fiber expressed excluding residual ash.

refusals were recorded both in the pen and respiratory chambers to determine the DM intake and feed conversion ratio. Samples of feed and PA were collected at the beginning of each period, whereas samples of refusals were collected each day when the animals were in respiratory chambers. All samples were stored at −20°C until further analysis.

2.2.1 Respiratory chamber description and gas sampling for methane and heat production measurements

Gas sampling was performed for three consecutive days, beginning from days 27 and 40 during the TMR and TMR-PA periods, respectively. Animals were placed in the chamber; the CH_4, CO_2, and oxygen (O_2) concentrations were measured using three indirect open-circuit whole-body respiratory chambers composed of steel frames and covered by detachable, clear polyvinyl chloride (PVC) sheets to enable visual communication between animals. The design and operating procedures of the respiratory chambers were developed based on those previously reported in Cesar and Garry (27). The outer dimensions of each chamber were 137 (width) × 356 (depth) × 200 (height) cm, resulting in a chamber volume of 9.7 m^3. Each chamber was equipped with a feeder and water bowl; the chamber floor was layered with a rubber mat containing a metal grated opening (80 × 80 cm) in the middle. Each chamber was fixed to the floor; the gap between the floor and the bottom of the chamber (36 cm) was sealed by covering the middle opening with a movable steel container for urine collection. All chambers were designed to maintain ambient temperature and relative humidity at 21°C and 60%, respectively, using an independent regulation system that contained an air refrigeration unit (model DK-C-150E; Dryer Korea) with a recirculating fan (model ALFFIZ-WBCAI-015H; Busung, Korea) and an air filter section. This unit was integrated into the chamber through 10-cm-diameter flexible thermo-insulating tubes from the ceiling. For recirculation, air left the chamber (0.6 m/s) through openings in the middle of the chamber ceiling; the dehumidified air was then recycled into the chamber (0.1 m/s) through openings at the back end of the chamber ceiling. The speed of air circulation was fixed to avoid loss of gases and maximize their recovery. Condensed water from the refrigeration unit was drained into the chamber through a capillary tube attached to the water traps.

Streams of ambient air from the barn were drawn through a 10-cm-diameter opening in the back door of each chamber by a sealed rotary pump connected to a flow meter (model LS-3D; Teledyne Technologies, Thousand Oaks, CA, USA) that provided a constant wet ventilation rate (wet VR) of 450 L/min. Each chamber was fitted with an air outlet containing a filter box in the front section of the ceiling, and the air was continuously drawn out next to the control room through a 10-cm-diameter polyvinyl chloride (PVC) pipe. Air outflow from each chamber was sampled from the middle of a PVC pipe between the chamber and the exhaust pump, through a gas tube connected to the analysis system.

All three chambers shared a common gas analysis system that consisted of a multiplexer (Metabolic Controller, B.S. Technolab, Seoul, Korea) connected to a gas sampling pump (Columbus Instruments, Columbus, OH, USA) and a gas analyzer (model VA-3000, Horiba Ltd., Tokyo, Japan) containing non-dispersive infrared CH_4 (0–2,000 ppm) and CO_2 (0–10,000 ppm) gas sensors, as well as a paramagnetic O_2 (0–25%) sensor. The multiplexer had a gas switching system with eight measuring channels that controlled the three chambers and background air samples; it delivered only one sample stream (from one of the chambers or background) to the analyzer set through a sample flow that was set to 0.6 L/min by the pump. The sample used to determine background gas concentrations was acquired near the inlet of each chamber. Before each air sample entered the analysis system, it was filtered and dried in cylindrical plastic columns filled with desiccants and an indicator (Mesh size 8, Drierite, Xenia, OH, USA). The sample stream was then sequentially delivered to the CH_4, CO_2, and O_2 analyzers. Calibrations of gas sensors (zero and span) were performed before the beginning of each measurement using standard gas mixtures with concentrations of 807 μmol/mol CH_4, 7,996 μmol/mol CO_2, and 19.99 %mol/mol O_2 in N (Air Korea, Seoul, Korea).

Data acquisition and analysis software (Metabolic System, SciTech, Seoul, Korea) was used to record the CH_4, CO_2, and O_2

concentrations, as well as the wet VR, during the measurement period. The time interval of multiplexer channel switching was also defined by the operator in the data acquisition software. Based on the stabilities of gas concentrations and numbers of operating chambers, the air was sampled every 10.5 min for each chamber, with background air and chamber air sampling sequences of 90 and 120 s, respectively. The average value of the final 20 s of sampling time was considered for calculations.

Before animal placement in respiratory chambers, the recovery rate of each chamber was tested in each period using a standard CH_4 gas mixture (25% mol/mol balance N_2; Air Korea). Briefly, a fixed volume of CH_4 (50 ml/min) was injected into each chamber through a gas tube connected to a portable mass flow meter. The gas tube was placed near the feeder to accurately simulate animal emissions during feeding. The gas was allowed to mix for 10 min to achieve an equilibrium state with the inlet and outlet air passages closed. After 10 min, the inlet and outlet air passages were opened; inlet and outlet gases were sampled as described above. The difference in CH_4 concentration between outlet and inlet gases ($DCH_{4\ ppm}$), known ventilation rate (wet VR), and the known injected CH_4 concentration ($InCH_{4ml/min}$) were used to calculate the gas recovery rates of the chamber using the following formula:

$$Theoretical\ CH_{4ppm}\left(ThCH_{4ppm}\right) = \left(InCH_{4mL/min}/wetVR_{mL/min}\right) \times 1,000,000$$

$$Gas\ recovery\ rate\ (\%) = \left(DCH_{4\ ppm}/ThCH_{4\ ppm}\right) \times 100$$

During the experiment, the same routine operations were conducted when animals were placed in the chamber, and CH_4 emission was calculated using the formula:

$$CH_4\ emission\ (L/min) = \left(wetVR_{L/min} \times \left(\left[DCH_{4ppm}\right]/1,000,000\right)\right)/gas\ recovery\ rate\ (\%)$$

The same formula and gas recovery rate (%) were used to determine CO_2 emissions by substituting the values of $DCH_{4\ ppm}$ with $DCO_{2\ ppm}$. For the determination of O_2 consumption, the oxygen $DO_{2\%}$ observed during the experiment was normalized and scaled up with the $DO_{2\%}$ observed during the recovery test.

2.2.2 Digestibility and N balance trial

Feces and urine collection were performed for four consecutive days beginning on days 29 and 42 of the TMR and TMR-PA periods, respectively, immediately after gas measurements. For collection of total feces and urine, clear PVC sheets were detached from the chamber and the chamber itself was used as a metabolic crate. The animals were housed on rubber mattresses and allowed free access to feed and fresh drinking water. The feces voided daily from each animal on the rubber mat were collected using a shovel and dragged into a stainless-steel box located at the end of each metabolic crate. The daily total feces output was collected at 09:00 each day and recorded. The feces were then homogenized; 10% of the total feces from each animal was sampled and stored at −20°C. The total urine excreted from each animal was also collected daily using a custom-made pyramidal silicone funnel attached to the animal's lower body. The funnel was connected to a rotary pump and the excreted urine drained directly into clean 25-L airtight plastic containers via polypropylene tubing. In each container, 300 ml of 4N H_2SO_4 were included daily to acidify urine upon entry. The daily total urine output was recorded and homogenized; 10% of the urine volume from each animal was sampled and stored at −20°C. All daily subsamples were mixed; 10% of the total mixed sample was retained and used for further analysis.

2.2.3 Chemical analyses

Feed, refusals, PA, and feces were dried in a forced-air oven at 65°C for 72 h, then ground to pass through a 1-mm screen (Thomas Scientific Model 4, NJ, USA). The ground samples were subjected to DM analysis by drying at 105°C for 3 h. The OM was determined after ashing at 600°C using a Nabertherm LE 14/11/R7 Compact Muffle Furnace (Bahnhofstr, Lilienthal, Germany) for 3 h (28). Ether extract (EE) was determined using an ANKOM^{XT15} Extractor (ANKOM Technology Corp., Fairport, NY, USA) after a filter bag procedure (29) with petroleum ether as the solvent. NDF and acid detergent fiber were measured using the ANKOM A2000 fiber analyzer (ANKOM Technology Corp.) filter bag technique. NDF content was determined with a heat-stable amylase and expressed exclusive of residual ash (amylase neutral detergent fiber organic matter [aNDFom] (30). Acid detergent fiber was measured using the method of Van Soest (31), and the results were presented excluding residual ash (aNDFom). The N contents of feeds, refusals, PA, feces, and acidified urine were determined using the Kjeldahl method (Kjeltec Auto Sampler System, 8400 Analyzer; Foss, Sweden) as described in AOAC method 981.10; 2016. CP was calculated as 6.25 × N. The gross energies (GEs) of feeds, refusals, PA, dried feces, and acidified urine were determined using an automatic isoperibol calorimeter (6400EF, Parr Instrument Company, Moline, IL, USA). FA concentrations in feed and PA were measured by the direct methylation method (32), using previously described analysis techniques (33).

The ammonium nitrogen (NH_3-N) concentration in rumen fluid obtained from experiment 2 (described below) was determined using a modified colorimetric method (34). For determination of volatile fatty acids (VFAs), a 5.0-ml aliquot of rumen fluid was mixed with 1.0 ml 25% HPO_3 and 0.2 ml 2% pivalic acid (35); the mixture was analyzed using previously described methods (36).

2.2.4 Calculations

Energy partitioning was determined by subtracting the energy losses in feces, urine, and CH_4, as well as daily heat production, from the GE intake as detailed in Sinz et al. (15). Heat energy (HE) was calculated with correction for the assumed CO_2 production from microbial fermentation based on the equation given in Chawlibog et al. (37).

2.3 Experiment 2: effect of PA on rumen fermentation and protozoal populations in Holstein steers

In experiment 2, five rumen fistulated Holstein steers with an initial BW of 744.8 ± 15.8 kg (mean ± standard error) were randomly allocated to individual feeders equipped with steel

stanchions. All animals were adapted to the same basal diet (Table 1) for 25 days (TMR) then supplemented with PA at 5% DM (TMR-PA) of the basal diet for 38 days. The diet was provided at 2% initial BW twice daily (09:00 and 18:00), and the animals had unrestricted access to water. Approximately 200 ml of ruminal fluid were collected from each animal through the cannula before feeding (0 h), and at 1.5 and 3 h after feeding, on the last day of each period; all fluid was strained through four layers of muslin. Immediately after the pH had been measured with a Seven Easy pH meter (Mettler-Toledo, Schwerzenbach, Switzerland), the ruminal fluid was transferred to a 50-ml centrifuge tube, snap-frozen in liquid N, and stored at −80°C until further analysis.

2.3.1 Quantification of rumen protozoal abundance using RT-qPCR

Rumen liquor collected after 3 h of morning feeding from cannulated Holstein steers was subjected to genomic DNA extraction using previously described methods (36). Real-time PCR assays to determine the relative abundances of major rumen protozoal species such as *Entodinium caudatum*, *Epidinium caudatum*, *Eudiplodinium maggii*, and *Polyplastron multivesiculatum* were performed using the SYBR Green Real-Time PCR Master Mix (Bioneer, Daejeon, Korea) and the CFX96 Touch™ Real-Time PCR Detection System with previously described analysis methods and PCR conditions (10). All primers (Table 2) were designed using the Primer-BLAST tool based on National Center for Biotechnology Information (NCBI) published sequences (www.ncbi.nlm.nih.gov, accessed on December 22, 2021). All primers were optimized for the annealing temperature with the highest product band intensity, which was determined by multiple gradient PCR assays. The primers targeted the 18S ribosomal region or other species-specific conserved coding region that lacked potential matches with other nucleotide sequences available in the NCBI. The $2^{-\Delta\Delta CT}$ method was used to determine relative fold-changes (38), and all data were normalized to the abundance of total protozoa.

2.4 Statistical analyses

A Shapiro–Wilk test was performed to check the normality of the data. When data were not normally distributed, they were transformed using the PROC RANK procedure of SAS (version 9.4, SAS Institute, Cary, NC, USA). Data were subjected to mixed-model analysis of variance in SAS, considering the treatment and period as fixed effects and animals as the random effect. Pairwise comparisons among least-squares means were performed by the Tukey-Kramer test. The ruminal fermentation characteristics (pH, VFA, and NH_3) were analyzed by repeated-measures analysis of variance. The Akaike information criterion was used to determine which covariance structure had the best fit; based on the results, an "autoregressive (AR1)" analysis was chosen for assessment of fermentation characteristics. Means were calculated using the LSMEANS function, then compared using the PDIFF option in SAS. Differences were considered statistically significant at $p < 0.05$, and trends were considered at $0.05 \leq p < 0.10$.

3 Results

3.1 Chemical composition of the PA and diet

The proximate, FA composition, and metabolite profile of PA are reported in our previous study (25). Briefly, the analysis of the FA composition of PA revealed enrichment of saturated FAs (SFAs; 3.5 g/kg DM), monounsaturated fatty acids (MUFAs; 2.3 g/kg DM), and PUFAs (5.1 g/kg DM) dominated by linoleic acid (C18:2; 4.5 g/kg DM). Analytes such as caffeic acid, chlorogenate, quercetin, quercetin-3-O-glucoside and dioctyl sulfosuccinate were present in high intensities (25). The actual concentrations of these metabolites are yet to be determined.

The TMR offered to animals in both periods had a similar nutrient composition, except that the EE content of TMR-PA was 8% greater than the EE content of the TMR (Table 1) because of the higher EE concentration in PA (25). This also led to greater polyunsaturated FA and total FA concentrations in the TMR-PA diet (Supplementary Table 1) compared with TMR.

3.2 Effects of PA in Hanwoo steers

3.2.1 Intake, digestibility, and performance

There were no significant differences ($p > 0.05$) in nutrient intake across dietary treatments (Table 3), but an increase ($p < 0.001$) in apparent nutrient digestibility was observed with maximum increases of 8.4% and 18.6% in OM and NDF digestibility, respectively in TMR-PA compared with TMR. There were no changes ($p > 0.05$) in ADG or feed conversion ratio in response to PA supplementation.

3.2.2 Methane emission

A pronounced decrease ($p = 0.079$) in daily absolute CH_4 production was observed in animals supplemented with PA at 5% DM intake (TMR-PA; Table 4). Relative decreases ($p < 0.05$) of 17.2% and 17.5% were observed when the results were expressed as CH_4 (g/kg OM) and CH_4 (g/kg NDF), respectively. The reduction in CH_4 yield was particularly pronounced ($p < 0.005$), with levels of 24.0% and 33.3% expressed for digestible OM and NDF intakes (Table 4). An 18.1% decrease in GE intake lost as CH_4 (Ym) was also observed in animals supplemented with PA, relative to animals fed only TMR.

3.2.3 N balance

The supplementation of PA at 5% DM intake (TMR-PA) did not affect ($p > 0.05$) daily fecal or urinary N excretion amount (Table 5). However, when expressed as the proportion of total N intake, a 7.9% and 7.6% decrease ($p < 0.05$) in fecal and urinary N excretion were noted, respectively. Overall, a 7.7% decrease ($p < 0.05$) in total N excretion and 30.3% increase ($p < 0.05$) in N retention in the body were observed in animals fed PA compared with the non-PA-fed group (Table 5).

TABLE 2 Oligonucleotide primers used for real-time PCR assays.

S. No	Primer name		Sequence	NCBI accession No.	Product size (bp)
1	*Entodinium caudatum*	FP	ATTCATCCAAGCCTCAAGAGAAGT	AM051654.1	144
		RP	TGTCATTTTCCCATTCGACCTTGA		
2	*Epidinium caudatum*	FP	ACTGAAATGGGAGCCACTGA	AB011273.1	186
		RP	TGGTTCAAAGGTTCCAGCAGA		
3	*Eudiplodinium maggii*	FP	TCAGGCTGCACTGGAAAACA	AM419456.1	185
		RP	CCCAATCCCATCCATTGCCT		
4	*Polyplastron multivesiculatum*	FP	CATTGATGGCAACCCTACATTT	AJ516958.1	114
		RP	AGTCCTTTACTCGTCCATGCT		
5	Total protozoa	FP	TTTGTACACACCGCCCGTC		135
		RP	GGTTCACCTACGGAAACCTTGT		

TABLE 3 Least-squares means of body weight, feed intake, and apparent total tract digestibility of Hanwoo steers ($n = 6$) supplemented with *Pharbitis nil* seeds at 5% DM (TMR-PA).

Item	TMR	TMR-PA	SEM	*p*-value
Initial BW, kg	459.01	480.67	26.12	
Final BW, kg	480.67	521.83	26.87	
Average daily gain, kg/day	1.03	0.91	0.10	0.190
Feed conversion ratio	9.68	9.24	1.33	0.231
Intake, kg/day				
Dry matter (DM)	7.72	7.93	0.35	0.169
Organic matter (OM)	7.20	7.40	0.33	0.158
Crude protein (CP)	1.26	1.33	0.06	0.029
Ether extract (EE)	0.37	0.41	0.02	0.001
Neutral detergent fiber (aNDFom)	2.99	3.08	0.14	0.117
Acid detergent fiber (ADFom)	1.43	1.45	0.06	0.289
Gross energy (GE, Mcal/day)	33.74	34.86	1.55	0.103
Digestibility, %				
DM	65.13	71.08	0.87	<0.0001
OM	67.46	73.22	0.82	0.001
CP	69.67	71.89	0.88	0.082
EE	73.49	70.57	1.65	0.255
aNDFom	51.58	63.41	1.21	<0.0001
ADFom	55.62	61.22	1.41	0.011
GE	67.12	71.97	0.92	0.003

TMR, total mixed ration; TMR-PA, TMR supplemented with Pharbitis nil seeds (5% DM). FCR, feed conversion ratio (kg DM intake/kg BW gain); aNDFom, neutral detergent fiber assayed with heat-stable amylase and expressed exclusive of residual ash; ADFom, acid detergent fiber expressed excluding residual ash; SEM, standard error of the mean.

TABLE 4 Effects of *Pharbitis nil* seeds supplemented at 5% DM (TMR-PA) on enteric methane emission from Hanwoo steers ($n = 6$).

Item	TMR	TMR-PA	SEM	*p*-value
Intake (chamber), kg/day				
Dry matter (DM)	7.27	7.73	0.29	0.001
Organic matter (OM)	6.78	7.22	0.27	0.001
Neutral detergent fiber (aNDFom)	2.82	3.01	0.11	0.001
Gross energy (GE, Mcal/day)	31.78	34.02	1.27	0.001
CH_4 emissions				
CH_4 (g/day)	165.92	145.05	7.65	0.079
CH_4 (g/kg DMi)	22.83	18.92	0.78	0.016
CH_4 (g/kg OMi)	24.46	20.26	0.85	0.015
CH_4 (g/kg NDFi)	58.92	48.60	2.03	0.014
CH_4 (g/kg dOMi)	36.31	27.61	1.53	0.004
CH_4 (g/kg dNDFi)	114.87	76.56	6.46	0.002
Ym	6.92	5.67	0.25	0.014

DMi, dry matter intake; OMi, organic matter intake; NDFi, neutral detergent fiber intake; dOMi, digestible organic matter intake; dNDFi, digestible NDF intake; Ym, CH_4 energy expressed as %GE intake; SEM, standard error of the mean.

3.2.4 Energy balance

No difference ($p > 0.05$) in CO_2 production was observed between the experimental groups (Table 6). The respiratory quotient was observed to be greater ($p < 0.05$) in TMR fed animals. Although the GE intake did not differ between treatments, the fecal, urine and CH_4 energy losses decreased ($p < 0.005$) when PA was included in the diet, thereby increasing ($p < 0.005$) the supplies of digestible energy (DE) and metabolizable energy (ME) by 7.2% and 14.8%, respectively, when expressed as % GEI (Table 7). However, only a numerical increase ($p > 0.05$; Table 7) in retained energy (RE) was observed in animals supplemented with PA. The relationship between ME and DE differed ($p < 0.005$) between the treatments; correlation coefficients of 0.81 and 0.87 were obtained for the TMR and TMR-PA groups, respectively. The utilization efficiencies of the ME to RE were not significantly different ($p > 0.05$); they averaged 0.21 and 0.25 for the TMR and TMR-PA groups, respectively (Table 7).

TABLE 5 Effects of *Pharbitis nil* seeds supplemented at 5% DM (TMR-PA) on nitrogen balance in Hanwoo steers ($n = 6$).

Item	TMR	TMR-PA	SEM	*p*-value
DMI, kg/day	7.72	7.93	0.35	0.169
Feces excreted, kg DM/day	2.69	2.29	0.14	0.010
Urine excreted, kg/day	10.03	9.22	1.08	0.391
Nitrogen (N) intake, g/day	202.01	213.23	9.01	0.026
Fecal N excretion, g/day	61.47	59.62	3.76	0.569
Urinary N excretion, g/day	99.80	97.67	5.78	0.420
Total N excretion, g/day	161.27	157.30	8.32	0.283
Body N retention, g/day	40.74	55.93	2.50	0.003
% N intake				
Fecal N	30.32	27.91	0.87	0.054
Urinary N	49.44	45.70	1.61	0.077
Total excreted N	79.75	73.61	1.18	0.003
Retained N	20.25	26.39	1.18	0.003
% total N excreted				
Fecal N	38.03	38.05	1.42	0.990
Urinary N	61.98	61.96	1.42	0.990

SEM, standard error of the mean.

TABLE 6 Effects of *Pharbitis nil* seeds supplemented at 5% DM (TMR-PA) on heat production in Hanwoo steers ($n = 6$).

Item	TMR	TMR-PA	SEM	*p*-value
O_2 consumption, L/day	3,180.83	3,619.88	249.35	0.033
CO_2 production, L/day	3,614.35	3,601.73	239.48	0.959
CH_4 production, L/day	232.17	203.03	15.28	0.079
RQ	1.14	1.00	0.03	0.025
Ratio CH_4/CO_2 in breath	0.07	0.06	0.00	0.004
Urinary N, g/day	99.80	97.67	5.78	0.420
HP, Mcal/day	14.45	16.40	1.13	0.049

RQ, respiratory quotient (CO_2 production/O_2 consumption); SEM, standard error of the mean; HP = heat production (Mcal/day) corrected for assumed CO_2 production from microbial fermentation = [0.01618 × O_2 (L/day) + 0.00502 × (CO_2 (L/day) − 3 × CH_4 (L/day)) − 0.00217 × CH_4 (L/day) − 0.00599 × urinary N (g/day)]/4.184.

3.3 Effects of PA on fermentation parameters and protozoal populations in Holstein steers

The average ruminal pH and NH_3-N concentration decreased ($p < 0.05$) in cannulated Holstein steers supplemented with PA (Table 8). The NH_3-N concentration was 56.3% lower in the TMR-PA group than in the TMR group after 1.5h feeding; it continued to remain lower after 3 h feeding. An increase ($p = 0.087$) in the total VFA concentration after 3 h of feeding, accompanied by a 26.6% increase ($p < 0.05$) in the propionate proportion, was also observed in the TMR-PA group. This clearly led to a decrease ($p < 0.005$) in the acetate/propionate ratio. Analysis of rumen protozoal populations revealed a 40% reduction ($p = 0.062$) in the relative abundance of the protozoal species *En. caudatum* among animals supplemented with PA (Figure 1). There were no significant differences in the abundances of other protozoal species.

TABLE 7 Effects of *Pharbitis nil* seeds supplemented at 5% DM (TMR-PA) on energy balance in Hanwoo steers ($n = 6$).

Item	TMR	TMR-PA	SEM	*p*-value
Gross energy (GE, Mcal/day)	33.74	34.86	1.55	0.103
Loss, Mcal/day				
Feces	11.11	9.78	0.63	0.033
Urine	1.98	1.38	0.11	0.004
Methane	2.33	1.98	0.13	0.012
Heat	14.45	16.40	1.13	0.049
Digestible energy (DE), Mcal/day	22.63	25.09	1.04	0.001
Metabolisable energy (ME), Mcal/day	18.32	21.73	1.00	0.001
Retained energy (NE), Mcal/day	3.87	5.34	0.77	0.192
Loss, %				
Feces, % GE	32.86	28.01	0.92	0.003
Urine, % GE	5.95	4.02	0.48	0.029
Methane, % GE	6.92	5.68	0.25	0.013
Heat, % GE	42.84	46.74	1.96	0.190
DE, % GE	67.14	71.99	0.92	0.003
ME, % GE	54.28	62.29	1.01	0.001
NE, % GE	11.45	15.56	2.23	0.180
GE, Mcal/kg DM	4.37	4.40	0.00	<0.0001
DE, Mcal/kg DM	2.94	3.17	0.04	0.002
ME, Mcal/kg DM	2.37	2.74	0.04	0.001
RE, Mcal/kg DM	0.50	0.68	0.10	0.172
ME/DE	0.81	0.87	0.01	0.003
RE/ME	0.21	0.25	0.04	0.403

SEM, standard error of the mean.

4 Discussion

Dietary strategies to mitigate CH_4 and N emissions from ruminants must be commercially practical, without negative impacts on digestibility and animal performance. In our previous *in vitro* evaluations (10) we demonstrated the anti-methanogenic potential of *P. nil* seeds that are widely distributed in East Asian countries. We also observed increases in the *in vitro* DM and NDF digestibility with increasing supplementation of PA; the maximum response was recorded at 9% DM supplementation (25). A consistent response was present in the current *in vivo* trial at a supplemental level of 5% DM; the magnitude of increase in digestibility was comparable with our previous *in vitro* observation (25). This increased digestibility led to increased DE intake for animals in the treatment period compared with the control period, although there were

no differences regarding GE intake. The increase in nutrient digestibility may be partially explained by the laxative effect of PA, which improved intestinal motility (24). There is evidence that altered intestinal motility and contractions create turbulence and convective currents that transport nutrients from the center of the lumen toward the epithelium, eventually improving nutrient absorption (39). Furthermore, dioctyl sulfosuccinate, an anionic surfactant substance traditionally recommended as a laxative and stool softener, was reported to be present in PA in our previous study (25). Another possible explanation is that better synchronization between carbohydrates and N occurred when less ammonia was available to rumen microbes in the treatment period, compared with the control period. This might have increased the growth of cellulolytic bacteria and enhanced fiber digestibility (40). The observed increase and decrease in rumen total VFA and pH in treatment period, respectively, provide further support to this hypothesis. Increases in fibrolytic bacterial populations were also observed in our previous study (25) when PA was incubated in the rumen of Holstein steers. Although the energy balance trial and rumen fermentation trial were performed in two different animal breeds, the basal diet fed was same in the both trials and hence relating their effects can be meaningful.

Greater NDF digestion stimulates acetate fermentation, which may enhance CH_4 and CO_2 emission (41). In this study, an inverse effect was observed that favored propionate production and decreased CH_4 yield, consistent with our previous study (25). This effect led to a decrease in CH_4 energy loss for animals in the treatment period, compared with the control period. Furthermore, the observed CH_4 yield and Ym from Hanwoo steers fed TMR in the experiment were comparable with previously reported values (42). The proportion of CH_4 energy loss to DE intake in the present study (10.3%) was comparable with values from growing Hanwoo steers fed different diets, as reported in our previous study (43). However, the magnitude of CH_4 reduction via PA supplementation in the present study was 2.5-fold greater than the magnitude observed in our previous *in vitro* study with a similar level of PA supplementation (25). There are several possible reasons for this, including differences in culture system (44), animal breed (42), and diet. Furthermore, CH_4 reduction in the treatment period may be partially explained by the greater polyunsaturated FA concentration, including dietary linoleic acid (C18:2n6c) from the supplemented PA (25). The role of the dietary FA concentration in ruminal CH_4 production is well-known (45). Beauchemin et al. (46) reported that a decrease in CH_4 yield (g/kg DM intake) was accompanied by a decrease in the number of rumen protozoa when cows were fed crushed linseed, similar to our results. Defaunation has been associated with lower levels of methanogenesis (26) due to the symbiotic relationship between methanogens and protozoa (47). Dohme et al. (48) also reported the antiprotozoal actions of FAs, including C18:2n6c, which produce considerable decreases in CH_4 production. However, in the present study, supplementation with PA alone increased the added FA concentration by 0.4%, which was insufficient to achieve the observed reductions in protozoal population and CH_4 (45). This result suggests that other metabolites in PA (e.g., quercetin and quercetin-3-O-glucoside) exhibit antiprotozoal action, as demonstrated in previous studies (49, 50). In our previous studies, we observed a moderate binding of these metabolites to the *cyclic guanosine monophosphate-dependent protein kinase* of *En. Caudatum* (25), and also a drastic decrease in total protozoal population in PA treated *in vitro* rumen buffer (10), further supporting our results. However, the concentrations of these metabolites in PA have not been quantified. Furthermore, *Entodinium* spp. reportedly constitute only 10–15% of the total protozoal population; they are dominated by *Ophryoscolex* spp. or other unclassified families (51, 52). Therefore, the decrease in *En. caudatum* abundance alone could not completely explain the decrease in CH_4 production observed in this study. Another possible factor may be the laxative effect of PA, which could have increased the rate of feed passage and requires further investigation. The elimination of rumen protozoa is consistently associated with an increase in the propionate proportion (2), which matches the observed 26.6% increase in propionate proportion during the treatment period in Holstein steers. This finding suggested that propionate synthesis was the major H_2 sink, thereby reducing H_2 available for CH_4 synthesis in animals supplemented with PA (41). Furthermore, defaunation has been related to a decrease in nutrient digestibility (26); such a decrease was not observed in the present study. This was possibly related to the lack of change in the abundances of cellulolytic genera such as *Epidinium*, *Polyplastron*, and *Eudiplodinium*, and a decrease in the abundance of *Entodinium*, which is weakly hemicellulolytic (53).

The increases in ruminal protein degradation and peptide deamination cause increased production of NH_3-N, which is absorbed through the rumen wall and excreted as urea in urine, leading to N losses in a highly volatile form (54). The 56% decrease in the rumen NH_3-N concentration in cannulated Holstein steers supplemented with PA could be related to the decreased urinary N excretion and increased N retention in Hanwoo steers in the current experiment. These findings suggest that the ruminal degradability of dietary CP is reduced, which may be related to the overall decrease in protozoa observed in our current and previous studies (25). However, there was no negative effect on the total tract digestibility of CP, which suggested hind gut digestion and CP utilization. Additionally, consistent with the present study, defaunation is associated with lower NH_3-N levels in rumen fluid (2), due to a decrease in the microbial grazing of *En. caudatum* (55), which deaminates the engulfed microbial protein to NH_3-N (26). Previous studies (49, 50) have also shown decreases in protozoal ammoniagenesis after the addition of plant extracts that (like PA) are rich in quercetin and quercetin-3-O-glucoside. The minimum required NH_3-N concentration for maximum microbial growth in the rumen is 5 mg/dl (56). A previous study showed that levels of 5–8 mg/dl were sufficient to favor fiber digestion, whereas levels of >8 mg/dl did not increase feed degradation in the rumen (57). The NH_3-N concentration of 5.5–11.9 mg/dl upon supplementation with PA would therefore meet the minimum requirement for microbial protein synthesis. However, studies on the rumen microbial protein synthesis should be carried out to further support the increased N utilization noted in PA fed animals. The proportion of N retained from N intake for Hanwoo steers fed only a basal diet (18%−20%) observed in this study was bit greater than proportions (16%−18%) observed in other breeds of beef cattle (13, 58, 59).

The decrease in urinary N excretion in animals supplemented with PA also led to a decrease in the urinary energy (UE) content

TABLE 8 Ruminal fermentation characteristics in cannulated Holstein steers supplemented with *Pharbitis nil* seeds at 5% DM ($n = 5$).

Item	TMR			TMR-PA			SEM	*p*-value		
Time interval	0 h	1.5 h	3 h	0 h	1.5 h	3 h		Treatment	Hours	Treatment × hours
pH	7.17	6.44	6.55	6.81	6.30	6.27	0.09	0.001	0.117	0.006
Total VFA, mM	63.54	121.13	104.46	72.01	121.32	118.10	6.14	0.087	<0.0001	0.419
VFA, %										
Acetate	66.50	57.86	60.75	64.78	54.93	56.24	1.05	0.019	<0.0001	0.136
Propionate	17.83	26.15	22.15	19.73	29.26	28.05	0.94	0.002	<0.0001	0.025
Iso-butyrate	1.89	1.28	1.34	1.83	1.27	1.24	0.07	0.184	<0.0001	0.820
Butyrate	9.30	10.47	11.35	8.90	9.72	9.85	0.36	0.060	<0.0001	0.067
Iso-valerate	3.10	2.19	2.32	3.29	2.64	2.33	0.21	0.303	<0.0001	0.290
Valerate	1.38	2.05	2.10	1.48	2.18	2.30	0.11	0.136	<0.0001	0.756
Acetate:Propionate	3.75	2.27	2.78	3.30	1.88	2.01	0.16	0.004	<0.0001	0.191
NH_3-N, mg/dl	10.98	25.38	17.43	5.49	11.09	11.89	1.48	<0.0001	<0.0001	0.006

SEM, standard error of the mean.

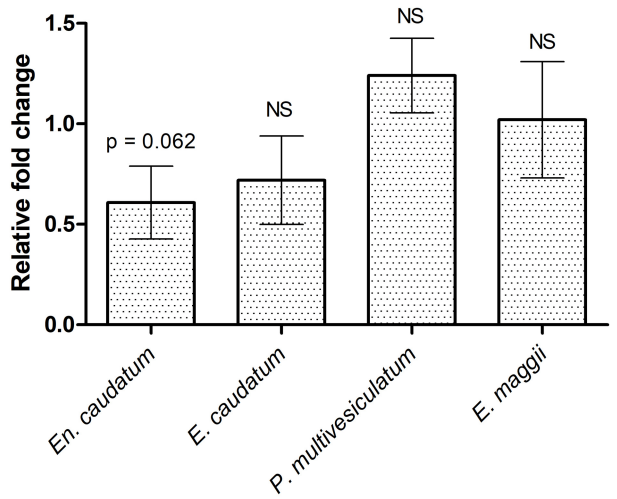

FIGURE 1
Effect of supplementing *Pharbitis nil* seeds at 5% DM intake on rumen protozoal abundance in cannulated Holstein steers ($n = 5$).

(from 6% GE intake to 4% GE intake) because UE is closely associated with UN due to the higher energy contents of urinary hippuric acids, amino acids, and creatinine (60). The ratio of UE losses to the GE and DE intakes (8.8%) in the present study was much higher than the ratio among Hanwoo steers in the early fattening stage, reported in our previous work (43, 61). This discrepancy may be related to the greater dietary CP content of 16.6% in the present study, compared with the value of 12.5% in the previous studies, which might have increased the UN excretion while enhancing UE loss.

The decreased ratio of energy intake to energy excretion in feces, urine, and enteric CH_4 resulted in greater ME intake in animals supplemented with PA compared with animals fed only TMR. The observed DE intake and ME intake for Hanwoo steers in the present study were comparable with the results in our previous work that consolidated many studies involving Hanwoo steers with different BWs and diets (43). Additionally, the DE intake and ME intake values were within the ranges of values reported for other beef cattle breeds (1.8–3.8 and 1.4–3.5 Mcal/kg of DM, respectively) (62). The observed efficiency of converting DE to ME in animals fed a basal diet in the present study (0.81) was similar to the efficiencies recommended by the Agricultural Research Council (63), National Research Council (64), and Commonwealth Scientific and Industrial Research Organization (65). However, it was below the efficiencies within the British feeding system (66) and other recent studies (43, 62, 67), which ranged from 0.86 to 0.90 for several beef cattle breeds (Hanwoo, Friesian, Hereford, Brahman, Thai, Angus, Hereford-Angus, Jersey, MARC II composite, Holstein, Charolais-cross, and Continental-British). Similarly, the utilization efficiency of ME (RE:ME) was 0.21 under 2.4 Mcal/kg DM of ME intake, which was lower than the reported value of 0.29 considering feeds with ME intakes of 2.0 Mcal/kg DM (64). However, this discrepancy may be due to differences in animal breeds and diets between experiments. Moreover, the ME:DE (0.87) and RE:ME (0.25) ratios for animals supplemented with PA were similar to the ratios for suggested values (43, 62, 67), presumably due to increases in DE intake and ME intake upon supplementation with PA.

Increases in ruminant productivity (e.g. ADG) via reduction of enteric CH_4 emissions and enhancement of energy conversion efficiency is the desired outcome of a CH_4 mitigation strategy. Similarly, Li and Guan (68) reported that increases in N metabolism and utilization contributed to increased feed efficiency in beef cattle. However, decreases in CH_4, N emission, and other energy losses in the treatment period did not significantly contribute to the increase in RE, may be due to the increased heat energy loss. This result might also be due to the digestion of metabolites such as phenols and tannins (chlorogenate in high intensities) in PA (25), which may require detoxification with the cost of additional energy (69). Furthermore, CH_4 mitigation and urinary N mitigation in the treatment period were insufficient for reduction of the calculated HE because small coefficients for CH_4 and urinary N were used

in the equation for HE estimation. As a result, RE only increased by 1.3 Mcal/day in the treatment period; which eventually did not contribute much to changes in the ADG and feed conversion efficiency. There were also individual animal variations in heat production that contributed to the lack of statistical significance for RE, suggesting that future studies should be conducted over a longer period with additional animal replicates.

5 Conclusions

Seeds of *P. nil* demonstrated promise for mitigating CH_4 emissions from *in vitro* rumen fermentation studies. They were very efficient in live animals, leading to a 17.2% decrease in CH_4 yield (g/kg OM) when supplemented at 5% DM. The supplemented feed was also palatable, demonstrated high nutritional value and a 7.6% decrease in urinary N loss, and exhibited a >10% increase in nutrient digestibility. Metabolizability (ME/GE) and ME utilization (RE/ME) were also improved, suggesting that PA was a valid alternative to ionophores or other growth promoters. Greater retention of dietary N and E may contribute to improvements in production parameters, thereby reducing age at slaughter, with environmental and economic benefits. However, the increase in heat production was a notable limitation. Future studies should focus on profiling rumen and blood metabolites to identify any potential PA-induced toxicity related to the increased HE. The rumen microbial metagenome should also be evaluated to better understand the beneficial effects of PA on digestibility and energy conversion efficiency.

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 approved by Committee for the Institutional Animal Care and Use of Seoul National University (SNU-210615-3). The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

RB: Conceptualization, Data curation, Investigation, Methodology, Software, Validation, Writing – original draft, Writing – review & editing, Formal analysis, Visualization. PX: Formal analysis, Resources, Writing – review & editing. WH: Formal analysis, Writing – review & editing. TK: Formal analysis, Writing – review & editing. JB: Formal analysis, Writing – review & editing. YL: Formal analysis, Resources, Writing – review & editing. BT: Formal analysis, Writing – review & editing. KK: Conceptualization, Funding acquisition, Project administration, Supervision, Writing – review & editing. RI: Formal analysis, Supervision, Writing – review & editing.

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Supplementary material

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Optimal fermentation of Shuanghuanglian and its effects on production performance of laying hens

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Background: Shuanghuanglian is a Chinese medicine composed of *Honeysuckle Flower*, *Baical Skullcap Root*, and *Fructus Forsythiae*. It has various effects, including anti-inflammatory, antibacterial, antiviral, and immunomodulatory effects. The fermented product of Shuanghuanglian can be used as an antibiotic alternative, as it has similar efficacy, which may improve the immunity, feed intake and utilization efficiency of laying hens, thus improving their production performance. The aim of this study was to optimize the fermentation conditions for Shuanghuanglian using single factor and response surface methodology, evaluate the chemical and microbial composition of the Shuanghuanglian fermentation liquor (SFL), and explore the effects of SFL on the production performance of laying hens.

Methods: A total of 288 Xinyang black-feather laying hens (50 week-old) were randomly allocated to four treatments with nine replicates, each replicate containing eight hens, for a total of 37 days trial (including a 7-day adaptation period). The treatments included a control group (0% SFL in drinking water) and drinking water supplemented with 0.3, 0.5, or 0.7% SFL.

Results: The fermentation optimization conditions for Shuanghuanglian were selected as a solid-to-liquid ratio at 1:7, 3% inoculation quantity, fermentation temperature at 28°C for 5 days, initial pH of 7, 60 mesh (sieved), and rotation speed of 150 r/min. Various bioactive compounds, such as myrtenol, 2-hexyn-1-ol, arsenous acid tris(trimethylsilyl) ester, 3(10)-caren-4-ol, and oxime-, methoxy-phenyl, were detected in SFL. The most abundant bacterial phyla in SFL were Proteobacteria and Firmicutes, with *Acinetobacter* being the most abundant genus. The most abundant fungal phyla were Phragmoplastophyta and *Magnoliophyta*. The 0.5 and 0.7% SFL supplementation in water increased egg weight and laying rate, while decreasing the feed-to-egg ratio of laying hens compared with the control group ($p<0.05$). Additionally, 0.3, 0.5, and 0.7% SFL supplementation in water increased ($p<0.05$) the Haugh unit, but there were no significant differences ($p>0.05$) in albumen height, egg shape index, egg thickness, and yolk color of the eggs.

Conclusion: Supplementation of SFL under optimized conditions had a positive impact on the production performance of laying hens, especially when the supplementation amount reached 0.5%. This study provides a theoretical basis for the application of Shuanghuanglian in the commercial egg industry.

KEYWORDS

bioactive compounds, egg quality, optimized fermentation, response surface methodology, Chinese medicine

1 Introduction

Shuanghuanglian is a Chinese medicine that consists of Chinese natural herbs, including *Honeysuckle Flower*, *Baical Skullcap Root*, and *Fructus Forsythiae* in a 1:1:2 ratio (1). It has been found to have various biological functions, such as antipyretic, anti-inflammatory, antibacterial, antiviral, and immune-enhancing effects, and is widely used for respiratory tract infections, mild pneumonia, and common cold treatments caused by virus and bacterial infection (2, 3). Recent studies have also shown that Shuanghuanglian can regulate the structure and function of the intestinal microbiota in rats (3), indicating that it may have potential to improve the intestinal health of poultry and livestock and therefore influence their production performance. Although Shuanghuanglian is widely used as a safe drug in human medicine (4), there is limited information available regarding its use in animal husbandry.

A previous study found that supplementing drinking water with Shuanghuanglian liquid improved the immune performance of broilers (5). Therefore, we hypothesized that Shuanghuanglian might have positive effects on the laying performance and egg quality of laying hens by reducing diseases. Since Shuanghuanglian is commonly used as an oral liquid (2–5) and the cell walls of Chinese herbal medicines typically limit the release of bioactive components (6), we used lignin-degrading bacteria (*Bacillus amyloliquefaciens-c4*) to ferment Shuanghuanglian to release the bioactive components into Shuanghuanglian fermentation liquor (SFL). Consequently, we optimized the fermentation conditions of Shuanghuanglian using single factor and response surface methodology, evaluated the compound and microbial composition of SFL, and further supplemented SFL in drinking water for laying hens. Albumen height, egg shape index, eggshell thickness, Haugh unit, and yolk color were determined as important indexes of egg quality (7, 8). This study will provide a theoretical basis for the application of Shuanghuanglian in laying hens.

2 Materials and methods

2.1 Sources of Shuanghuanglian and microbial inoculum

Honeysuckle Flower, *Baical Skullcap Root*, and *Fructus Forsythiae* were provided by Fuyang Normal University, Fuyang, Anhui, China. The microbial inoculum (*Bacillus amyloliquefaciens-c4*; preservation number CGMCC No. 15178) was provided by the Biological Inoculation Research and Development Center of Northeast Agricultural University, Harbin, Heilongjiang, China.

2.2 Optimization of microbial fermentation conditions by single factor experiment

Shuanghuanglian [not sieved; solid-to-liquid ratio (SLR) of 1:5] was added with 5% brown sugar. It was then inoculated with 5% microbial inoculum and fermented at 28°C for 7 days using a constant temperature shaker (Shidan Equipment Inc., Shanghai, China) with a rotational speed of 180 r/min.

The following conditions were used to determine fermentation optimization were as follows: (1) the SLR was adjusted to 1:1, 1:3, 1:5, 1:7, and 1:9, while keeping other fermentation conditions unchanged, (2) the inoculation quantity of microbial inoculum was adjusted to 1, 3, 5, 7, and 9%, while keeping other conditions unchanged, (3) the fermentation temperature was set to 24°C, 28°C, 33°C, 37°C, and 42°C using the constant temperature shaker (Shidan Equipment Inc., Shanghai, China), while keeping other conditions unchanged, (4) the fermentation time was set to 1, 3, 5, 7, and 9 days, while keeping other conditions unchanged, (5) the initial pH value was adjusted to 5, 6, 7, 8, and 9 using a pH meter (Gaozhi Precision Instrument Inc., Shanghai, China), while keeping other conditions unchanged, (6) the particle size was set to dp (decoction pieces form without sieving), 20, 40, 60, and 80 mesh using sieves (ZhenXing Inc., Guangzhou, China), while keeping other conditions unchanged, and (7) the rotational speed was set to 0, 120, 150, 180, and 210 r/min using the constant temperature shaker (Shidan Equipment Inc., Shanghai, China), while keeping other conditions unchanged. Each fermentation condition was replicated three times. Finally, the surface morphology of the three component medicines, chemical and microbial compositions, and feeding effects of Shuanghuanglian on laying hens were evaluated based on the optimized fermentation conditions.

2.3 Chemical compound composition analysis

The dry matter content of the Shuanghuanglian was analyzed using previously described methods by Esfahani and Goli (9). Chlorogenic acid, baicalin, and phillyrin were extracted using the ChP method (1). Briefly, 1 mL of SFL was added to 5 mL of 50% (*w*/*v*) methanol and sonicated for 25 min using a multifunctional constant-temperature ultrasonic extractor (Scientz-1000C, Xinzhi Biotechnology Inc., Ningbo, China) to obtain the supernatant for analysis. The chlorogenic acid, baicalin, and phillyrin contents were measured as previously described by Li et al. (10) using an ultraviolet spectrophotometer (T6 New Century, Puxi General Instrument Inc., Beijing, China). Additionally, the supernatants obtained from sonication were concentrated 100 times using a rotary evaporator (Hei-VAP Advantage ML/HB/G3, Heidolph, Schwabach, Germany) and filtered through a 0.22 μm filter membrane to determine the chemical composition of the SFL using a gas chromatography–mass spectrometer (Agilent 7890A/5975C, Agilent, Santa Clara, United States). The chemical composition of the SFL was analyzed in triplicate.

2.4 Observation of surface morphology

The surface morphologies of *Honeysuckle Flower*, *Baical Skullcap Root*, and *Fructus Forsythiae* were examined using a field emission scanning electron microscopy (FESEM; model SU8010; Hitachi, Japan), as previously described by Yi et al. (11). Images representing the average characteristics of the surface morphology were screened at a magnification of 1,000×.

2.5 Analysis of microbiota composition

The composition of the microbiota was analyzed following the methods previously described by Yi et al. (11). Briefly, the total microbial DNA of the SFL was extracted using the TIANamp Stool DNA Kit (Tiangen Biochemical Technology Inc., Beijing, China). The concentration and purity of the extracted DNA were determined using a NanoDrop ND-2000 spectrophotometer (Thermo Fisher Inc., Waltham, MA, United States). The V3–V4 region of the 16S rRNA genes was amplified using the bacterial universal primers 341F (5′-ACTCCTACGGGAGGCAGCAG-3′) and 806R (5′-GGACTACH VGGGTWTCTAAT-3′). Similarly, the V4 region of the 18S rRNA genes was amplified using the fungal universal primers F (5′-CCAGCASCYGCGGTAATTCC-3′) and R (5′-ACTTTCGTTCTT GATYRA-3′). The sequencing of the 16S rRNA and 18S rRNA was performed using the Illumina NovaSeq platform (Illumina Inc., San Diego, CA, United States) by Boyuezhihe Biology Science and Technology Co., Ltd. (Wuhan, China). The analysis of the microbiota composition of the SFL was performed in triplicate.

2.6 Animals, treatment, and feeding

The animal trial was conducted at Duoduoli Agricultural Science and Technology Co., Ltd., Fuyang, China. A total of 288 healthy Xinyang black laying hens, 50 week-old (laying rate < 80%) and with similar body weight, were selected for the feeding trial. After a 7-days adaptation period, the laying hens were divided into four groups using a completely randomized block design. Each group was fed with different SFL levels in water: 0 (control), 0.3, 0.5, and 0.7%. Each group consisted of nine replicates with eight hens per replicate. The composition and nutrient levels of the basal diets are presented in Table 1. The experimental laying hens were reared at the Ancient West Lake Modern Agricultural Science and Technology Demonstration Park in Fuyang, China, and were fed twice daily at 06:00 and 18:00. All the hens had free access to water at all times. The SFL was mixed with drinking water before feeding. The experiment lasted for 37 days, including the adaptation period.

TABLE 1 Ingredients and chemical compositions of basal diet (as DM basis).

Item	Value
Ingredient, g/kg	
Corn grain	645.08
Soybean meal	225.50
Limestone powder	88.90
DL-methionine	0.52
Premix[a]	40.00
Nutrient levels[b], g/kg	
Available phosphorus	3.30
Calcium	32.30
Crude protein	168.0
Metabolizable energy, MJ/kg	11.54
Lysine	6.70
Methionine	3.10
Total phosphorus	4.60

[a]Providing the following amounts of vitamins and minerals per kg of a complete diet: vitamin A, 10, 000 IU; vitamin D_3, 2, 500 IU; vitamin E, 18 IU; vitamin K_3, 1 mg; vitamin B_1, 2 mg; vitamin B_2, 6 mg; vitamin B_6, 3.5 mg; vitamin B_{12}, 15 μg; nicotinic acid, 63 mg; pantothenic acid, 18 mg; folic acid, 0.4 mg; biotin 0.15 mg; Fe, 80 mg; Cu, 9 mg; Zn, 70 mg; Mn, 80 mg; I, 0.6 mg; and Se, 0.3 mg.
[b]The data of crude protein was analyzed in triplicate according to AOAC methods No. 990.03, the calcium and total phosphorus were analyzed in triplicate according to AOAC methods No. 968.08 (12). Available phosphorus, metabolizable energy, lysine, and methionine levels were calculated.

2.7 Determination of laying performance and egg quality

The egg production, egg weight, feed offered, and refusals were recorded daily to calculate average egg weight, average daily feed intake (ADFI), feed-to-egg ratio, and laying rate.

Eggshell thickness without egg membrane was measured as the mean of the thickness at either end and in the middle of the shell using a screw micrometer (211-115, Sanliang Measuring Tool Inc., Dongguan, China) to calculate the eggshell thickness. The albumin height and yolk color were measured with a multifunctional egg quality meter (EA-01, Tenovo International Inc., Beijing, China). The egg shape index was calculated from the longitudinal and transverse diameters of the eggs using Vernier calipers (171-502, Sanliang Measuring Tool Inc., Dongguan, China). The Haugh units of eggs were calculated using the albumen height and egg weight.

2.8 Response surface experimental design and statistical analysis

The response surface experiments were designed and analyzed using Design-Expert 13.0 software, following the methods described by Mohammadabadi et al. (13). The independent variables were the initial pH and temperature, initial pH and rotational speed, and temperature and rotational speed. The initial pH, temperature, and rotational speed ranges were 6–7, 24–32°C, and 120–180 r/min, respectively. The dependent variables included chlorogenic acid, baicalin, and phillyrin. Regression models for these variables were constructed based on the data obtained in this study. The accuracy of the regression models was assessed using R^2, R^{adj}, C.V.%, and Adep precision values.

The data for the optimized fermentation conditions and the production performance of laying hens were analyzed using one-way ANOVA, and the comparative analysis among different treatment groups was analyzed by Duncan's multiple range test with SPSS software (version 26.0; SPSS Inc., Chicago, IL, United States). The data for the optimized fermentation conditions are presented as means and standard errors, and the data for the production performance of hens are presented as means and standard errors of the mean (SEM). Statistical significance was set at $p < 0.05$.

3 Results

3.1 Optimization of microbial fermentation conditions of Shuanghuanglian by single factor experiments

The results of the optimized fermentation conditions of Shuanghuanglian in a single-factor experiment are shown in Figure 1.

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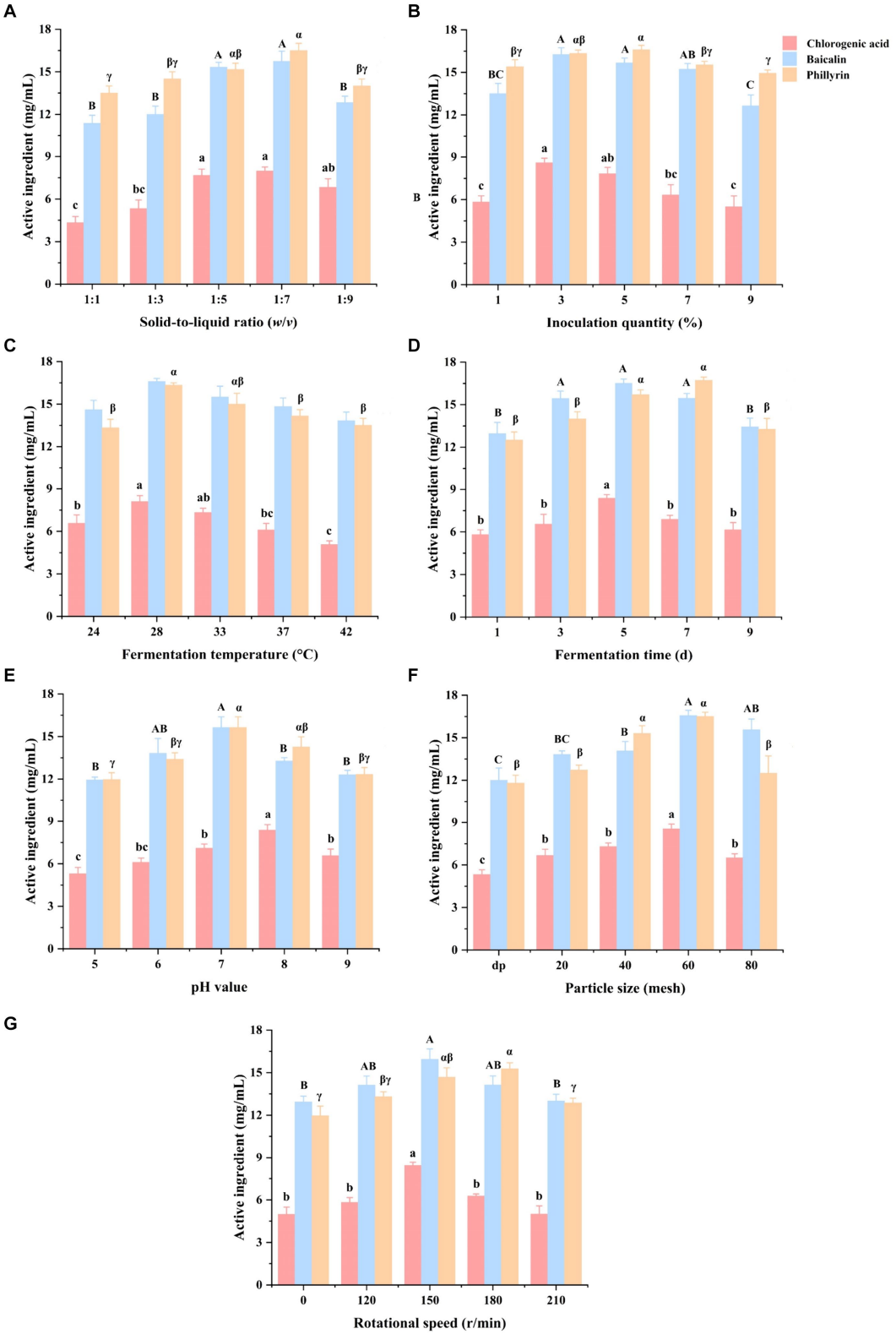

FIGURE 1
Effects of solid-to-liquid ratio **(A)**, inoculation quantity **(B)**, temperature **(C)**, time **(D)**, initial pH **(E)**, particle size **(F)**, and rotational speed **(G)** on chlorogenic acid, baicalin, and phillyrin concentrations of Shuanghuanglian fermentation liquor ($n=3$). d, days; dp, decoction pieces form without sieving; data are presented as means±standard error. Different letters in the same index indicate significant differences ($p<0.05$).

Compared with the 1:5 and 1:7 SLR, Shuanghuanglian fermented at 1:1 and 1:3 SLR had a lower ($p < 0.05$) chlorogenic acid concentration, while Shuanghuanglian fermented at 1:1, 1:3, and 1:9 SLR had a lower ($p < 0.05$) baicalin concentration. Furthermore, Shuanghuanglian fermented at 1:7 SLR had a higher ($p < 0.05$) phillyrin concentration than the 1:1, 1:3, or 1:9 SLR (Figure 1A).

In term of inoculation quantities, Shuanghuanglian fermented with 3% inoculation had a higher ($p < 0.05$) chlorogenic acid concentration than the 1, 7, and 9% inoculation. Shuanghuanglian fermented with 3 and 5% inoculation quantities had a higher ($p < 0.05$) baicalin concentration compared to the 1 and 9% inoculation. Moreover, Shuanghuanglian fermented with 5% inoculation had a higher ($p < 0.05$) phillyrin concentration than the 1, 7, and 9% inoculation (Figure 1B).

Compared with the fermentation temperatures at 24°C, 37°C, and 42°C, Shuanghuanglian fermented at 28°C had higher ($p < 0.05$) concentrations of chlorogenic acid and phillyrin. However, there was no significant difference ($p > 0.05$) in baicalin concentration among the different fermentation temperatures (including 24°C, 28°C, 33°C, 37°C, and 42°C) (Figure 1C).

Among the different fermentation times, Shuanghuanglian fermented for 5 days had a higher ($p < 0.05$) chlorogenic acid concentration compared to the other fermentation times (1, 3, 7, and 9 days). Shuanghuanglian fermented for 3, 5, and 7 days had a higher ($p < 0.05$) baicalin concentration compared to those fermented for 1 or 9 days. Furthermore, Shuanghuanglian fermented for 5 and 7 days had a higher ($p < 0.05$) phillyrin concentration than the 1, 3, and 9 days (Figure 1D).

When considering the initial pH values, Shuanghuanglian fermented with an initial pH of 8 had a higher ($p < 0.05$) chlorogenic acid concentration compared to the other initial pH values (5, 6, 7, and 9). Shuanghuanglian fermented with an initial pH of 7 had a higher ($p < 0.05$) baicalin concentration than the pH values of 5, 8, or 9. Moreover, Shuanghuanglian fermented with an initial pH value of 7 had a higher ($p < 0.05$) phillyrin concentration than the initial pH values of 5, 6, or 9 (Figure 1E).

Among the different particle sizes, Shuanghuanglian fermented with a particle size of 60 mesh had a higher ($p < 0.05$) chlorogenic acid concentration than other particle sizes (dp, 20, 40, and 80 mesh). Furthermore, Shuanghuanglian fermented with a particle size of 60 mesh had a higher ($p < 0.05$) baicalin concentration than the dp, 20, and 40 mesh, while Shuanghuanglian fermented with 40 and 60 mesh particle sizes had a higher ($p < 0.05$) phillyrin concentration compared to dp, 20, and 80 mesh (Figure 1F).

Compared with the 150 r/min, Shuanghuanglian fermented at 0, 120, 180, and 210 r/min had a lower ($p < 0.05$) chlorogenic acid concentration. Shuanghuanglian fermented at 150 r/min had a higher ($p < 0.05$) baicalin concentration than at the 0 and 210 r/min. Furthermore, Shuanghuanglian fermented at a rotational speed of 180 r/min had a higher ($p < 0.05$) phillyrin concentration than the 0, 120, and 210 r/min (Figure 1G).

3.2 Variance analysis of response surface model

Based on the single-factor experiments, three factors (initial pH, temperature, and rotational speed) were considered to verify the response surface experiments with significant differences. The design and results of the optimized fermentation of Shuanghuanglian by the response surface experiments are shown in Tables 2, 3.

The variance analysis of chlorogenic acid is presented in Table 4. The regression model for chlorogenic acid is $Y = 8.33 - 0.14A - 0.25B + 0.053C + 0.023AB - 0.14AC + 0.16BC - 1.42A^2 - 0.88B^2 - 0.84C^2$. Where Y represents the chlorogenic acid concentration (mg/mL), and A, B, and C represent initial pH, temperature (°C), and rotational speed (r/min), respectively. There was a significant difference ($p < 0.05$) in the regression model and no difference ($p > 0.05$) in the lack-of-fit. The factors A, B, C, AC, BC, A^2, B^2, and C^2 had significant differences ($p < 0.05$), indicating that they had a significant influence on the chlorogenic acid concentration. The R^2 and R^{adj} values of the regression model were 0.999 and 0.998, respectively. The C.V.% and Adep Precision were 0.70 and 72.39, respectively, indicating that the model has high accuracy and credibility.

The variance analysis of baicalin is shown in Table 5. The regression model of baicalin is $Y = 17.20 - 0.19A + 1.20B + 0.35C - 0.33AB - 0.96AC + 0.19BC - 2.50A^2 - 2.14B^2 - 2.35C^2$. Where Y represents the baicalin concentration (mg/mL), and A, B, and C represent initial pH, temperature (°C), and rotational speed (r/min), respectively. There was a significant difference ($p < 0.05$) in the regression model and no difference ($p > 0.05$) in the lack-of-fit. The factors A, B, C, AB, AC, A^2, B^2, and C^2 had significant differences ($p < 0.05$), indicating that they had a great effect on the baicalin concentration. The R^2 and R^{adj} values of the regression model were 0.996 and 0.992, respectively. The C.V.% and Adep Precision were 1.58 and 35.45, respectively, indicating that the model has high accuracy and credibility.

The variance analysis of phillyrin is presented in Table 6. The regression model for phillyrin is $Y = 17.10 + 0.31A - 0.84B - 0.41C - 0.15AB - 0.78AC + 0.19BC - 2.65A^2 - 1.45B^2 - 1.67C^2$. Where Y represents the phillyrin concentration (mg/mL), and A, B, and C represent initial pH, temperature (°C), and rotational speed (r/min), respectively. There was a significant difference ($p < 0.05$) in the model and no difference ($p > 0.05$) in the lack-of-fit. The factors A, B, C, AC, A^2, B^2, and C^2 had significant differences ($p < 0.05$), indicating that they had great effects on the phillyrin concentration. The R^2 and R^{adj} values of the regression model were 0.987 and 0.969, respectively. The C.V.% and Adep Precision were 2.49 and 18.98, respectively, indicating that the model has high accuracy and credibility.

3.3 Optimization of microbial fermentation conditions of Shuanghuanglian by response surface experiment

The contour lines and response surface of the optimized fermentation of Shuanghuanglian are shown in Figures 2–4. The effects of the interaction between initial pH and temperature on the

TABLE 2 Factors and levels of response surface experiment.

Factors	Levels		
	–1	0	1
pH	6	7	8
Temperature (°C)	24	28	32
Rotational speed (r/min)	120	150	180

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TABLE 3 Design and results of response surface experiment.

Design				Results		
Serial number	initial pH	Temperature (°C)	Rotational speed (r/min)	Chlorogenic acid (mg/mL)	Baicalin (mg/mL)	Phillyrin (mg/mL)
1	0	1	1	6.58	14.48	12.75
2	1	−1	0	6.11	11.46	14.08
3	1	0	0	8.38	16.89	17.54
4	0	1	−1	6.14	13.32	13.18
5	−1	0	1	6.39	13.77	12.80
6	1	0	1	5.83	11.55	11.93
7	0	0	0	8.37	17.39	16.95
8	1	1	0	5.65	13.20	12.45
9	0	0	0	8.26	17.59	16.5
10	0	0	0	8.26	17.10	17.35
11	0	0	0	8.36	17.05	17.17
12	0	−1	1	6.75	11.71	14.39
13	0	−1	−1	6.95	11.32	15.59
14	−1	0	−1	6.03	11.23	12.06
15	−1	1	0	5.89	14.32	12.21
16	1	0	−1	6.01	12.84	14.31
17	−1	−1	0	6.44	11.25	13.24

TABLE 4 Variance analysis of response surface model for chlorogenic acid.

Item	Sum of variance	Freedom	Mean square	*F*-values	*p*-values
Model	17.23	9	1.91	824.24	<0.001
A	0.17	1	0.17	71.19	<0.001
B	0.50	1	0.50	213.17	<0.001
C	0.02	1	0.02	9.50	0.018
AB	2.03×10^{-4}	1	2.03×10^{-3}	0.87	0.382
AC	0.07	1	0.07	31.39	0.001
BC	0.10	1	0.10	44.10	<0.001
A^2	8.52	1	8.52	3670.33	<0.001
B^2	3.28	1	3.28	1412.94	<0.001
C^2	2.97	1	2.97	1280.16	<0.001
Residual	0.02	7	2.32×10^{-3}		
Lack of fit	5.75×10^{-4}	3	1.92×10^{-4}	0.05	0.984
Error term	0.02	4	3.92×10^{-3}		
Summation	17.24	16	1.91		

A, initial pH; B, temperature; C, rotational speed.

concentrations of chlorogenic acid, baicalin, and phillyrin in SFL are shown in Figure 2. The contour lines for chlorogenic acid were circular, indicating that the interaction between pH and temperature had no significant effect on chlorogenic acid. However, the concentration of chlorogenic acid reached its highest point, and pH and temperature had significant effects on the concentration of chlorogenic acid. The contour lines for baicalin and phillyrin were elliptical, and the interaction between pH and temperature significantly affected baicalin and phillyrin. Temperature and pH had more significant effects on the concentration of baicalin than the phillyrin. Compared with the effects of pH on baicalin and phillyrin concentrations, temperature had greater effects, and there was also a significant interaction between pH and temperature. When the pH value was fixed, the concentrations of chlorogenic acid, baicalin, and phillyrin increased initially and then decreased with increasing temperature. At the same temperature, the concentrations of the three bioactive components increased initially and then decreased, and the concentrations reached a maximum with increasing pH value. Overall, the highest concentrations of chlorogenic

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TABLE 5 Variance analysis of response surface model for baicalin.

Item	Sum of variance	Freedom	Mean square	*F*-values	*p*-values
Model	94.12	9	10.46	215.67	<0.001
A	0.29	1	0.29	5.96	0.045
B	11.47	1	11.47	236.59	<0.001
C	0.98	1	0.98	20.21	0.003
AB	0.44	1	0.44	9.12	0.019
AC	3.67	1	3.67	75.63	<0.001
BC	0.15	1	0.15	3.06	0.124
A^2	26.38	1	26.38	544.13	<0.001
B^2	19.34	1	19.34	398.88	<0.001
C^2	23.32	1	23.32	480.88	<0.001
Residual	0.32	4	0.08		
Lack of fit	0.34	7	0.05		
Error term	0.02	3	7.57×10^{-3}	0.10	0.959
Summation	94.46	16			

A, initial pH; B, temperature; C, rotational speed.

TABLE 6 Variance analysis of response surface model for phillyrin.

Item	Sum of variance	Freedom	Mean square	*F*-values	*p*-values
Model	66.06	9	7.34	57.33	<0.001
A	0.76	1	0.76	5.91	0.045
B	5.63	1	5.63	43.96	<0.001
C	1.34	1	1.34	10.44	0.014
AB	0.09	1	0.09	0.70	0.430
AC	2.43	1	2.43	19.01	0.003
BC	0.15	1	0.15	1.16	0.318
A^2	29.67	1	29.67	231.77	<0.001
B^2	8.88	1	8.88	69.36	<0.001
C^2	11.77	1	11.77	91.96	<0.001
Residual	0.90	7	0.13		
Lack of fit	0.25	3	0.08	0.52	0.689
Error term	0.64	4	0.16		
Summation	66.96	16			

A, initial pH; B, temperature; C, rotational speed.

acid, baicalin, and phillyrin were observed within the temperature range of 27–30°C and the pH value of 6.5–7.5.

The interactions between initial pH and rotational speed on the chlorogenic acid, baicalin, and phillyrin concentrations in the SFL are shown in Figure 3. The contour lines for chlorogenic acid, baicalin, and phillyrin observed as oval shapes, and the interaction between pH and rotational speed had significant effects on the chlorogenic acid, baicalin, and phillyrin concentrations. Compared to the rotational speed on chlorogenic acid concentration, the pH value had a greater effect on its concentration. Compared with the effects of pH on baicalin and phillyrin concentrations, the effects of rotational speed on their concentrations were more significant. When the pH value was constant, the chlorogenic acid, baicalin, and phillyrin concentrations displayed an increasing trend initially and then decreased with increasing rotating speed, whereas when the rotational speed was constant, the three bioactive components displayed an increasing trend initially and then decreased with increasing pH value. Overall, the highest concentrations of chlorogenic acid, baicalin, and phillyrin were observed at a rotational speed of 145–155 r/min and the pH value of 6.5–7.5.

The interactions between temperature and rotational speed on chlorogenic acid, baicalin, and phillyrin concentrations in SFL are shown in Figure 4. The contours lines for baicalin and phillyrin concentrations were circular, and the interaction between temperature and rotational speed had no significant effects on baicalin and phillyrin concentrations. However, baicalin and phillyrin reached their highest concentration points, and the rotational speed and temperature had significant effects on baicalin and phillyrin concentrations. Temperature had a more significant effects on baicalin and phillyrin concentrations

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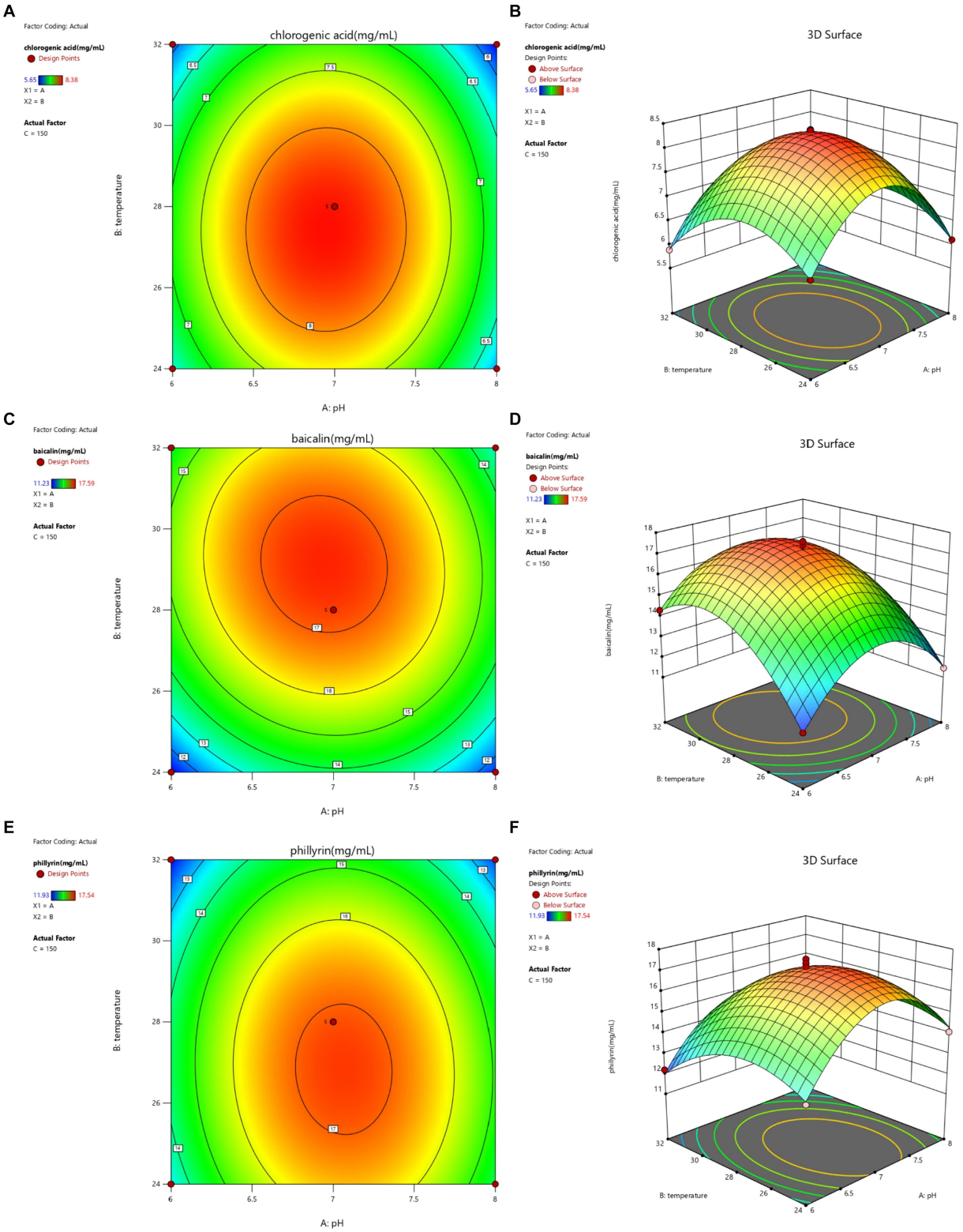

FIGURE 2
Contour lines and response surface of initial pH and temperature to chlorogenic acid **(A,B)**, baicalin **(C,D)**, and phillyrin **(E,F)** concentrations of Shuanghuanglian fermentation liquor.

than the rotational speed. The contour line for chlorogenic acid concentration was elliptical, and the interaction between temperature and rotational speed had significant effects on the chlorogenic acid concentration. Temperature had a more significant effect on chlorogenic acid concentration than the rotational speed. When the temperature was constant, the chlorogenic acid, baicalin, and phillyrin

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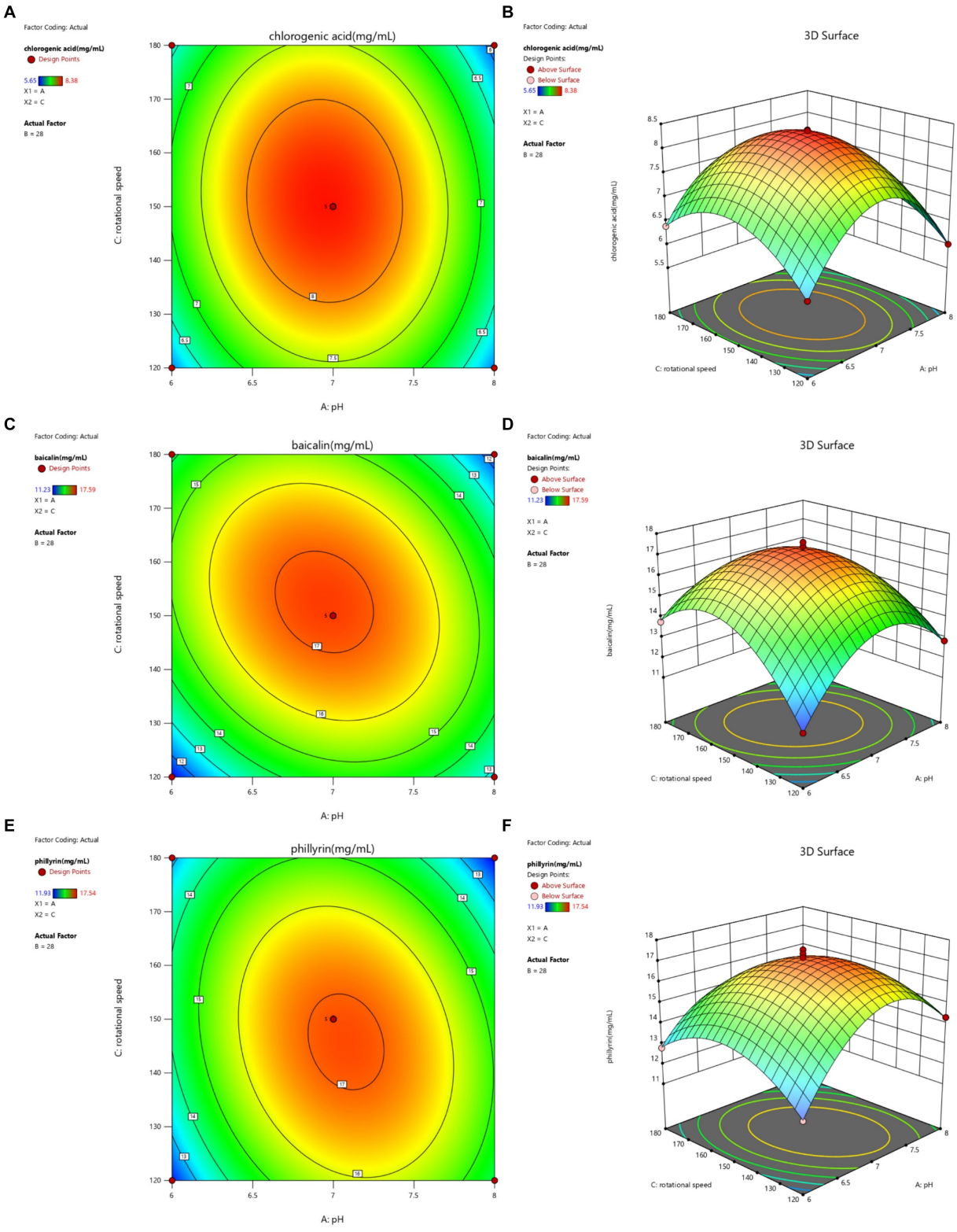

FIGURE 3
Contour lines and response surface of initial pH and rotational speed to chlorogenic acid **(A,B)**, baicalin **(C,D)**, and phillyrin **(E,F)** concentrations of Shuanghuanglian fermentation liquor.

concentrations displayed an increasing trend initially and then decreased with an increasing rotational speed. At a constant rotational speed, the three bioactive components initially increased and then decreased with the increasing temperature. Overall, the highest concentrations of chlorogenic acid, baicalin, and phillyrin appeared with a rotational speed of 145–155 r/min and temperature of 27–30°C.

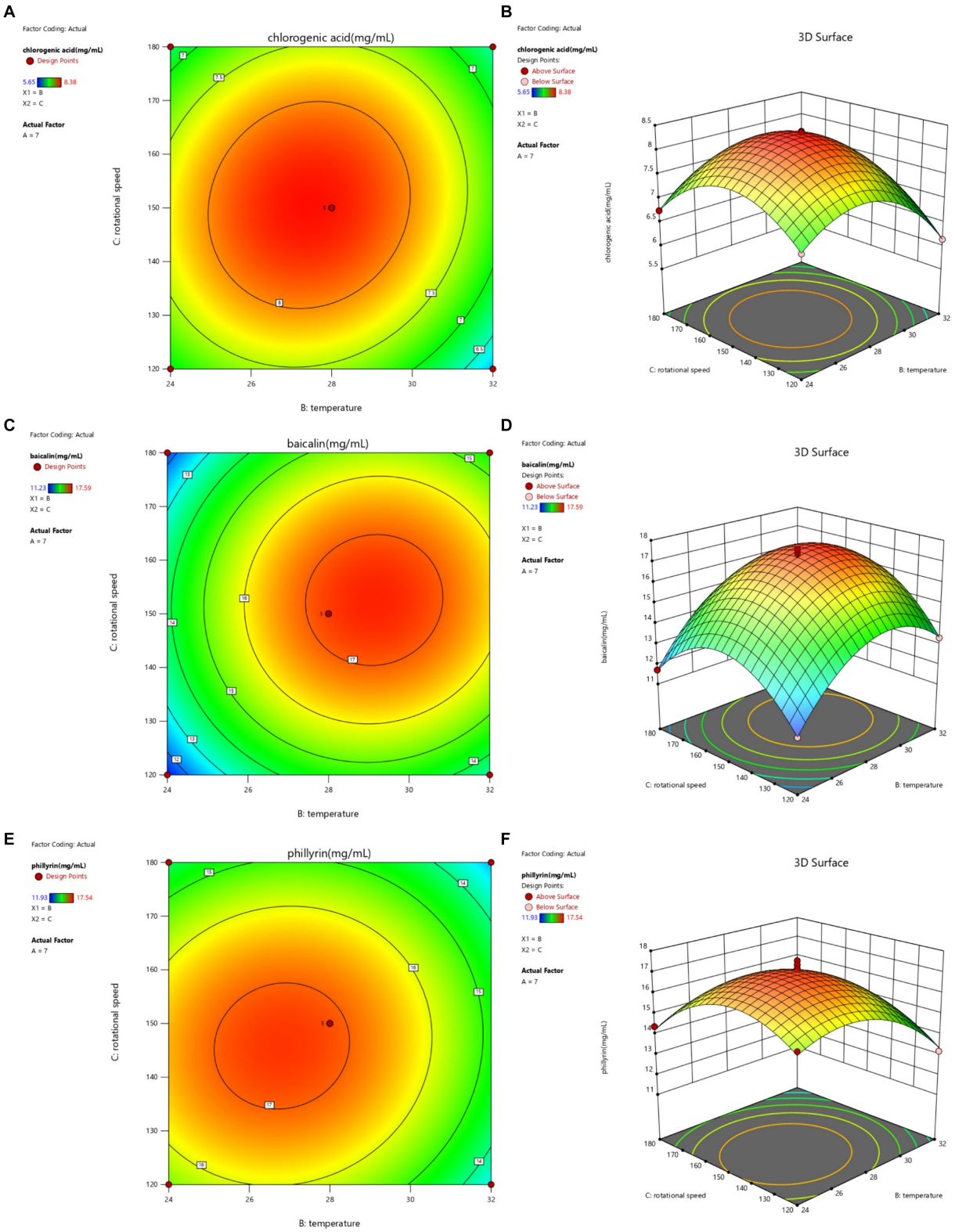

FIGURE 4
Contour lines and response surface of temperature and rotational speed to chlorogenic acid **(A,B)**, baicalin **(C,D)**, and phillyrin **(E,F)** concentrations of Shuanghuanglian fermentation liquor.

The optimized fermentation conditions were determined as follows: an initial pH of 6.99, temperature of 27.89°C, and rotational speed of 149.99 r/min. Considering the application of production, the fermentation conditions were set with an initial pH of 7, temperature at 28°C, and rotational speed of 150 r/min. Under these conditions, the concentrations of chlorogenic acid, baicalin, and phillyrin were 9.36, 18.52, and 17.63 mg/

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TABLE 7 Compound compositions (% of total compounds) of Shuanghuanglian fermentation liquor.

Item	Value
(2E,5E)-2,5-Heptadiene	4.80
Myrtenol	4.45
2,6-Di-tert-butylnaphthalene	3.25
Decamethylcyclopentasiloxane	2.60
2-Hexyn-1-ol	2.11
Arsenous acid tris(trimethylsilyl) ester	2.04
3(10)-Caren-4-ol	1.81
Decamethyltetrasiloxane	1.67
3-Hydroxymethylbenzamide	1.39
2-Isopropylidenecycloheptanone semicarbazone	1.19
Oxime-, methoxy-phenyl	1.09
Others	73.62

mL, respectively. These concentrations were not significantly different from those predicted by the response surface model (concentrations of chlorogenic acid, baicalin, and phillyrin were 8.33, 17.17, and 17.12 mg/mL, respectively). Compared to Shuanghuanglian fermented under basic conditions, the concentrations of chlorogenic acid, baicalin, and phillyrin were increased by 75.60, 43.23, and 47.40%, respectively, indicating that the optimized technological parameters were accurate and reliable, and the response surface model could accurately predict the concentrations of chlorogenic acid, baicalin, and phillyrin in SFL.

Finally, the optimized fermentation conditions for Shuanghuanglian were selected as a 1:7 SLR, 3% inoculation quantity, 28°C fermentation for 5 days, initial pH of 7, 60 mesh particle size, and a rotational speed of 150 r/min.

3.4 Chemical composition of Shuanghuanglian fermentation liquor

The chemical composition of SFL is presented in Table 7. Compounds with relative proportions ≤1.0% and qualitative values ≤50% were excluded from further analysis. The baicalin, phillyrin, and chlorogenic acid concentrations in SFL were 18.52, 17.63, and 9.36 mg/mL, respectively. Furthermore, (2E,5E)-2,5-heptadiene had the highest proportion (4.80%) in SFL, followed by myrtenol (4.45%), 2,6-di-tert-butylnaphthalene (3.25%), decamethylcyclopentasiloxane (2.60%), 2-hexyn-1-ol (2.11%), arsenous acid tris(trimethylsilyl) ester (2.04%), 3(10)-caren-4-ol (1.81%), decamethyltetrasiloxane (1.67%), 3-hydroxymethylbenzamide (1.39%), 2-isopropylidenecycloheptanone semicarbazone (1.19%), and oxime-, methoxy-phenyl (1.09%).

3.5 The surface morphology of *honeysuckle flower*, *Baical skullcap root*, and *Fructus Forsythiae*

Scanning electron microscopy analyses showed that the cell walls of *Honeysuckle Flower* (Figures 5A,B), *Baical Skullcap Root* (Figures 5C,D), and *Fructus Forsythiae* (Figures 5E,F) were more broken after fermentation compared to before fermentation.

3.6 Microbiota composition of Shuanghuanglian fermentation liquor

The bacterial composition in SFL is shown in Figure 6. Phyla with relative abundances ≤1.0% were not included in further analyses. Proteobacteria (58.27%) and Firmicutes (27.79%) were the most dominant phyla in SFL, followed by Bacteroidota (5.65%), Deinococcota (2.75%), Actinobacteria (2.13%), and Spirochaetota (2.08%) (Figure 6A). Genera with relative abundances ≤1.0% were excluded from further analysis. *Acinetobacter* (31.35%) was the most dominant genus in SFL, followed by *Porphyrobacter* (3.45%), *Subdoligranulum* (2.80%), *Deinococcus* (2.75%), *Brevundimonas* (2.65%), *Lactobacillus* (2.24%), *Sphingomonas* (2.15%), *Treponema* (2.08%), *Enhydrobacter* (2.08%), *Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium* (1.91%), *Clostridia_UCG-014* (1.89%), *Blautia* (1.57%), *Methylobacterium-Methylorubrum* (1.53%), *Sphingobium* (1.44%), *Muribaculaceae* (1.39%), *Blastomonas* (1.24%), *Agathobacter* (1.10%), *Streptococcus* (1.08%), and *Prevotella* (1.01%) (Figure 6B).

The fungal composition of SFL is shown in Figure 7. Phyla with relative abundances ≤1.0% were not considered for further analyses. The relative abundances of Phragmoplastophyta and Arthropoda in SFL were 97.48 and 1.04%, respectively (Figure 7A). Genera with relative abundances ≤1.0% were not included in further analyses. The relative abundances of *Magnoliophyta* and *Dryopteris* in SFL were 91.49 and 5.89%, respectively (Figure 7B).

3.7 Laying performance and egg quality

The effects of SFL on laying performance and egg quality are presented in Table 8. Supplementation of 0.5 and 0.7% SFL in drinking water increased ($p < 0.05$) egg weight and laying rate and decreased ($p < 0.05$) feed-to-egg ratio compared with the control group. However, there was no significant difference ($p > 0.05$) in the ADFI among different groups.

Regarding egg quality traits, hens supplemented with 0.3, 0.5, and 0.7% SFL in drinking water had a higher ($p < 0.05$) Haugh unit than the control group, whereas there were no significant differences ($p > 0.05$) in albumen height, egg shape index, eggshell thickness, and yolk color among all groups.

4 Discussion

Shuanghuanglian has found to have anti-inflammatory, antibacterial, antiviral, and immunomodulatory effects (1–3). However, there is still a lack of research on the use of Shuanghuanglian in animal husbandry, particularly in the layer industry. In this study, fermentation was conducted with lignin-degrading bacteria (*Bacillus amyloliquefaciens-c4*) to enhance the bioactive components in Shuanghuanglian. This study optimized the fermentation conditions for Shuanghuanglian, investigated the compounds and microbial composition of SFL, and evaluated its effects on the production performance of laying hens. The results showed that supplementing SFL had positive effects on the production performance of laying hens. The bioactive components present in SFL, such as chlorogenic acid, baicalin, forsythin, myrtenol, 2-hexyn-1-ol, arsenous acid

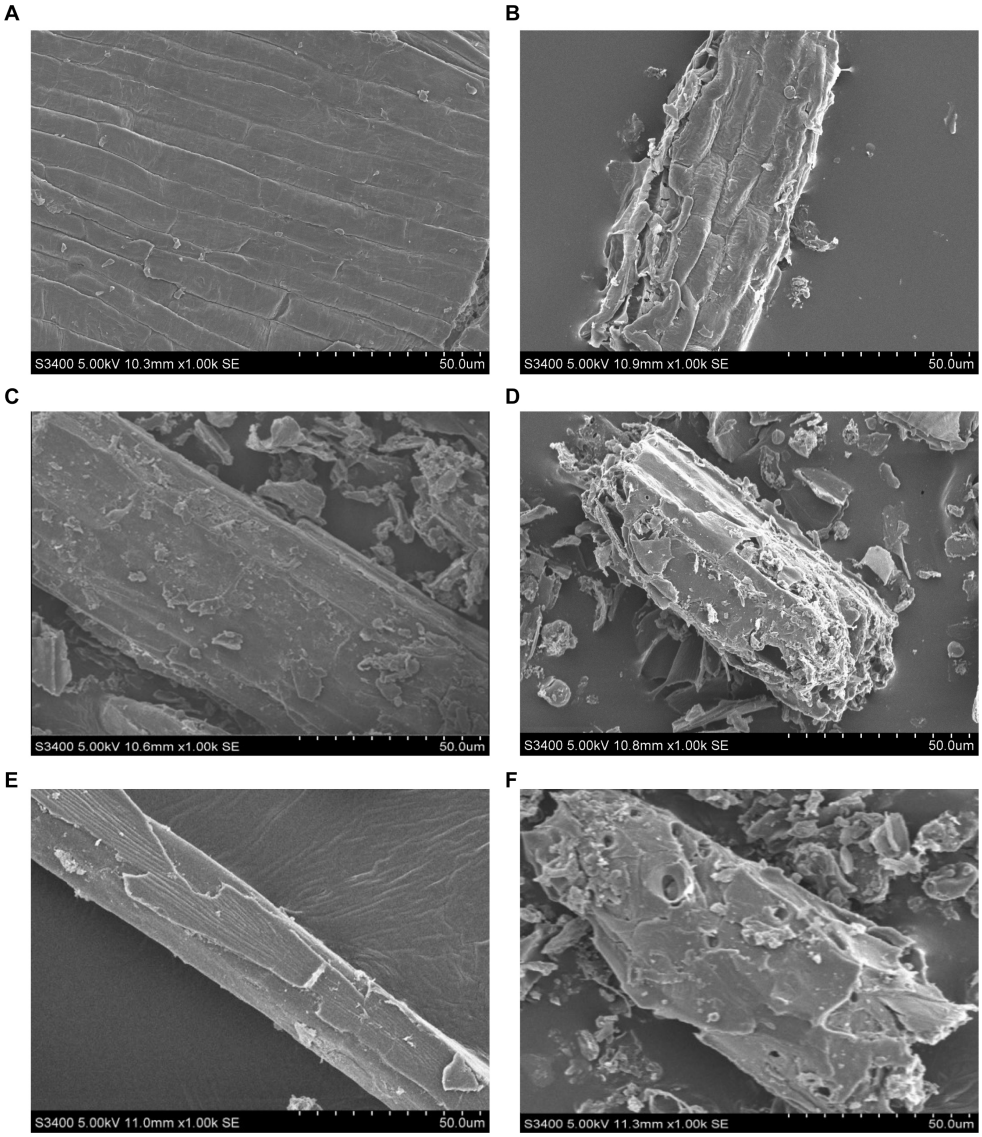

FIGURE 5
Scanning electron microscopy images of *Honeysuckle Flower* **(A,B)**, *Baical Skullcap Root* **(C,D)**, and *Fructus Forsythiae* **(E,F)** before and after fermentation.

tris(trimethylsilyl) ester, 3(10)-caren-4-ol, and oxime-, methoxy-phenyl, may play a positive role in improving the production performance of laying. These components can also improve the immunity of laying hens due to their antibacterial, antiviral, and anti-inflammatory effects.

Chlorogenic acid, baicalin, and phillyrin are the main bioactive components of *Honeysuckle Flower*, *Baical Skullcap Root*, and *Fructus Forsythiae* (1), and have various biological effects such as antibacterial, antiviral, anti-inflammatory, anti-oxidative, and intestinal health-improving effects in laying hens (14–16). Thus, chlorogenic acid, baicalin, and phillyrin concentrations in SFL were assessed as representative indices to determine the optimal conditions for Shuanghuanglian. Moisture content, inoculation quantity, initial pH, particle size, rotational speed, fermentation time, and fermentation temperature are the main factors that could affect the concentrations of bioactive components present in Chinese medicine after fermentation (11, 17). Thus, in this study, we optimized these fermentation conditions. The concentrations of chlorogenic acid, baicalin, and phillyrin reached the highest values when the following parameters, including solid–liquid ratio, fermentation time, and particle size, were 1:7, 5 days, and 60 mesh. Therefore, the optimized fermentation conditions of solid–liquid ratio, fermentation time, and particle size for Shuanghuanglian were selected as a 1:7 SLR, 5 days, and 60 mesh through single factor experiments. However, Shuanghuanglian fermented with 3% inoculation had higher chlorogenic acid and baicalin concentrations, whereas fermented with 5% inoculation had higher baicalin and phillyrin concentrations in SFL. Shuanghuanglian fermented with 3% inoculation is more cost-effective than the 5% inoculation. Thus, this study used 3% inoculation for Shuanghuanglian fermentation. The optimized fermentation conditions of initial pH, temperature, and rotational speed were 6.99, 27.89°C, and 149.99 r/min,

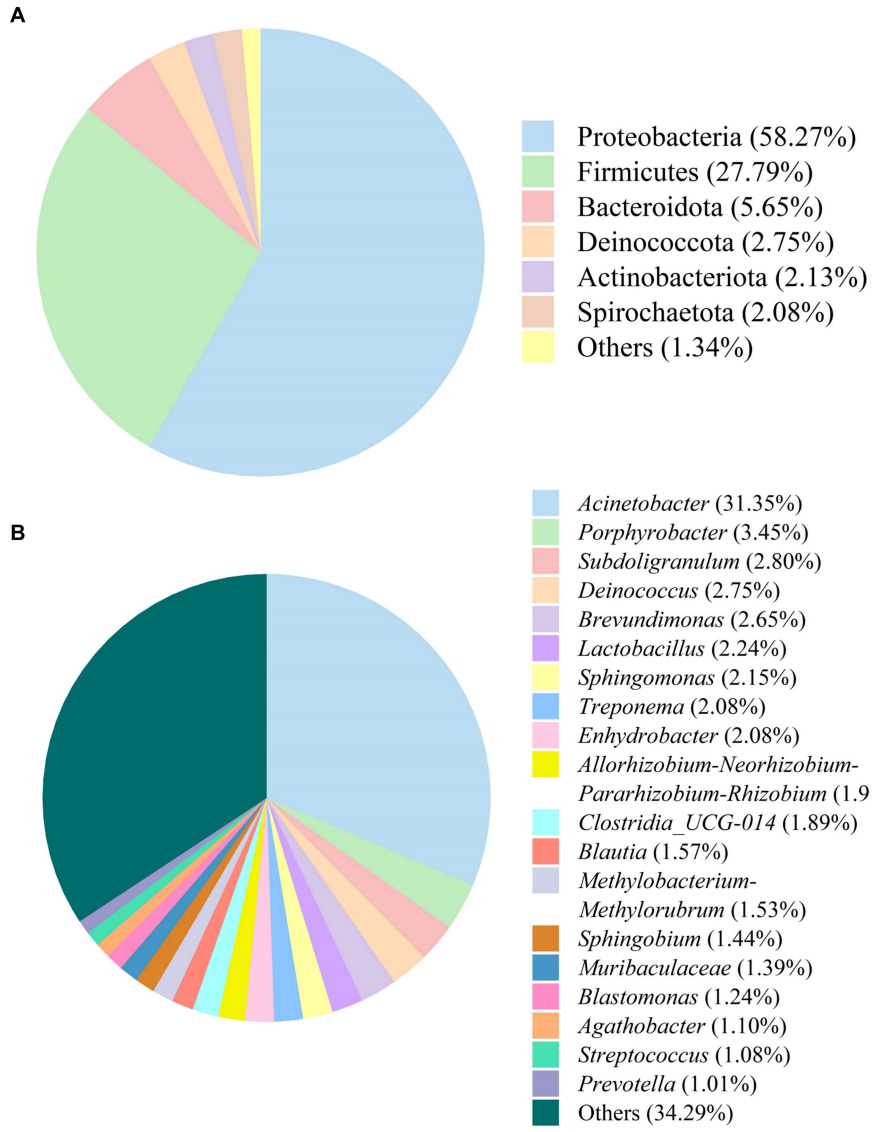

FIGURE 6
The bacteria composition of Shuanghuanglian fermentation liquor at the phylum **(A)** and genus **(B)** levels.

respectively, determined with response surface experiment. Considering the economical production, the optimal fermentation conditions of initial pH value, temperature, and rotational speed were set at 7, 28°C, and 150 r/min, respectively. Finally, the optimized fermentation conditions for Shuanghuanglian were selected as a 1:7 SLR, inoculation quantity 3%, initial pH 7, 60 mesh particle size, rotational speed 150 r/min, and 28°C fermentation for 5 days through single factor and response surface experiments.

We further determined the composition of SFL and identified various compounds with important biological efficacy. Among these compounds, myrtenol has been reported to have antibacterial, antibiofilm, anti-inflammatory, and antinociceptive effects (18, 19). In the present study, SFL contained higher myrtenol content, indicating that SFL may reduce disease incidence and damage to poultry. In addition, 2-hexyn-1-ol contributes to the antibacterial activity (20), and arsenous acid tris(trimethylsilyl) ester is a flavor-related compound (21, 22). Similar to the present study, arsenous acid tris(trimethylsilyl) ester and 3(10)-caren-4-ol have been detected in various Chinese herbal medicines in previous studies and exhibited antibacterial effects (23–26). Moreover, oxime-, methoxy-phenyl is also a flavor-related compound (27) and has been found to have antitumor and anticancer effects (28). Therefore, supplementing SFL to laying hens may increase disease resistance and feed intake, thereby enhancing production performance.

Proteobacteria and Firmicutes were the most abundant phyla in SFL, which is consistent with the bacterial phyla composition of Chinese herbal medicines fermented by *Bacillus amyloliquefaciens-c4* (11). *Acinetobacter* was the most abundant genus detected in SFL and can produce various umami peptides (29). Thus, a higher relative abundance of *Acinetobacter* in SFL may increase the feed intake in poultry. However, there was no significant change in the abundance of *Bacillus* in the present study despite inoculation with 3% *Bacillus amyloliquefaciens-c4*, which is inconsistent with a previous study

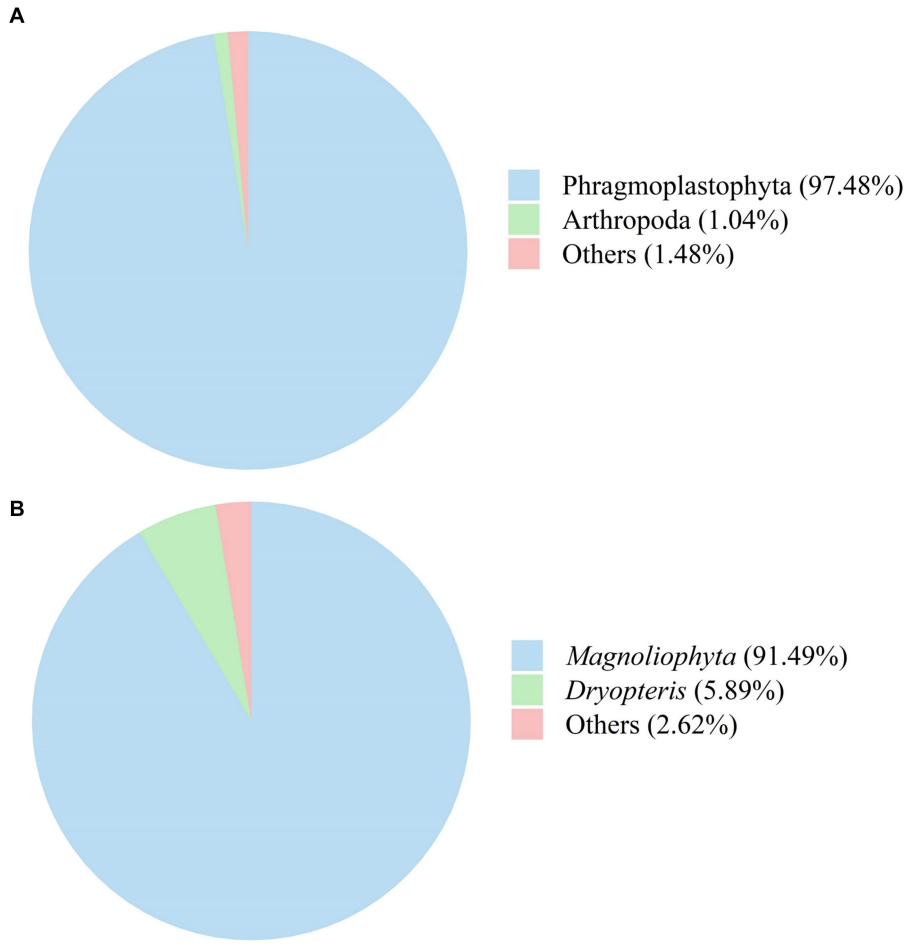

FIGURE 7
The fungi composition of Shuanghuanglian fermentation liquor at the phylum **(A)** and genus **(B)** levels.

TABLE 8 Effects of Shuanghuanglian fermentation liquor (SFL) on laying performance and egg quality of laying hens.

Item	SFL levels in water				SEM	*p*-values
	0%	0.3%	0.5%	0.7%		
Laying performance						
ADFI (g/d)	105.60	104.88	109.19	108.87	1.471	0.684
Egg weight (g)	51.26^{c}	54.35^{bc}	59.99^{a}	57.90^{ab}	1.095	0.007
Feed-to-egg ratio	2.07^{a}	1.93^{ab}	1.82^{b}	1.88^{b}	0.031	0.014
Laying rate (%)	70.85^{b}	75.86^{ab}	79.14^{a}	78.26^{a}	1.132	0.022
Egg quality						
Albumen height (mm)	4.50	4.80	5.00	4.83	0.101	0.404
Egg shape index	1.28	1.30	1.30	1.30	0.009	0.832
Eggshell thickness (mm)	0.36	0.36	0.37	0.37	0.004	0.797
Haugh unit	68.53^{b}	72.17^{a}	73.30^{a}	72.20^{a}	0.620	0.007
Yolk color	4.33	4.70	5.30	5.57	0.247	0.300

ADFI, average daily feed intake. Different letters indicate significant differences ($p < 0.05$).

(11). This inconsistency may be due to *Bacillus amyloliquefaciens-c4*, being a lignin-degrading bacterium, attaching primarily to the cell wall of Shuanghuanglian rather than releasing it to SFL. This statement is also supported by the more disrupted surface morphology of the *Honeysuckle Flower*, *Baical Skullcap Root*, and *Fructus Forsythiae* after fermentation in the present study. In addition, Phragmoplastophyta was the main fungal phylum, and *Magnoliophyta* was the main fungal genus in SFL. The findings are consistent with

the findings of Yi et al. (11), who reported that Chinese herbal medicines fermented with *Bacillus amyloliquefaciens-c4* had higher abundances of Phragmoplastophyta and *Magnoliophyta*. The genera *Magnoliophyta* and *Dryopteris* belong to the phylum Phragmoplastophyta and exist widely in the air and soil environments (30), which may explain their abundance in SFL.

To evaluate the effects of SFL, different levels of SFL were supplemented with drinking water in laying hens. The results showed that 0.5 and 0.7% SFL supplementation increased egg weight and laying rate while decreasing the feed-to-egg ratio of laying hens. The increased production performance of laying hens is possibly due to the antibacterial and anti-inflammatory effects of SFL. In addition, supplementation of SFL increased the Haugh units of eggs, indicating that the bioactive components present in SFL improved the albumen and storage quality of eggs (31, 32). Wang et al. (14) also found that supplementation of *Honeysuckle Flower* and *Baical Skullcap Root* extracts in laying hens' diet alleviated intestinal disorders and performance impairment caused by *Salmonella pullorum* infection. Interestingly, SFL supplementation had no effects on the ADFI of laying hens, although it included various flavor-related compounds, which may have been caused by the addition of SFL in drinking water rather than in the diet. Furthermore, supplementation of 0.3% SFL had no effects on the laying performance of hens, indicating that SFL supplementation in drinking water should be at least 0.5%. Overall, the findings indicate that SFL supplementation had beneficial effects on the production performance of laying hens, especially when supplementation reached 0.5%.

5 Conclusion

The optimal fermentation conditions for Shuanghuanlian were determined as follows: a SLR ratio of 1:7, 3% inoculation quantity, fermentation at 28°C for 5 days, initial pH of 7, a 60 mesh particle size, and a rotational speed of 150 r/min. These conditions are crucial in ensuring consistent product quality and composition for future testing and application. Supplementing at least 0.5% SFL in drinking water had positive effects on the production performance of laying hens, including increased egg weight, laying rate, and Haugh unit and decreased feed-to-egg ratio, which indicate that SFL based on the optimized fermentation conditions could positively influence the production performance of laying hens. Further in-depth studies are necessary to explore the long-term effects of SFL, the mechanisms of bioactive components in SFL, and the combination effects with other feed additives or drugs on the production performance of laying hens. Overall, these findings provide a solid foudation for further investigation into the use of Shuanghuanglian as a natural feed addition to improve the productivity and sustainability of egg production.

Data availability statement

The data presented in the study are deposited in the Figshare repository, accession number is doi: 10.6084/m9.figshare.27050017.v1.

Ethics statement

The animal study was approved by the Animal Care and Use Committee of the Institute of Subtropical Agriculture, Chinese Academy of Sciences, Changsha, China (ISA-2018-071). The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

YX: Data curation, Formal analysis, Visualization, Writing – review & editing. SY: Formal analysis, Visualization, Writing – original draft. XX: Data curation, Methodology, Writing – review & editing. MZ: Data curation, Methodology, Writing – review & editing. YC: Methodology, Writing – review & editing. WL: Methodology, Writing – review & editing. FL: Conceptualization, Funding acquisition, Writing – review & editing. XK: Conceptualization, Funding acquisition, Writing – review & editing.

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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 potential conflicts of interest.

The author(s) declared that they were an editorial board member of Frontiers, at the time of submission. This had no impact on the peer review process and the final decision.

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Effects of *Lagenaria siceraria (Molina) Standl* polysaccharides on growth performance, immune function, cecum microorganisms and short-chain fatty acids in broilers

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In this study, *Lagenaria siceraria (Molina) Standl* polysaccharides (LSP) was prepared using the water-alcohol precipitation method to evaluate its effects on growth performance, slaughter performance, cytokines, immune organ indices, cecal short-chain fatty acids (SCFAs), and microbial community structure in broiler chickens when added to the basal diet. Seventy-five broiler chickens were selected and randomly divided into five groups, with 15 chickens per group. All groups were fed a basal diet for 7 days. From 7 days of age, the control group continued to receive the basal diet, while the positive drug group was fed a diet supplemented with Astragalus polysaccharides (APS, 100 g/kg) in addition to the basal diet. The experimental groups were fed diets containing different concentrations of LSP (50, 100, and 200 g/kg) in addition to the basal diet, and the supplementation continued for 42 days. The findings indicated that the incorporation of LSP into the feed significantly enhanced average daily weight gain (ADWG), average daily feed intake (ADFI), feed to gain ratio (F/G), dressing percentage, percentage of breast muscle, percentage of leg muscle, and percentage of abdominal fat while concurrently reducing drip loss rate and cooking loss rate ($p < 0.01$) in comparison to the control group. Additionally, it significantly augmented the levels of interleukin-4 (IL-4) and interleukin-12 (IL-12) in cytokines, secreted immunoglobulin A (SIgA) and immunoglobulin G (IgG) in immunoglobulins, as well as immune organ indicators ($p < 0.05$). Furthermore, LSP also modulated the intestinal microbiome composition by increasing the abundance of *Bacteroides* species and significantly changing concentrations of specific short-chain fatty acids (SCFAs) such as propionic acid, isobutyric acid, acetic acid, and isovaleric acid ($p < 0.01$). These results suggest that dietary supplementation with LSP can effectively regulate intestinal microbiome composition while promoting short-chain fatty acid production. The alterations in microbial characteristics ultimately contribute to improved intestinal immunity and immune organ development as well as enhanced production performance and immune function in broilers.

KEYWORDS

growth performance, immune function, intestinal microbiota, *Lagenaria siceraria (Molina) Standl* polysaccharides, short-chain fatty acids

1 Introduction

Intestinal health is a prerequisite for the optimal growth and development of livestock and poultry. The intestinal well-being of these animals is determined by factors such as the composition of their gut microbiota, host immunity (including mucosal barrier function), nutritional intake, and environmental conditions (1). Issues related to intestinal health in livestock and poultry farming often arise from dysbiosis within the gut microbiota, impairment of the mucosal barrier integrity, or inflammatory responses (2). Although antibiotics, vaccines, chemical drugs, and immune stimulants have been utilized in farming with significant effectiveness, their excessive use has resulted in numerous side effects (3). For instance, overuse of antibiotics in farming can contribute to antibiotic resistance development, disrupt the microbial environment, and cause drug residues that contaminate the environment (4). Therefore, there is an urgent requirement for a novel feed additive that possesses safety, efficiency, absence of residues, and immune-enhancing properties to ensure the robust development of the breeding industry.

Lagenaria siceraria, belonging to the Cucurbitaceae family, is a plant with both medicinal and nutritional value (5). In traditional medicine, it has been widely used to treat various diseases. The pulp of the *Lagenaria siceraria* can serve as both an emetic and a laxative, and it also has diuretic properties (6). The vines and tendrils of the plant, which share similar medicinal properties with the flowers, can be used to treat skin conditions like leprosy. The seeds are bitter, cold in nature, and toxic, and they can be used to treat dental diseases, facial swelling, and urinary retention (7). Additionally, *Lagenaria siceraria* is rich in nutrients, containing polysaccharides, proteins, various trace elements, vitamin C, and trypsin inhibitors. These components help enhance immunity, promote antibody synthesis, and improve disease resistance in animals (8).

Polysaccharides derived from natural plants exhibit anti-tumor, antioxidant, anti-inflammatory, hypoglycemic, blood pressure-lowering, immune-regulating, properties, among others. Simultaneously, they possess the advantages of non-toxicity, absence of residue in animal bodies and products, and resistance to occurrence. These attributes play a pivotal role in promoting the sustainable development of modern animal husbandry (9). As a significant active ingredient in *Lagenaria siceraria*, *Lagenaria siceraria (Molina) Standl* polysaccharide (LSP) has progressively garnered attention from researchers. Studies have revealed that LSP hold potential for treating jaundice, diabetes, ulcers, hemorrhoids while also alleviating symptoms associated with colitis (10). Furthermore it has been found to possess antipsychotic and antihypertensive properties along with its ability to alleviate congestive heart failure and skin diseases (11). The molecular weight of LSP was determined to be 78 kDa by Kaushik Ghosh et al., who identified structural fragments such as 1–4 linked α-D-galacturonic acid, 1–2 linked 3-O-acetylmethyl-α-D-galacturonic acid and 1–4 linked β-D-galactose within the polysaccharide structure (12). Zhou et al. demonstrated that LSP50 exhibits immunomodulatory effects on immune organ indices, H9N2-specific IgG levels, cytokine profiles (including IFN-γ, IL-2, IL-4, and IL-5), and the CD3eCD8a T cell ratio. Furthermore, sequencing analysis revealed that LSP50 regulates PLA2G12B and PTGDS genes involved in arachidonic acid pathway to modulate immune response (13). Wusiman et al. (14) used polysaccharide extracts as vaccine adjuvants for chicken immunity. It was found that the administration of LSP led to an increase in HI titers, enhanced production of antigen-specific IgG NDV antibodies, increased proliferation of splenic lymphocytes, and elevated immune organ indices (14).

Therefore, the objective of this study is to assess the potential of LSP as a natural dietary supplement for enhancing chicken health, with a specific focus on its impact on growth performance, slaughter performance, immune function, gut microbiota composition, and short-chain fatty acid concentration. The effects of LSP on chicken growth and slaughter performance were evaluated by monitoring changes in body weight, feed consumption, carcass weight, and net body weight. Furthermore, the influence of LSP on intestinal functionality in chickens was analyzed by investigating its effects on intestinal morphology, immune function markers such as globulin content and immune organ index values, short-chain fatty acid concentration levels, and gut microbial composition.

2 Materials and methods

2.1 Preparation and structural analysis of LSP

The crude polysaccharides were extracted using water decoction and ethanol precipitation methods, followed by protein removal through the Sevag method. Subsequently, the crude polysaccharides underwent a process of separation and purification involving elution with deionized water. The elution fractions were collected, merged, and then subjected to freeze-drying to obtain a pure and homogeneous form of LSP (14). The monosaccharide composition of LSP was analyzed using Fourier-transform infrared spectroscopy and high-performance liquid chromatography techniques.

2.2 Animal grouping and immunization procedures

A total of 75 newly hatched Sanhuang broilers, aged 0 days, were randomly allocated into five groups with 15 broilers per group after a seven-day period of domestication. The control group was fed a basal diet daily (Tiankang Biotech Co., Ltd.). The LSP treatment group was further divided into three subgroups, each receiving different doses of LSP in the basal diet: LSP-low (LSP-l, 50 g/kg), LSP-mid (LSP-m, 100 g/kg), and LSP-high (LSP-h, 200 g/kg). The positive drug group received a basal diet supplemented with 100 g/kg of APS (Sichuan Dingjian Animal Pharmaceutical Co., Ltd.). At day 8, the broilers were intranasally immunized with a commercially available inactivated H9N2 vaccine (Urumqi Center for Disease control and Prevention) followed by a secondary immunization at day 22. The control group received intranasal administration of physiological saline solution. Dissection was performed at day 50 with *ad libitum* feeding throughout the experimental period. Animal welfare guidelines for experimental animals were strictly adhered to during the study (Table 1).

2.3 Sample collection

At 50 days of age, blood samples were collected from the broilers' hearts and centrifuged at 3000 rpm for 15 min. The resulting serum was stored at −80°C. Following ether inhalation anesthesia, the broilers were euthanized by cervical vertebrae dislocation. The immune organs including thymus, spleen, bursa of fabricius, cecal tonsils, and jejunum were harvested to calculate the immune organ index. [immune organ index = organ weight (g) / body weight (kg)]. A portion of these tissues underwent fixation in a paraformaldehyde solution for histological observations while another portion was stored at −80°C for future use (15).

2.4 Measurement of growth performance

The broiler chickens were weighed at 8 and 50 days, following a 12-h fasting period, in order to determine the average daily feed intake (ADFI), average daily weight gain (ADWG), and feed to gain ratio (F/G) (16).

2.5 Determination of slaughtering performance

The following parameters were calculated at 50 days: dressing percentage, percentage of half-eviscerated yield, percentage of eviscerated yield, percentage of breast muscle, percentage of leg muscle, percentage of abdominal fat, drip loss rate, and cooking loss rate (17).

2.6 Determination of immunoglobulins

Establishment of ELISA Method: 1.59 g of sodium carbonate and 2.93 g of sodium bicarbonate were weighed into a beaker and dissolved in 800 mL of distilled water. The pH was adjusted to 9.6 using sodium hydroxide, and then water was added to make up to 1,000 mL, preparing a carbonate buffer solution (PBS) for later use. The H9N2 avian influenza antigen was diluted in PBS with a pH of 9.6 and coated on the ELISA plate overnight at 4°C. After washing the plate with PBST, the ELISA plate was blocked with a 1% bovine serum albumin solution at 37°C for 2 h. The diluted test serum or intestinal juice was added and incubated for 1 h, followed by adding HRP-labeled IgG or IgA antibody and incubating for another hour. Color development was achieved using TMB substrate solution in the dark, and finally terminated by adding 2 M H_2SO_4 before measuring absorbance at A450 (14).

2.7 Cytokine assay

Serum samples were collected and stored at −80°C until analysis. Prior to the assay, samples were thawed at room temperature and diluted as necessary. In accordance with the manufacturer's instructions, commercially available enzyme-linked immunosorbent assay (ELISA) kits (FanKew, Shanghai, China) were utilized to quantify the concentrations of Interleukin-4, Interleukin-5, Interleukin-12, and Interferon-gamma (IL-4, IL-5, IL-12, and IFN-γ) in serum samples.

2.8 Histological change detection

The thymus, spleen, fabricius, cecal tonsils, and jejunum were immersed in a 4% paraformaldehyde fixative for fixation purposes. Subsequently, the tissues underwent histological processing to obtain sections that were stained using the hematoxylin and eosin (HE) method for observing alterations in their histological morphology (14).

2.9 Microbial diversity of the cecum and determination of SCFAs

On the 50th day, fecal samples were collected from the control group, APS group, and LSP group receiving the most effective treatment and stored in liquid nitrogen tanks for subsequent transportation to the laboratory for DNA extraction. The DNA concentration and quality were evaluated using a spectrophotometer. The V3-V4 region of bacterial 16S rRNA gene was amplified by PCR using universal primers, followed by purification. Subsequently, a sequencing library was established and accurate quantification was performed using a Qubit fluorometer. PANOMIX Biotech conducted computer sequencing analysis on gut microbiota and SCFA-related indicators.

2.10 Statistical analysis

The growth performance, slaughter performance, cytokines, and SCFAs concentrations in cecal digesta were subjected to one-way analysis of variance (ANOVA) using SPSS 24.0 statistical software. To assess significant differences among treatment means, Duncan's multiple range test was utilized. All reported findings represent the mean values accompanied by standard errors of the mean (SEM). Graphs were created employing GraphPad Prism 5 and Origin 2022 software.

3 Results

3.1 Composition and structure of LSP

The total LSP was obtained through water extraction and ethanol precipitation, followed by purification using a DEAE column and elution with NaCl to isolate the main polysaccharide fractions (Figure 1). The HPLC analysis revealed that the composition of LSP consisted of galactose (28.60%), rhamnose (28.83%), arabinose (15.76%), xylose (11.11%), mannose (8.55%) and glucose (7.16%). Rhamnose and galactose were identified as the predominant monosaccharides in LSP, collectively accounting for 57.43% of the total monosaccharide content (Table 1).

The infrared spectral analysis results are presented in Figure 1D. The intense absorption peak at 3440 cm^{-1} is attributed to the stretching vibration of O-H, while the absorption peak at 2940 cm^{-1} corresponds to C-H stretching vibration (18). The presence of carbonyl groups in the polysaccharide is suggested by an absorption peak around 1740 cm^{-1}, and the C-O stretching causes an absorption peak near 1,630 cm^{-1}. Peaks between 1,200 and 1,000 cm^{-1} represent the stretching vibration of C-O-C bonds, confirming the presence of glucuronic acid and adjacent acetyl groups (19).

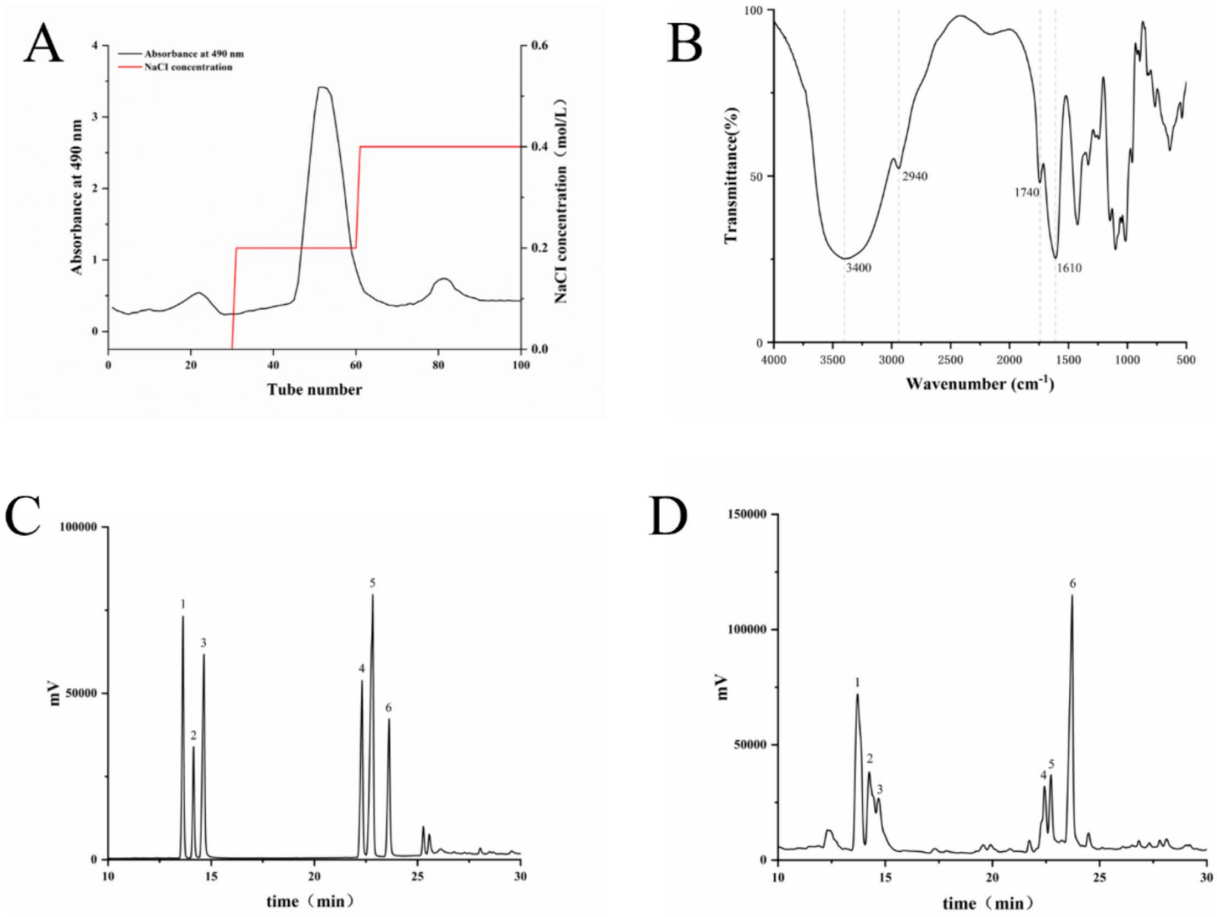

FIGURE 1
Composition and structural analysis of LSP **(A)** Elution curve of LSP, **(B)** Fourier-transform infrared spectroscopy (FT-IR) analysis of LSP, **(C)** Mixed standard, **(D)** Monosaccharide composition of LSP. Numbers 1–6 represent xylose, arabinose, mannose, galactose, glucose, and rhamnose, respectively.

3.2 Effect of LSP on broiler growth performance

The impact of LSP on the growth performance of broilers is presented in Table 2. The average daily weight gain (ADWG), average daily feed intake (ADFI), and feed to gain ratio (F/G) were significantly higher in the LSP-h, LSP-m, and LSP-l groups compared to the control group ($p < 0.05$). There was no significant difference observed among all groups regarding LSP levels ($p > 0.05$). Moreover, the F/G of the LSP-l group showed a more pronounced improvement, with no significant difference compared to the APS group ($p > 0.05$).

3.3 Effect of LSP on the slaughtering performance of broiler chickens

The impact of LSP on the slaughter performance of broiler chickens is demonstrated in Table 3. Compared to the control group, the inclusion of LSP in the feed significantly increased various parameters including dressing percentage, percentage of half-eviscerated yield, percentage of eviscerated yield, percentage of breast muscle, percentage of leg muscle, drip loss rate, and cooking loss rate ($p < 0.01$). Among them, the LSP-l group exhibited significantly greater improvements in the percentage of half-eviscerated yield, percentage of eviscerated yield, percentage of breast muscle, and percentage of leg muscle compared to the LSP-m and LSP-h groups ($p < 0.01$). When compared with the positive control drug APS group, there were no significant differences observed in dressing percentage, percentage of half-eviscerated yield, percentage of eviscerated yield, percentage of breast muscle, percentage of abdominal fat, and drip loss rate ($p > 0.05$).

3.4 Effects of LSP on immune function

The impact of LSP on cytokines, immunoglobulins, and immune organ indices is illustrated in Figure 2. In comparison to the control group, supplementation with LSP significantly stimulated the production of IL-4, IL-12, SIgA, and IgG. Among the different doses of LSP groups, there were no significant differences observed in the expression levels of other indexes except for IgG. The expressions of IL-12, IFN-γ, and SIgA did not exhibit

TABLE 1 Basic feed ingredients and nutrient levels (Dry basis).

Ingredient	1–4 Weeks (%)	5–9 Weeks (%)	Nutrient level	1–4 Weeks (MJ/kg)	5–9 Weeks (MJ/kg)
Corn	62.30	67.20	Metabolizable Energy (MJ/kg)	12.96	13.89
Soybean meal	23.70	19.70	Crude Protein (%)	20.00	18.50
Cottonseed meal	4.73	5.26	Lysine (%)	1.00	0.80
Soybean oil	3.26	3.71	Methionine (%)	0.40	0.30
Limestone	1.27	1.18	Methionine + Cysteine (%)	0.76	0.84
Corn gluten meal	2.23	3.12	Calcium (%)	0.90	0.90
Dicalcium phosphate	1.76	1.65	Available Phosphorus (%)	0.40	0.30
Salt	0.32	0.30			
L-Lysine	0.25	0.21			
DL-Methionine	0.10	0.10			
Choline Chloride	0.08	0.05			

Vitamin A 2700 IU, Vitamin D 2000 IU, Vitamin E 10 IU, Vitamin K 0.5 mg, Vitamin B12 0.015 mg, Riboflavin 6 mg, Pantothenic Acid 10 mg, Niacin 40 mg, Pyridoxine 3 mg, Biotin 0.15 mg, Choline 300 mg, Iron 50 mg, Copper 4 mg, Manganese 50 mg, Zinc 40 mg, Iodine 0.35 mg, Selenium 0.18 mg.

TABLE 2 Effect of LSP on growth performance of broilers.

Items	Groups						
	Control	APS	LSP-l	LSP-m	LSP-h	SEM	*p*-value
Average daily feed intake(g)	17.54^{c}	26.43^{a}	23.21^{b}	22.68^{b}	24.31^{b}	0.83	< 0.01
Average daily weight gain(g)	103.00^{c}	125.25^{a}	114.00^{b}	114.25^{b}	117.50^{ab}	4.13	< 0.01
Feed to gain ratio(g/g)	5.89^{a}	4.74^{c}	4.91^{bc}	5.04^{b}	4.84^{bc}	0.10	< 0.01

Different lowercase letters on the data shoulder tags indicate significant differences ($P < 0.05$).

significant differences compared to the APS group ($p > 0.05$). Furthermore, thymus, spleen, and bursa of fabricius indexes were significantly increased in the LSP group when compared to the control group ($p < 0.05$). Notably, this enhancement effect was more pronounced in the LSP-l group than in both the LSP-m and LSP-h groups; However it did not differ significantly from that observed in the APS group ($p > 0.05$).

3.5 HE stained section analysis

To evaluate the impact of LSP on broiler chickens' immune organs and intestines, we examined tissue changes in the thymus, spleen, fabricius, cecal tonsils, and ileum. The results depicted in Figure 3 demonstrate that HE staining observations revealed no discernible alterations in the structure of immune organs and intestinal tissues among all experimental groups of broiler chickens. All groups exhibited a normal physiological morphology.

3.6 The influence of LSP on intestinal microbial abundance

The impact of LSP on the abundance of intestinal microbiota in broiler chickens is depicted in Figure 4. To ensure accurate representation of microbial composition in cecal content, observed species curves and Shannon curves were employed to assess sequencing depth (20). The results indicated that a sequencing depth of 50,000 reads covered the majority of microbial species, and even with further sequencing, no new species were identified, ensuring the reliability of the data obtained through sequencing (Figures 4A,B). The Rank abundance curve illustrated the distribution pattern of high-abundance and rare ASV/OTU within the microbial community (21). The graph shows initial fluctuations in samples, but the curves stabilize after reaching a richness value of 3,000 (Figure 4C). This suggests a uniform overall distribution of microbial species in each sample, providing a robust foundation for further comprehensive analysis.

The Venn diagram in Figure 4D illustrates the composition of the intestinal microbial community, revealing that there were 9,709 OTUs

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TABLE 3 Effect of LSP on slaughter performance indexes of broiler chickens.

Items	Groups						
	Control	APS	LSP-l	LSP-m	LSP-h	SEM	*P*-value
Dressing percentage	93.94^{b}	97.12^{a}	96.44^{a}	95.83^{a}	96.98^{a}	0.64	<0.01
Percentage of half-eviscerated yield	91.87^{c}	94.77^{a}	93.83^{ab}	93.04^{bc}	93.44^{b}	0.59	< 0.01
Percentage of eviscerated yield	73.71^{c}	77.73^{a}	76.35^{ab}	74.61^{bc}	75.20^{bc}	0.78	< 0.01
Percentage of breast muscle	11.15^{d}	17.35^{a}	15.87^{ab}	13.97^{c}	15.35^{bc}	0.77	< 0.01
Percentage of leg muscle	11.74^{c}	17.60^{a}	15.55^{b}	15.06^{b}	15.51^{b}	0.77	< 0.01
Percentage of abdominal fat	2.27^{a}	1.43^{b}	1.61^{b}	1.69^{b}	1.54^{b}	0.12	< 0.01
Drip loss rate	2.58^{a}	1.34^{b}	1.56^{b}	1.52^{b}	1.56^{b}	0.11	< 0.01
Cooking loss rate	45.01^{a}	38.19^{c}	41.59^{b}	42.03^{b}	40.63^{b}	0.81	< 0.01

Different lowercase letters on the data shoulder tags indicate significant differences ($P < 0.05$).

for the LSP group, 9,538 for the APS group, and 9,689 for the control group. Among these groups, a total of 2000 OTUs were shared, with 1,078 OTUs shared between the LSP group and control group, and 1,007 OTUs shared between the APS group and control group.

3.7 The impact of LSP on the diversity of intestinal microbiota

To acquire additional knowledge regarding the influence of LSP on the variety of gut microbiota, we utilized the Chao1 and Observed species indices to assess richness, and employing the Shannon and Simpson indices to evaluate diversity. Furthermore, we measured evolutionary diversity using the Faith PD index, assessed evenness through the Pielou e index, and evaluated coverage using the Goods coverage index. As shown in Figure 5A, the Chao1, Observed species, and Faith PD indices in the LSP group were higher than those in the control group, but the differences were not statistically significant ($p > 0.05$). The Simpson, Shannon, Pielou e, and Goods coverage indices in the LSP and APS group were lower than those in the control group, but the differences were not statistically significant ($p > 0.05$). The Principal Coordinates Analysis (PCoA) based on Bray-Curtis distances was conducted for the control, LSP, and APS groups. As depicted in Figure 5B, Axis 1 and Axis 2 eigenvalues accounted for 23.8 and 14.4% of the variation in sample community composition, respectively. When projected onto a two-dimensional plane within a 95% confidence interval, distinct separation between the LSP and control groups was observed, indicating disparities in intestinal microbiota between these two groups. Partial overlap was noted between the LSP and APS groups, suggesting that the dissimilarities in intestinal microbiota between these two groups were not statistically significant. To further investigate dissimilarities among groups, we conducted Non-Metric Multidimensional Scaling (NMDS) analysis, as depicted in Figure 5C, which revealed a substantial distance between the LSP and control groups, indicating their pronounced dissimilarity in terms of intestinal microbiota.

3.8 The impact of LSP on the microbial community composition at the phylum and genus levels

The composition of the broiler chicken intestinal microbiota at the phylum level is illustrated in Figure 6A, wherein Firmicutes and *Bacteroidota* emerge as the dominant phyla, collectively constituting over 75% of the microbial community. Notably, Firmicutes stands out as the predominant phylum. Comparatively, the LSP group exhibits an elevated abundance within the *Bacteroidota* phylum while displaying a reduced prevalence of Firmicutes. Conversely, the APS group demonstrates an increased predominance of Firmicutes accompanied by a diminished abundance of *Bacteroidota*. At the genus level, as depicted in Figure 6B, the prevailing genera comprise Bacteroides, Streptococcus, *Ruminococcus*, Lactobacillus, and *Faecalibacterium*; with Bacteroides being the most dominant genus. In comparison to the control group, the LSP group exhibited reduced abundances of Streptococcus, *Ruminococcus*, Lactobacillus, and *Oscillospira*; while there was an increase in the abundance of *Phascolarctobacterium*. The APS group displayed a decreased abundance of *Bacteroides* and increased abundances of *Streptococcus*, *Ruminococcus*, and *Lactobacillus*.

The LEfSe analysis results, as depicted in Figures 6C,D, revealed a total of 21 feature taxa, encompassing 2 at the class level, 4 at the order level, 7 at the family level, and 8 at the genus level. *Clostridium*, *Dorea*, *Alphaproteobacteria*, and *Odoribacter* were identified as characteristic taxa associated with the control group. *Ruminococcus*, *Alistipes*, *Streptococcaceae*, *Comamonadaceae RF32* and *Allobaculum* were found to be distinctive taxa linked to the APS group. *Bacteroidales Phascolarctobacterium Succinispira Turicibacterales Turicibacter gTuricibacter Turicibacteraceae Rhodobacterales Acidimicrobiia Mycobacteriaceae Mycobacterium* and *Veillonellaceae* were recognized as characteristic taxa associated with the LSP group. These findings further underscore that dietary supplementation with LSP induces alterations in microbial abundance across various taxonomic levels.

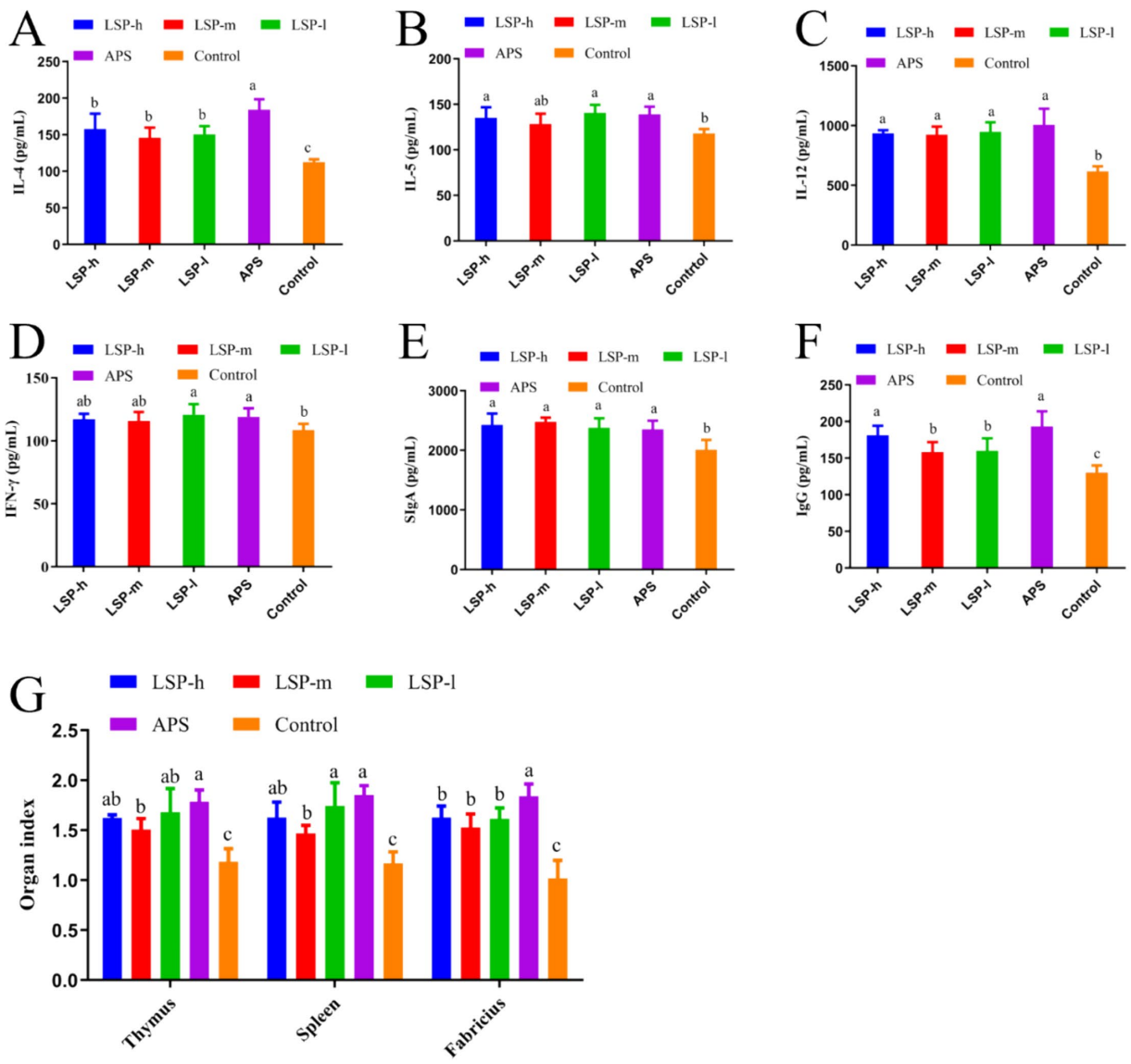

FIGURE 2
Repercussions of LSP on cytokine levels and immune organ indices. **(A)** IL-4 Content. **(B)** IL-5 Content. **(C)** IL-12 Content. **(D)** IFN-γ Content. **(E)** SIgA Content. **(F)** IgG Content. **(G)** Immunological organ index. Bars with different superscripts **(A–D)** indicate significant differences ($p < 0.05$).

3.9 Effect of LSP on short-chain fatty acids

The levels of short-chain fatty acids (SCFAs) are presented in Table 4. Compared to the control group, the LSP group exhibited a significant increase in the concentrations of propionic acid, isobutyric acid, acetic acid, isovaleric acid, and caproic acid ($p < 0.05$). Furthermore, the addition of LSP to the diet significantly elevated the content of acetic acid in the cecum of broiler chickens when compared to both the control group and the APS group ($p < 0.05$).

4 Discussion

The intestine is an open ecological system that directly interacts with the external environment. It serves as the largest digestive, absorptive, and immune organ within the animal body (22). Inhabiting the intestine is a vast array of microorganisms, which play a crucial role not only in host nutrient digestion and metabolism but also in organism development and their close associations with the host's immune system and diseases. Simultaneously, intestinal microbes serve as essential factors in stimulating both the "intestinal mucosal immune system" and the "systemic immune system," ensuring proper immune system functioning (23). As an immunomodulator derived from *Lagenaria siceraria*, LSP has been previously demonstrated to induce persistent high hemagglutination (HI) titers, antigen-specific IgA-NDV and IgG-NDV antibodies, splenic lymphocyte proliferation, and increased immune organ index to enhance immune response (14). However, there is a lack of research on the impact of incorporating LSP into broiler feed orally. This study aims to address this gap by examining the influence of dietary supplementation with LSP on growth performance, immune function, and intestinal microbial composition in broilers.

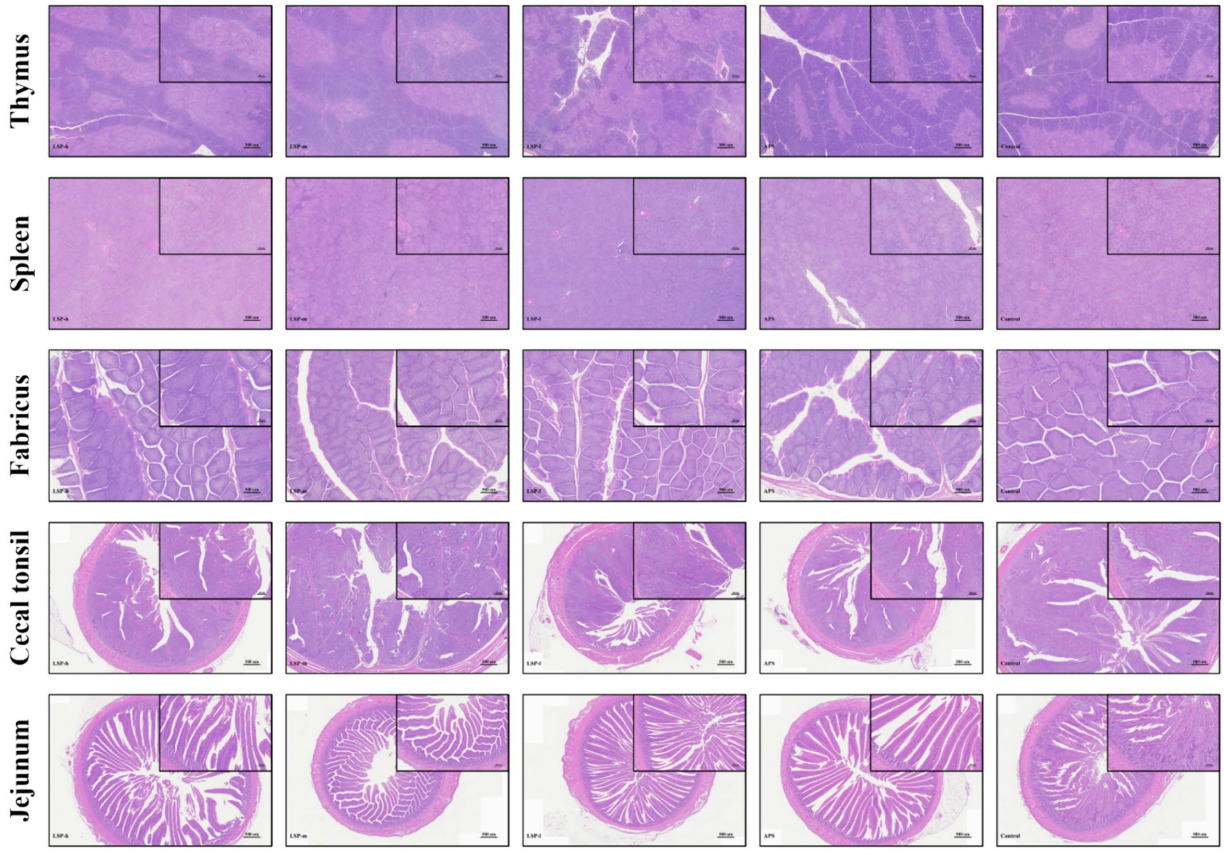

FIGURE 3
Tissue section analysis results.

Growth performance and slaughter performance are crucial indicators for evaluating animal growth rate and achieving economic profitability. Enhanced growth performance implies reduced feed consumption and more significant weight gain in animal farming production. Slaughter performance is reflected in the processing of slaughtering, where lower losses during slaughter lead to higher production profits (24). Therefore, the poultry industry emphasizes key performance indicators such as ADWG, ADFI, F/G, and slaughter rate when processing broiler chickens. This study discovered that incorporating LSP into the feed significantly increased the average weight of broiler chickens, thereby improving both ADWG and F/G (Table 2). The effect of low dose LSP was more significant than that of medium and high dose LSP, which increased percentage of half-eviscerated yield, percentage of eviscerated yield, percentage of breast muscle, and percentage of leg muscle (Table 3). Indicating that LSP positively influences the growth and development as well as muscle quality of broiler chickens, thus enhancing both growth performance and slaughter performance.

Inflammatory cytokines have a crucial role in initiating immune responses and eliminating pathogens within the host (25). Previous research has shown that various feed additives can enhance the proliferation of T helper 1/T helper 2 (Th1/Th2) cells and stimulate the secretion of both pro-inflammatory and anti-inflammatory cytokines, thereby impacting the poultry immune system. IL-12 and IFN-γ, secreted by Th1 cells, are crucial for immune regulation (26). IL-12 promotes Th1 cell proliferation and IFN-γ production, which has been shown to be essential in resolving inflammation caused by primary *Salmonella* infection in chickens (27). Conversely, IL-4 induces Th2 cell proliferation while down regulating IL-12 production and inhibiting Th1 differentiation. Elevated levels of IL-5 contribute to lymphocyte activation, promoting bone marrow cell proliferation and differentiation as well as enhancing both cellular and humoral immunity levels (28). Secretory Immunoglobulin A (SIgA) is formed through Immunoglobulin A (IgA) aggregation within the intestinal mucosa; it plays a critical role in the intestinal mucosal immune response by selectively transferring to other mucosal tissues, establishing a broad immune response, stabilizing the mucosal barrier system, and preventing pathogenic microorganism invasion. Immunoglobulin IgG reflects virus-induced humoral immunity levels (29). In this study, supplementation of LSP significantly increased serum inflammatory cytokine levels (IL-4, IL-5, IL-12, IFN-γ), SIgA concentrations, and IgG titers (Figure 2). Suggesting that LSP can concurrently induce Th1/Th2 immunity while enhancing cellular, humoral, and intestinal mucosal immunity levels thereby reinforcing broiler chickens' resistance against infectionand inflammation.

The thymus, spleen, and fabricius are pivotal immune organs in avian species. B lymphocytes undergo maturation within the fabricius, thereby acquiring a diverse repertoire of antibodies (30). In broiler chickens, the fabricius is more susceptible to exogenous feed influences compared to other organs (31). Wu et al. observed an increase in the relative weight of the thymus, spleen, and fabricius in broiler chickens when polysaccharide components were added to their

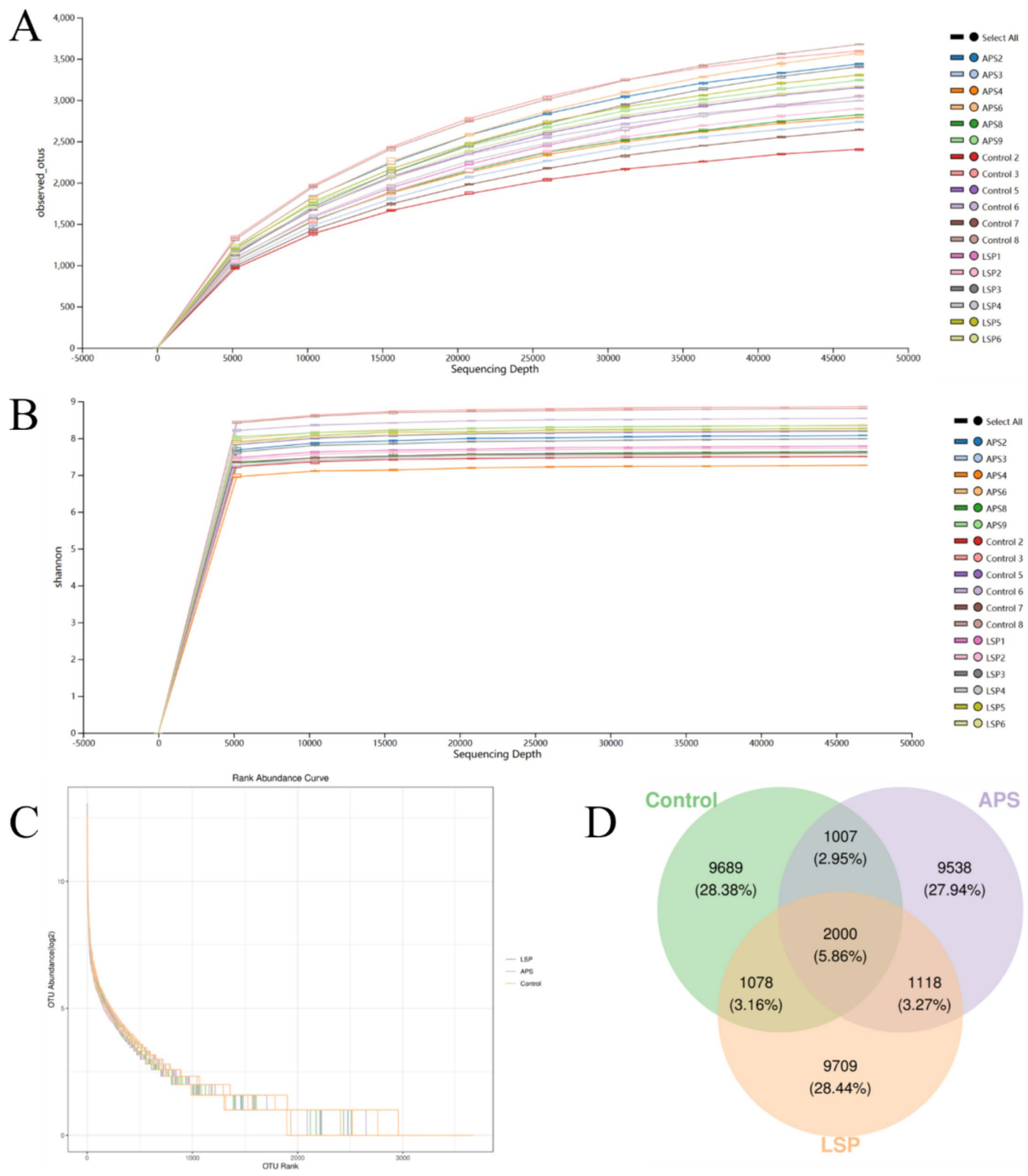

FIGURE 4
The influence of LSP on intestinal microbial abundance **(A)** Observed_species curve. **(B)** Shannon curve. **(C)** Rank abundance curve. **(D)** Venn diagram of ASVs/OTUs.

daily diet (29). Our study observed no lesions or inflammatory infiltrations in various immune organs (thymus, spleen, fabricius) as well as cecal tonsils and ileum when supplemented with LSP. This indicates the safety profile of LSP supplementation. In addition, the indexes of thymus size, spleen weight and fabricius size of broilers supplemented with LSP were significantly increased, and the enhancement effect of low-dose LSP group was more significant. We propose that LSP might comprise compounds that facilitate the proliferation of advantageous microorganisms like lactic acid bacteria and spore-forming bacteria, thereby fostering immune organ development. Alternatively, it is possible that LSP contains prebiotic-like compounds (oligosaccharides) which facilitate development within broiler chicken's immune organs (30).

The host's digestion, intestinal development, nutrient absorption, and both innate and adaptive immune systems are regulated by the symbiotic relationship maintained with the gastrointestinal microbiota

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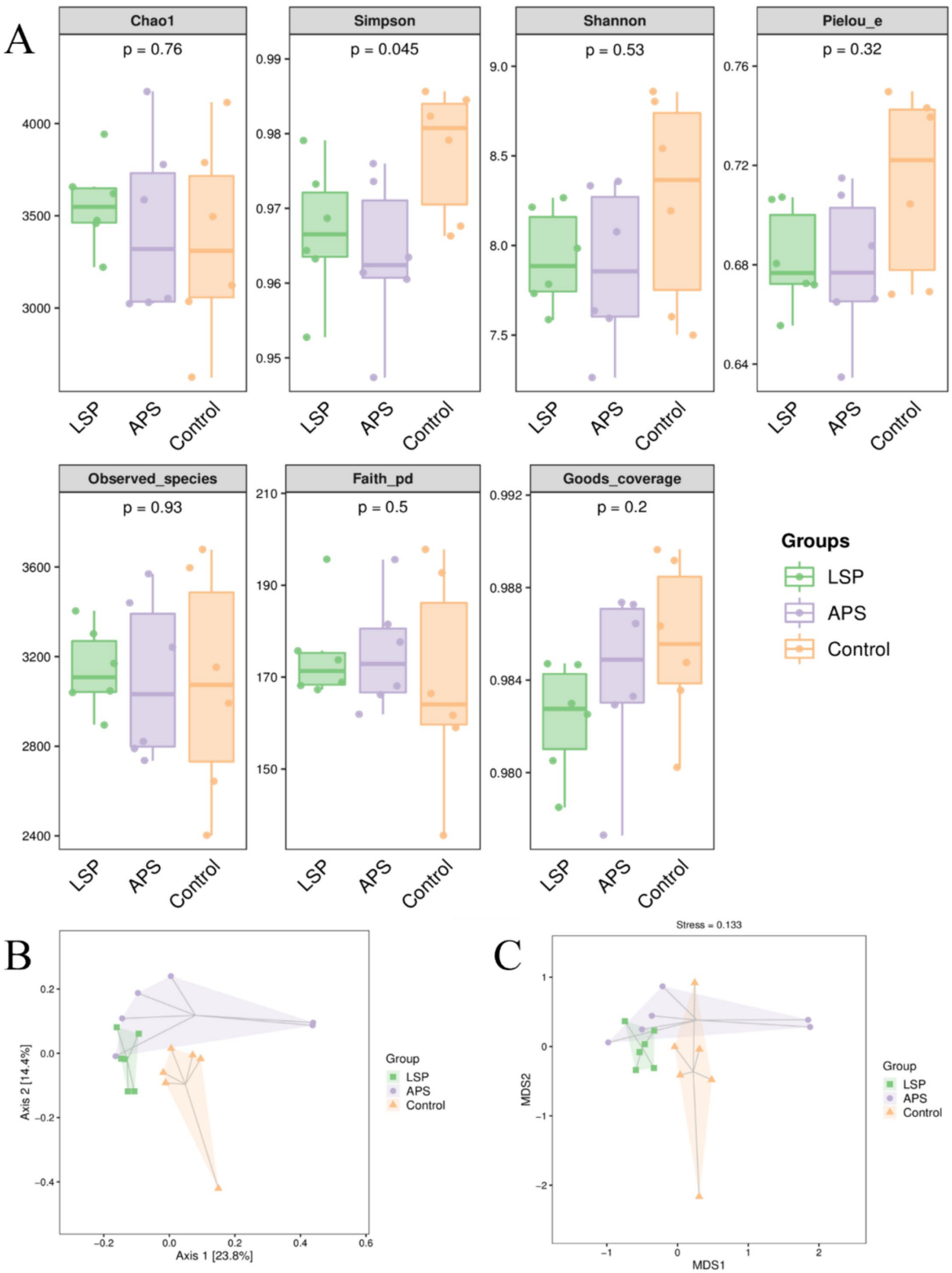

FIGURE 5
The impact of LSP on the diversity of intestinal microbiota **(A)** Grouped boxplot of alpha diversity indices. **(B)** Two-dimensional PCoA analysis plot. **(C)** Two-dimensional NMDS plot.

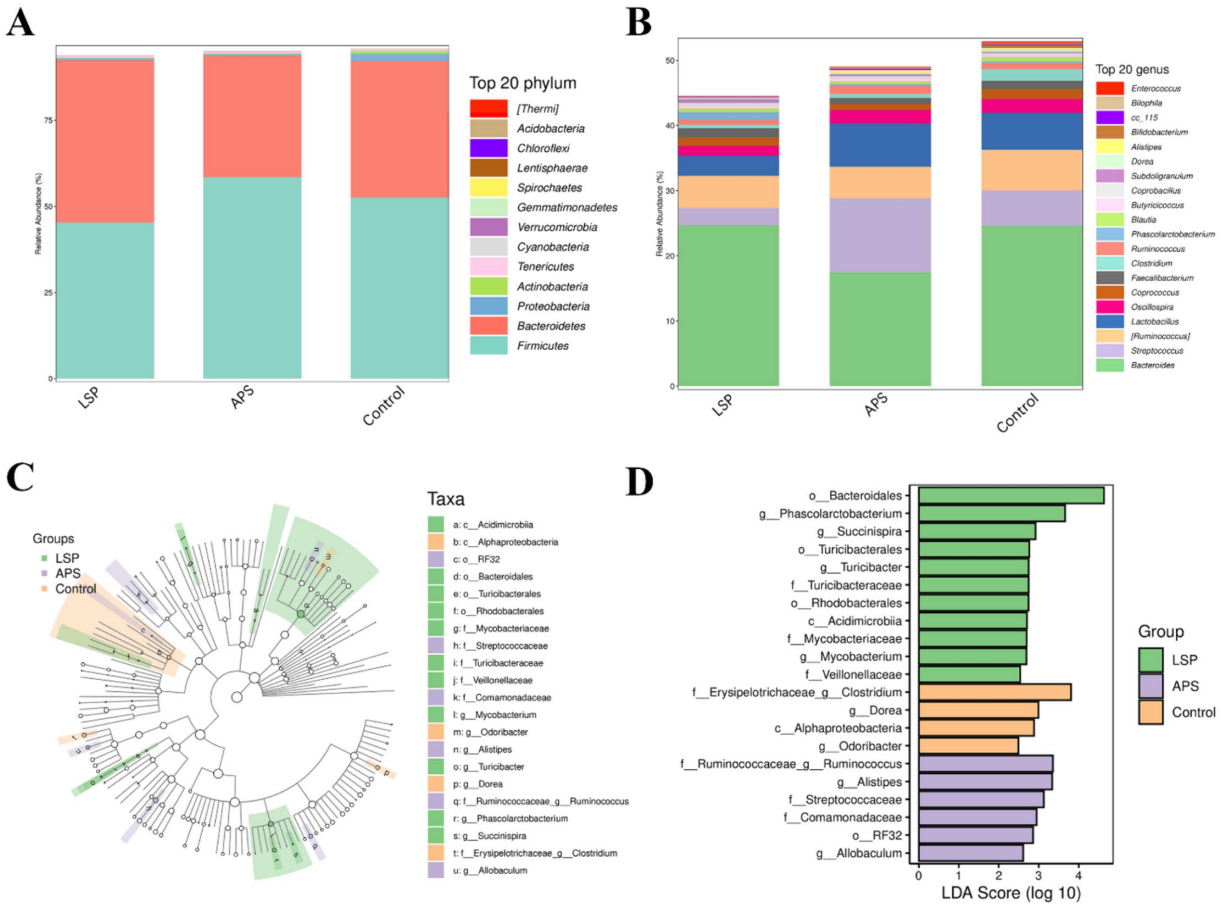

FIGURE 6
The influence of LSP on the composition of microbial communities. **(A)** bar plot of phylum-level species composition. **(B)** Bar plot of genus-level species composition. **(C)** Intergroup differences in taxonomic units based on classification tree. **(D)** Bar plot of LDA effect sizes for indicator species.

TABLE 4 Content of different SCFAs in each group.

Items	Groups			SEM	*P*-value
	Control	APS	LSP		
Propionic acid	409.06^{b}	564.58^{a}	545.43^{a}	46.91	<0.01
Isobutyric acid	42.76^{b}	64.40^{a}	62.09^{a}	5.92	<0.01
Acetic acid	1605.80^{b}	1645.07^{b}	2186.21^{a}	178.98	<0.01
Butyric acid	234.97^{a}	254.84^{a}	263.84^{a}	33.49	0.692
Isovaleric acid	26.96^{c}	53.42^{a}	40.02^{b}	5.28	<0.01
Valeric acid	55.98^{a}	69.16^{a}	54.68^{a}	6.87	0.091
Caproic acid	0.69^{b}	0.82^{ab}	0.94^{a}	0.11	0.098

Different lowercase letters on the data shoulder tags indicate significant differences ($P < 0.05$).

(32). The cecum, characterized by slow intestinal motility, low levels of antimicrobial substances, and weak alkaline pH, serves as a primary colonization site for microbiota (33). In this study, we investigated differences in the composition of microbial communities by supplementing LSP in the daily diet of broiler chickens. The results revealed an increased number of OTUs in the LSP group compared to the control group and APS group (Figure 4D). To further explore changes in the composition of the intestinal microbiota, this study conducted an analysis at the phylum and genus levels. At the phylum level, *Firmicutes* and *Bacteroidetes* dominated the intestinal microbiota, with a total abundance exceeding 75% (Figure 6A). These phyla play crucial roles in feed digestion and utilization, as they possess the ability to produce carbohydrate-active enzymes (CAZymes) responsible for breaking down complex indigestible polysaccharides, thereby promoting fiber degradation and carbohydrate utilization (34). At the genus level, the abundance of

Bacteroides and *Phascolarctobacterium* increased in the LSP group (Figure 6B). *Bacteroides*, a group of Gram-negative, non-spore-forming, anaerobic, rod-shaped bacteria, ferment polysaccharides to produce volatile fatty acids (35). Additionally, *Phascolarctobacterium*, an obligate anaerobic, Gram-negative bacterium, produces short-chain fatty acids, including acetate and propionate (36). These findings align with previous research, suggesting that the bacterial community may become enriched and diversified through the degradation of LSP.

Short-chain fatty acids (SCFAs), including acetate, propionate, and butyrate, are the most prevalent and extensively investigated metabolites generated during bacterial fermentation (37). Research has demonstrated that SCFAs possess anti-inflammatory and immune-modulatory properties. Specifically, the fortification of the intestinal epithelium's defensive role is heightened through the conversion of acetate into its corresponding salt structure. Propionate has been found to inhibit intestinal inflammation (38). Moreover, conversion of butyrate to its salt form not only stimulates mucin secretion in the animal intestine but also serves as a direct energy source for the intestinal mucosa, ensuring intestinal structural integrity and enhancing immune function (39). In this study, we observed a significant increase in the levels of propionic acid, isobutyric acid, acetic acid, isovaleric acid, and caproic acid in the LSP group compared to the control group (Table 4). This indicates that incorporating LSP into feed enhances the secretion of short-chain fatty acids within the gastrointestinal tract of broiler chickens.

5 Conclusion

In summary, LSP comprises monosaccharides such as galactose, rhamnose, arabinose, xylose, mannose, and glucose. Supplementation of LSP in feed induces alterations in the intestinal flora composition of broilers and enhances the secretion of short-chain fatty acids (propionic acid, isobutyric acid, acetic acid, isovaleric acid, and caproic acid), thereby promoting the development of immune organs like thymus, spleen and fabricius. These changes not only lead to increased levels of IL-4, IL-12, IgG and SIgA, but also positively impact parameters such as average daily weight gain (ADWG), average daily feed intake (ADFI), feed to gain ratio (F/G), percentage of half-eviscerated yield, percentage of eviscerated yield, percentage of breast muscle and percentage of leg muscle. Ultimately these improvements contribute to enhanced growth performance and immune function in broilers.

Data availability statement

The data presented in the study are deposition in the online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ncbi.nlm.nih.gov/bioproject/PRJNA1164933.

Ethics statement

All experimental procedures were approved by the Animal Welfare Ethics Committee of Xinjiang Agricultural University (Approval No. 2022016). The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

ZZ: Data curation, Methodology, Validation, Writing – review & editing. SD: Data curation, Methodology, Validation, Writing – review & editing. JL: Conceptualization, Data curation, Investigation, Methodology, Writing – review & editing. MA: Conceptualization, Data curation, Investigation, Methodology, Writing – original draft. PH: Formal analysis, Methodology, Writing – review & editing. SA: Formal analysis, Methodology, Writing – review & editing. ZM: Formal analysis, Methodology, Writing – review & editing. DL: Formal analysis, Methodology, Writing – review & editing. AW: Data curation, Investigation, Methodology, Supervision, Validation, Writing – review & editing.

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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.2024.1428623/full#supplementary-material

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Maternal chitosan oligosaccharide supplementation during late gestation and lactation optimizes placental function in sows and intestinal function in 21-day-old IUGR suckling piglets

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Maternal dietary supplementation with chitosan oligosaccharide (COS) has been considered as a potential intervention to mitigate the occurrence of intrauterine growth restriction (IUGR) and improve postnatal growth. The present study investigated the effect of COS as a dietary supplement for sows during late gestation and lactation on their productivity, placental function, and the intestinal health of IUGR piglets. From day (d) 85 of late gestation to d 21 of lactation, 30 sows were randomly divided into either a control group (basal diet) or a COS group (basal diet + 100 mg kg^{-1} COS). At d 21 of lactation, eight normal and eight IUGR littermates from eight litters belong to control sows, as well as eight IUGR littermates from COS sows, were selected for further analysis. The results showed a significant reduction in the number of stillbirths and mummies in COS groups ($p < 0.05$). Maternal dietary supplementation with COS also significantly up-regulated the expression levels of *GLUT1*, *GLUT3*, and *VEGFA* mRNA in the placenta of IUGR piglets compared to those in control group ($p < 0.05$). Furthermore, there was a significant decrease in MDA content and a significant increase in GSH content in the placenta of IUGR piglets from COS sows compared to those from control group ($p < 0.05$). Additionally, the expression levels of *MUC2* and *occludin* mRNA as well as claudin1 protein significantly up-regulated in the jejunum of 21-day-old IUGR piglets from COS sows group compared to those from control group ($p < 0.05$). Moreover, *IL-10* mRNA expression level was significantly increased while MDA content was significantly reduced in the jejunum of 21-day-old IUGR piglets from COS sows group compared to those from control group ($p < 0.05$). The results indicated that maternal dietary COS supplementation during late gestation effectively reduced the incidence of stillbirths and mummies, potentially linked to enhanced placental function, reduced oxidative stress, and improved immune status. Furthermore, maternal dietary COS supplementation exhibited positive impact on intestinal digestive and absorptive function, intestinal barrier integrity, intestinal antioxidant capacity and immune status in 21-day-old suckling IUGR piglets.

KEYWORDS

chitosan oligosaccharide, intrauterine growth retardation, sows, piglets, placenta, intestine

1 Introduction

Intrauterine growth retardation (IUGR) in mammals refers to abnormal fetal growth and organ development in the uterus, resulting in low birth weight and impaired growth and development (1). Various factors, including maternal, fetal, placental or genetic influences, contribute to the formation of IUGR (2). In multiparous animals, we considered neonates whose birth weights did not align with the mean litter weight distribution and were less than twice the standard deviation as IUGR animals (3). The placenta plays a crucial role as a mediator between the mother and fetus. Therefore, oxidative stress, poor developmental status, and impaired nutrient transport of the placenta can collectively contribute to the occurrence of IUGR (4). IUGR adversely impacts neonatal morbidity and mortality, leading to impaired nutrient absorption and abnormalities in body composition, such as reduced muscle mass (5, 6). IUGR has become an issue that cannot be ignored in both nutritional human obstetrics as well as animal production. Recent studies have shown that nutritional interventions in sows can effectively reduce the incidence of IUGR. For instance, Pieszka et al. (7) showed the effectiveness of supplementing sow diets with pancreatic-like enzymes, while Wu et al. (8) found that supplementation with adenosine was also effective in reducing the occurrence of IUGR. However, current IUGR research primarily emphasizes direct maternal effects on the fetus and the characterization of the IUGR placenta. Our aim was to connect the mother, fetus, and placenta, and investigate how IUGR affects the offspring's gut health.

Chitosan oligosaccharide (COS) is a degradation product of chitosan, typically prepared using physical, chemical, enzymatic, and other methods. COS has been demonstrated to have biological effects such as antioxidant (9), anti-inflammatory (10), antibacterial (11, 12), and anti-tumor activity (13). Study has proved that supplementing COS in the diet of pregnant sows can enhance sows health status, provide an improved growth environment for fetuses and increase the survival rate of newborn piglets (14). Additionally, supplementing sow feed with COS enhanced the antioxidant and anti-inflammatory abilities as well as the nutritional composition of sow milk, thereby improving the growth performance of piglets (15, 16). Furthermore, study has also indicated that COS improves amino acid transport capacity in the placenta and promotes nutrient transport from sow to fetuses (17). Existing studies focusing on reproductive performance (14), milk composition (18), antioxidant capacity (17), and placental capacity of sow with supplementation of COS to their diets have rarely investigated the effects of feeding COS to sows on IUGR piglets.

Therefore, we hypothesized that maternal COS supplementation during late gestation and lactation can improve placental function, reduce the incidence of IUGR, and improve the intestinal health of IUGR piglets. Thus, the aim of this study was to evaluate the effects of maternal supplementation with COS during late gestation and lactation on reproductive performance, placenta function, immune response, and antioxidant capacity of placenta in sows. Furthermore, digestive enzyme activity, intestinal barrier function, cytokine levels and antioxidant indicators were evaluated in piglet jejunum.

2 Materials and methods

2.1 Animal ethics

This experiment was performed in accordance with the Laboratory Animal-Guideline for ethical review of animal welfare of the People's Republic of China (GB/T 35892-2018) and approved by Sichuan Agricultural University Animal Care and Use Committee.

2.2 Chitosan oligosaccharide

The effective content of COS used in this study is 10% with Maltose dextrin as the carrier, MW <1,000 Da, and degree of deacetylation (DD) is about 95%, which was provided by Zhongke Runxin (Suzhou) Biological Technology Co., Ltd. The amount of COS added was calculated based on the effective content.

2.3 Animals, diets and experimental design

This study was conducted in Zitong Original Breeding Pig Farm, which is a member of Sichuan Mianyang Jinchiyang Agriculture and Animal Husbandry Co., Ltd. A total of 30 sows (Landrace × Yorkshire) with specific criteria for back fat, parity, and expected delivery date close to 85 days of pregnancy, were randomly allocated to 2 groups: control group (control, sows fed basal diet) and COS group (COS, sows fed basal diet + 100 mg kg^{-1} COS) (14). The basal diets were formulated to meet the nutrient requirements of gestating sows or lactation sows, respectively, as recommended by the National Research Council (NRC, 2012), and their compositions and nutrients levels were shown in Tables 1, 2.

After delivery, the piglets were fostered to other gilts within the same treatment group to ensure each litter included 10–11 piglets, and the number of sows in both COS group and control group was adjusted to 10.

The piglets were breastfed until weaning at day 21. Piglets with a birth weight below 0.9 kg were classified as IUGR piglet. Newborn piglets categorized as NBW weigh 1.50 kg on average, while those

Abbreviations: ADFI, Average daily feed intake; ADG, Average daily weight gain; ANG, Angiogenin; CAT, Catalase; COS, Chitosan oligosaccharides; DD, Degree of deacetylation; GLUT, Glucose transport protein; GSH, Glutathione; GSH-PX, Glutathione peroxidase; GSSG, Oxidized glutathione; IFN-γ, Interferon-γ; IL-10, Interleukin-10; IL-1β, Interleukin-1β; IL-6, Interleukin-6; IL-8, Interleukin-8; IUGR, Intrauterine growth inhibition; MDA, Malondialdehyde; MUC, Mucin; NBW, Normal body weight; PepT1, Oligopeptide transporter 1; SNAT, Basolateral transporters system A; T-AOC, Total antioxidant capacity; TNF-α, Tumor necrosis factor; VEGFA, Vascular endothelial growth factor; ZO-1, Zona occludens-1.

TABLE 1 Composition and nutrient levels of the basal diet for sow during late gestation.

Ingredient	Content, %	Nutrient levels[b]	Content
Corn	13.00	CP, %	16.5
Soybean meal (≥43%)	5.50	DE, Mcal/kg	3.00
Wheat	30.00	Ca, %	0.85
Rice bran	10.00	Available P, %	0.28
Puffed flaxseed powder	3.00	D-Lys	0.67
Beet pulp	4.00		
Saccharomyces cerevisiae ferments distiller's grains	9.70		
Soybean hulls	6.00		
Bran (wheat bran)	8.60		
Fermented soybean meal	4.00		
Fish meal	1.00		
Expanded soybean	2.00		
Sodium chloride	0.37		
Calcium carbonate	1.11		
Dicalcium phosphate	0.82		
Sodium bicarbonate	0.10		
Acidifier	0.15		
Choline chloride	0.25		
Phytase	0.02		
Feed compound enzyme	0.03		
DL-α-tocopherol acetate	0.02		
Potassium chloride	0.03		
Mineral and vitamin premix[a]	0.30		
Total	100		

[a]Premix supplied the following per kg of diet: copper ($CuSO_4$), 10 mg; iron ($FeSO_4$), 80 mg; zinc (ZnO), 50 mg; manganese ($MnSO_4$), 25 mg; selenium (Na_2SeO_3), 0.15 mg; iodine (KIO_3), 0.14 mg; vitamin A, 4,000 IU; vitamin D_3, 800 IU; vitamin E, 441 IU; menadione, 0.6 mg; thiamine, 1.0 mg; riboflavin, 3.35 mg; vitamin B_6, 1.0 mg; vitamin B_{12}, 15 μg; niacin, 10 mg; pantothenic acid, 12 mg; folic acid, 1.3 mg; D-biotin, 200 μg.
[b]Nutrient levels were calculated values.

TABLE 2 Composition and nutrient levels of the basal diet for sow during lactation.

Ingredient	Content, %	Nutrient levels[b]	Content
Corn	47.00	CP, %	17.00
Soybean meal	15.10	DE, Mcal/kg	3.32
Beet pulp	1.00	Ca, %	1.00
Wheat flour	8.00	Available P, %	0.35
Saccharomyces cerevisiae ferments distiller's grains	3.00	D-Lys	1.10
Soybean hulls	1.50		
Bran (wheat bran)	5.00		
Fish meal	2.00		
Expanded soybean	2.00		
Five corn peptide	8.00		
Sucrose	2.00		
Soybean oil	1.00		
Sodium chloride	0.40		
Calcium carbonate	1.03		
Dicalcium phosphate	1.46		
Acidifier	0.30		
Choline chloride	0.15		
L-lysine HCL	0.52		
DL-methionine	0.03		
L-threonine	0.13		
L-tryptophan	0.02		
Phytase	0.02		
DL-α-tocopherol acetate	0.02		
Mineral and vitamin premix[a]	0.32		
Total	100		

[a]Premix supplied the following per kg of diet: copper ($CuSO_4$), 10 mg; iron ($FeSO_4$), 80 mg; zinc (ZnO), 50 mg; manganese ($MnSO_4$), 25 mg; selenium (Na_2SeO_3), 0.15 mg; iodine (KIO_3), 0.14 mg; vitamin A, 4,000 IU; vitamin D_3, 800 IU; vitamin E, 441 IU; menadione, 0.5 mg; thiamine, 1.0 mg; riboflavin, 3.16 mg; vitamin B_6, 0.90 mg; vitamin B_{12}, 12.5 μg; niacin, 7 mg; pantothenic acid, 11 mg; folic acid, 1.3 mg; D-biotin, 180 μg.
[b]Nutrient levels were calculated values.

categorized as IUGR weigh 0.84 kg. By day 21, their weights have increased to 6.00 kg and 3.17 kg, respectively.

2.4 Sample collection

Based on the birth weight of piglets, the placenta was classified into two categories: those corresponding to normal weight piglets and those corresponding to IUGR piglets. Following the onset of sow delivery, before cutting the umbilical cord as it emerged from the birth canal, three different colored silk threads (white for normal body weight piglet's placenta in control group, NBW; pink for IUGR piglet's placenta in control group, IUGR); and (black for IUGR piglet's placenta in COS group, IUGR + COS) were used to tie off the respective umbilical cords of varying weight piglets. The marked umbilical cord with the silk threads were then retracted back into the sow's body (the white and pink silk threads are of different materials, making it easier to distinguish them during sample collection). Once all placentas had been discharged by the sows post-delivery, fresh placental samples were collected from a 5 cm distance near the umbilical cord based on their color markings and stored in frozen tubes for subsequent testing after freezing in liquid nitrogen.

When piglets reach 21-day-old, those were anesthetized and euthanized, followed by a rapid opening of the abdominal cavity. The small intestine was then dissected, and tissue samples from the jejunum were promptly frozen in liquid nitrogen and stored at −80°C for subsequent analysis.

2.5 Sow reproduction

Record the changes in backfat of sows at days 85 and 111 of gestation, and day 21 of lactation, as well as the average litter size, live

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litter size, stillbirth, mummy size and the number of piglets with a birth weight below 1 kg. Calculate the average birth weight, live litter weight, percentage of IUGR, weaned litter size and weight, average weaned weight and daily gain during lactation for piglet, also calculate daily feed intake and backfat loss during lactation for sow. The percentage of IUGR refers to the proportion of piglets with BW <1.0 kg among the total born. Backfat loss was defined as the difference in backfat thickness between farrowing and weaning for each sow.

2.6 Antioxidant status of placenta in sows and jejunum in piglets

The levels of ATP, MDA, glutathione (GSH), and oxidized glutathione (GSSG) in the placenta, as well as the content of MDA, GSH, and GSSG and the activity of CAT, T-AOC, GSH-PX in the jejunum of piglet were determined using the commercial kits provided by Nanjing Jiancheng Bioengineering Institute (Nanjing, China). The total protein content in both the placenta and the piglet's jejunum was measured using the same kit.

2.7 Placental cytokines concentrations

The levels of placental cytokines IL-1β, IL-10 and TNF-α were quantified using a swine ELISA kit (Beijing 4A Biotech Co., Ltd).

2.8 Activity of digestive enzyme in jejunum of piglets

The activities of trypsin, lipase, maltase, sucrase and lactase in piglet jejunum were determined using the commercial kits provided by Nanjing Jiancheng Bioengineering Institute (Nanjing, China).

2.9 Quantitative real-time RT-PCR analysis

The expression of nutrient transport related genes (*GLUT1*, *GLUT2*, *SNAT1*, *SNAT2*, *SNAT4*, *PepT1*, *ANG2*, and *VEGFA*) in placental tissue, intestinal barrier related genes (*MUC1*, *MUC2*, *claudin1*, *occludin*, and *ZO-1*) in jejunal tissue, and inflammation related genes (*IL-1β*, *IL-6*, *IL-8*, *IL-10*, *TNF-α* and *IFN-γ*) in jejunal tissue were detected by q-PCR. The gene primer sequences were shown in Table 3. Tissue samples were lysed using TRIzol and homogenized with a homogenizer. Total RNA was extracted from jejunal mucosa using TRIzol reagent (TaKaRa Biotechnology Co, Ltd., Dalian, China) according to the manufacturer's instructions. The concentration and purity of RNA was analysed spectrophotometrically (Beckman Coulter DU800; Beckman Coulter Inc.), taking into account the ideal absorbance ratio (1.8 A260/280 2.0). Reverse transcription was performed using the PrimeScript™ RT Reagent (TaKaRa, Japan) kit and stored at −20°C for testing. Reference TB Green™ Premix Ex Taq™ II (TaKaRa, Japan) instruction manual was used with QuantStudio5 (Applied Biosystem) detection system for real-time fluorescence quantitative PCR. The

TABLE 3 Primer sequences of the target genes.

Gene	Primer sequences (5′–3′)	Accession No.
MUC1	F: GTGCCGCTGCCCACAACCTG	XM_001926883.5
	R: AGCCGGGTACCCCAGACCCA	
GLUT1	F: GCCTGAGACCAGTTGAAAGCAC	XM_021096908.1
	R: CTGCTTAGGTAAAGTTACAGGAG	
GLUT3	F: TGCACGGGCTTTGTGCCGATG	XM_021092391.1
	R: AAGGAGGTGAAGATTAGGAA	
SNAT1	F: AAGAACCTGGGCTATCTCGG	XM_003355629
	R: TGTTGCGTTAGGACTCGTTG	
SNAT2	F: GTTACCTTTGGTGATCCAGGC	NM_018976
	R: ACCAATGACACCAGCAGAACC	
SNAT4	F: GTTCTTTGCCTTCACTCACTA	XM_003355630
	R: GACCCAAGCCTCCAGATT	
ANG2	R: AATAACTGTCCATCCACCTCCA	NM_213808.1
	F: AGCCTTCAGGAACAAGAGTGC	
VEGFA	F: GAACTTTCTGCTCTCTTGGGT	NM_214084.1
	R: GGTTTCTGGTCTCCTTCTGCC	
GLUT2	F: GACACGTTTTGGGTGTTCCG	NM_001097417.1
	R: GAGGCTAGCAGATGCCGTAG	
PepT1	F: GCCAAAGTCGTCAAGTGC	NM_214347
	R: GGTCAAACAAAGCCCAGA	
IL-1β	F: CGTGCAATGATGACTTTGTCTGT	NM_214055.1
	R: AGAGCCTTCAGCATGTGTGG	
IL-6	F: TTCACCTCTCCGGACAAAAC	NM001252429.1
	R: TCTGCCAGTACCTCCTTGCT	
IL-8	F: AGTTTTCCTGCTTTCTGCAGCT	NM213867.1
	R: TGGCATCGAAGTTCTGCACT	
IL-10	F: TAATGCCGAAGGCAGAGAGT	NM_214041.1
	R: GGCCTTGCTCTTGTTTTCAC	
TNF-α	F: CGTGAAGCTGAAAGACAACCAG	NM_214022.1
	R: GATGGTGTGAGTGAGGAAAACG	
IFN-λ	F: TGCATCACATCCACGTCGAA	NM001142837.1
	R: GCAGCCTTGGGACTCTTTCT	
MUC2	F: GGTCATGCTGGAGCTGGACAGT	XM021082584.1
	R: TGCCTCCTCGGGGTCGTCAC	
Claudin-1	F: GCATCATTTCCTCCCTGTT	NM001161638.1
	R: CATGACTTCTGCCCTGACGA	
Occludin	F: AACTTCCACTGATGTCCCCCGT	NM001163647.2
	R: CCTAGACTTTCCTGCTCTGCCC	
ZO-1	F: CGTGTCAACGCCACTATCA	XM021098896.1
	R: TTGTCTTCCAAAGCCCCT	
β-actin	F: GGATGACGATATTGCTGCGC	XM003124280.5
	R: GATGCCTCTCTTGCTCTGGG	

ANG, angiogenin; GLUT, glucose transport protein; IFN-*λ*, interferon-*λ*; IL-10, interleukin-10; IL-1β, interleukin-1β; IL-6, interleukin-6; IL-8, interleukin-8; MUC, mucin; PepT1, oligopeptide transporter 1; SNAT, basolateral transporters system A; TNF-α, tumor necrosis factor; VEGFA, vascular endothelial growth factor; ZO-1, zona occludens-1.

cycling conditions were as follows predenaturation at 95°C for 30 s and 40 cycles of denaturation at 95°C for 5 s, annealing for 30 s and extension at 60°C for 34 s.

2.10 Western blotting

The western blot (WB) method was used to detect the expression of claudin1 and occludin protein in jejunal tissue. Jejunal tissue samples were mechanically and ultrasonically crushed, centrifuged, and the supernatant was collected for determination of total protein concentration using the BCA protein assay (Beyotime, Beijing, China). Equal amounts of protein were separated on 10% SDSPAGE gels, and then electrically transferred to polyvinylidene difluoride (PVDF) membranes. The PVDF membranes were blocked with 5% skimmed milk powder for 1 h at room temperature. Subsequently, they were incubated with the different primary antibody [Abcam (Cambridge, MA, United States)] at 4 degrees for 12 h. Then the PVDF membranes were incubated with corresponding secondary antibodies [Santa Cruz Biotechnology (Santa Cruz, CA, United States)] for 1 h at room temperature, followed by testing the target protein bands using ECL Luminescence Detection Kit (Beyotime, Beijing, China). Image Lab 5.1 was used for exposure photography and strip grayscale value analysis. Antibody concentration of claudin1, occludin, β-actin are 1:1,000.

2.11 Statistical analysis

Statistical differences were analyzed by the independent samples *t*-test in SPSS 22.0 statistics software (Chicago, IL, United States). Independent sample *t*-tests were performed for pairwise comparisons between NBW, IUGR, and IUGR + COS. Firstly, the comparison between NBW and IUGR aimed to verify the successful establishment of the IUGR piglet injury model. Secondly, the analysis between IUGR and IUGR + COS was conducted to evaluate the improvement effect of COS supplementation on IUGR piglets. Lastly, the comparison between NBW and IUGR + COS assessed the remaining gap, if any, between IUGR piglets after COS supplementation and normal piglets. The results were expressed as mean ± standard error, with significance indicated by $p < 0.05$ and a trend indicated by $0.05 \leq p < 0.10$.

3 Results

3.1 Sows' reproductive performance

The results of sows' reproductive performance were shown in Table 4. The supplementation of COS in sow diet had no significant effect on the total born, born alive, mean BW and mean litter weight at birth, litter weight and average pig weight at weaning, as well as average daily gain of piglets during lactation ($p > 0.05$), but significantly reduced the number of stillbirths and mummies ($p < 0.05$). Furthermore, COS group exhibited a 35.22% reduction in the number of piglets weighing less than 1 kg compared to the control group. After supplementing with COS, the incidence of IUGR decreased by 8.57% compared to the control group.

TABLE 4 Effects of maternal chitosan oligosaccharides supplementation during late gestation and lactation on the reproductive performance of sows.

Item	Control	COS	*p*-value
Birth, piglets/litter, *n*=15			
Total born, *n*	12.47 ± 0.71	12.53 ± 0.56	0.94
Born alive, *n*	11.00 ± 0.49	11.93 ± 0.65	0.26
Born dead, *n*	0.93 ± 0.18	0.40 ± 0.13	0.02
Stillborn, *n*	0.60 ± 0.19	0.13 ± 0.09	0.04
BW <1.0 kg, *n*	2.47 ± 0.64	1.60 ± 0.49	0.29
Percentage of IUGR, %	20.59 ± 6.43	12.02 ± 3.71	0.26
Mean BW at birth, kg	1.34 ± 0.09	1.40 ± 0.06	0.63
Mean litter weight at birth, kg	14.37 ± 0.86	16.42 ± 1.03	0.14
Weaning, *n*=10			
Litter size after cross-foster, *n*/litter	10.80 ± 0.13	10.70 ± 0.26	0.74
Litter size at weaning, piglets/litter	9.00 ± 0.30	9.10 ± 0.23	0.80
Litter weight after cross-foster, kg	16.02 ± 0.95	14.85 ± 0.55	0.30
Litter weight at weaning, kg	52.37 ± 2.61	54.89 ± 2.61	0.50
Average pig weight at weaning, kg	5.84 ± 0.27	6.03 ± 0.13	0.53
ADG for piglet, g	207.37 ± 13.21	220.96 ± 6.17	0.37
Lactation ADFI for sow, kg	5.05 ± 0.21	5.13 ± 0.22	0.79
Sow backfat thickness, mm			
Day 85 of gestation (*n* = 15)	13.64 ± 0.72	14.00 ± 0.47	0.68
Farrowing (*n* = 10)	14.27 ± 0.69	14.90 ± 0.53	0.49
Weaning (*n* = 10)	12.64 ± 0.49	13.80 ± 0.61	0.15
Decrease in backfat thickness during lactation (*n* = 10)	1.64 ± 0.34	1.10 ± 0.23	0.22

3.2 Placenta function-related genes expression of sows

As shown in Figure 1, the mRNA expression of glucose transport related genes (*GLUT1* and *GLUT3*), amino acid transport related genes (*SNAT1* and *SNAT4*), and angiogenesis gene *VEGFA* in the placenta of IUGR piglets (from control sows) were significantly lower than those in the placenta of normal piglets (from control sows) ($p < 0.05$); However, the expression of *GLUT1*, *GLUT3* and *VEGFA* mRNA in the placenta of IUGR piglets (from COS sows) was significantly higher than that in the placenta of IUGR piglets (from control sows) ($p < 0.05$). The expression of *ANG2* mRNA in the placenta of IUGR piglets (from COS sows) was significantly lower than that in the placenta of IUGR piglets (from control sows) ($p < 0.05$).

3.3 Placental antioxidant status and cytokine of sows

As shown in Table 5, the levels of ATP, GSH, IL-10 and GSH/GSSG in the placenta of IUGR piglets (from control sows) were

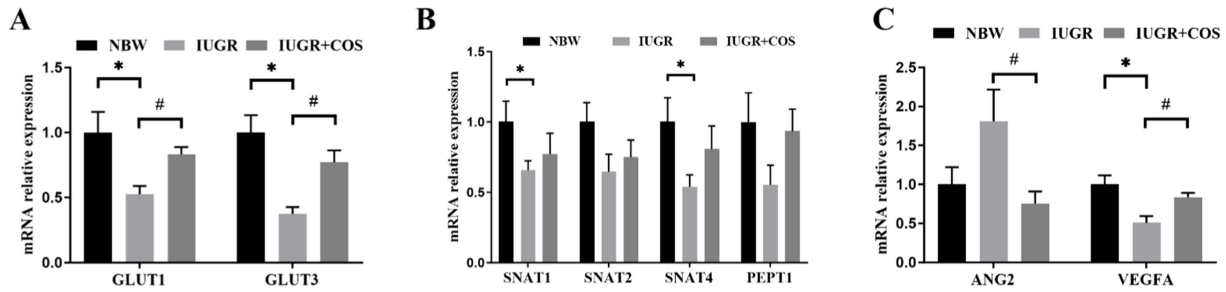

FIGURE 1
Effects of maternal chitosan oligosaccharides supplementation during late gestation on the placenta function-related genes expression of sows. (A) Glucose transporters: *GLUT1* and *GLUT3*. (B) Amino acid transporters: *SNAT1*, *SNAT2*, *SNAT4*, and *PepT1*. (C) Angiogenesis: *ANG2* and *VEGFA*. *Means significant difference between NBW vs. IUGR/IUGR + COS. #Means significant difference between IUGR vs. IUGR + COS, $p < 0.05$, $n = 10$.

TABLE 5 Effects of maternal chitosan oligosaccharides supplementation during late gestation on the placenta oxidative parameters and cytokines levels of sows.

Item	Control		COS	*p*-value		
	NBW	IUGR	IUGR	*p*1	*p*2	*p*3
ATP, nmol/mg prot	483.28 ± 61.76	239.82 ± 18.76	236.47 ± 50.80	0.01	0.95	0.01
MDA, μmol/mg prot	3.66 ± 0.17	5.01 ± 0.15	4.28 ± 0.12	<0.01	0.01	0.01
GSH, μmol/mg prot	2.52 ± 0.20	1.30 ± 0.07	1.70 ± 0.12	<0.01	0.01	<0.01
GSSG, μmol/mg prot	9.53 ± 1.76	16.54 ± 2.71	12.96 ± 1.45	0.04	0.26	0.15
GSH/GSSG	0.40 ± 0.09	0.12 ± 0.03	0.15 ± 0.02	0.02	0.47	0.02
IL-1β, ng/mg prot	14.56 ± 0.90	14.77 ± 1.40	13.77 ± 0.94	0.90	0.56	0.55
IL-10, ng/mg prot	107.21 ± 4.98	71.73 ± 7.64	76.21 ± 6.82	<0.01	0.67	0.02
TNF-α, pg./mg prot	129.16 ± 6.63	135.75 ± 11.61	135.28 ± 9.19	0.63	0.98	0.60

p-value means significant difference between two groups, *p*1: NBW vs. IUGR, *p*2: IUGR vs. IUGR + COS, *p*3: NBW vs. IUGR + COS, $n = 10$. MDA, malondialdehyde; GSH, glutathione; GSSG, oxidized glutathione; IL-1β, interleukin-1β; IL-10, interleukin-10; TNF-α, tumor necrosis factor-α.

significantly lower than those in the placenta of normal body weight piglets (from control sows), whereas the concentrations of MDA and GSSG exhibited an opposite trend ($p < 0.05$). Furthermore, maternal dietary COS supplementation resulted in a significant decrease in placental MDA levels in IUGR piglets, while there was a significant increase in GSH levels ($p < 0.05$). Compared to normal sow placenta (NBW), the levels of ATP, GSH, GSH/GSSG, and IL-10 remained significantly lower ($p < 0.05$), whereas MDA was significantly higher ($p < 0.05$) after COS supplementation of sow diets (IUGR + COS).

3.4 Jejunal digestive enzyme activities of 21-day-old suckling piglets

As shown in Figure 2, the activities of jejunal trypsin, lactase and maltase in 21-day-old IUGR piglets were significantly lower compared to those of normal body weight piglets ($p < 0.05$). Maternal dietary COS supplementation during late pregnancy and lactation significantly increased the activities of jejunal trypsin and lactase in 21-day-old IUGR piglets ($p < 0.05$), and significantly reduced sucrase activity ($p < 0.05$).

3.5 Jejunal barrier function of 21-day-old suckling piglets

As shown in Figure 3, compared with normal body weight piglets, the expression of *MUC1*, *MUC2*, *claudin1* mRNA and claudin1 protein in the jejunum of 21-day-old IUGR piglets (from control sows) were significantly reduced ($p < 0.05$). Supplementing COS to the diet of sows during late gestation and lactation resulted in a significant increase in the expression of *MUC2* and *occludin* mRNA as well as claudin1 protein in the jejunum of 21-day-old IUGR piglets ($p < 0.05$).

3.6 Gene expression levels of cytokines in the jejunum of 21-day-old suckling piglets

As shown in Figure 4, the expression of *IL-1β*, *IL-6*, *IL-8*, and *TNF-α* mRNA in the jejunum of 21-day-old suckling IUGR piglets was significantly increased compared to normal body weight piglets ($p < 0.05$), while the expression of *IL-10* mRNA was significantly reduced ($p < 0.05$). Supplementation of COS to the diet of sows during late pregnancy and lactation resulted in a significant increase in the

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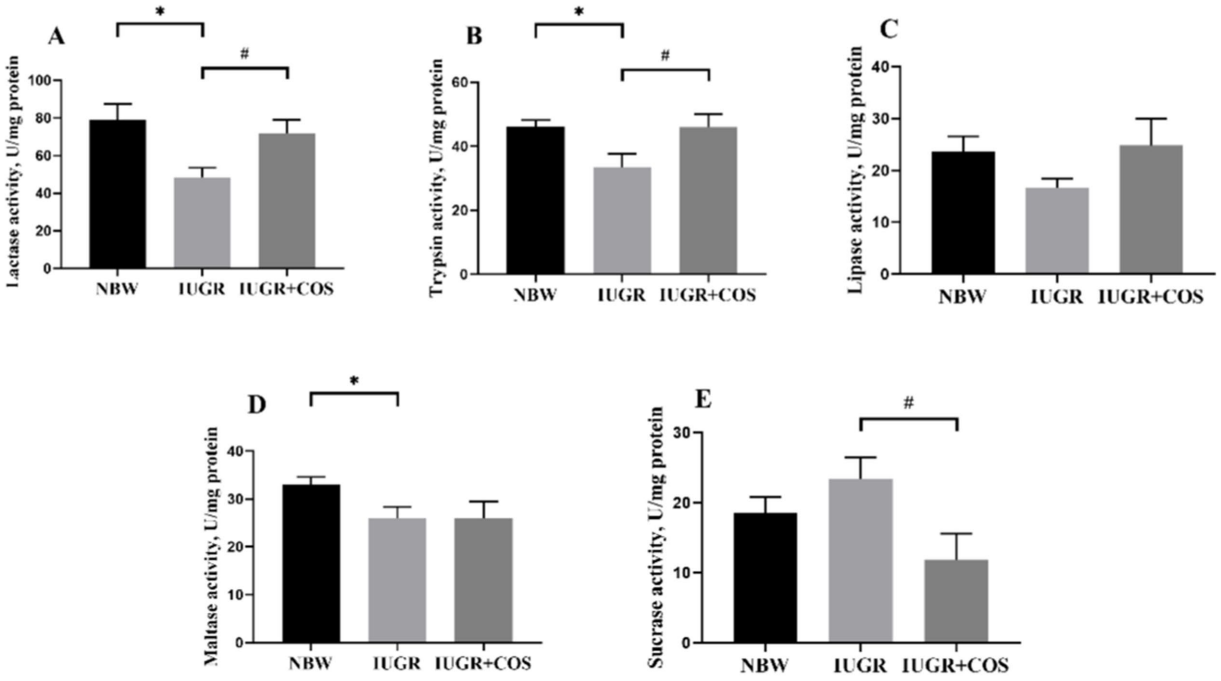

FIGURE 2
Effects of maternal chitosan oligosaccharides supplementation during late gestation and lactation on the jejunal digestive enzyme activities of 21-day-old suckling piglets. **(A)** lactase activity; **(B)** trypsin activity; **(C)** lipase activity; **(D)** maltase activity; **(E)** sucrase activity. *Means significant difference between NBW vs IUGR/IUGR + COS, #Means significant difference between IUGR vs IUGR + COS, $p<0.05$, $n=8$.

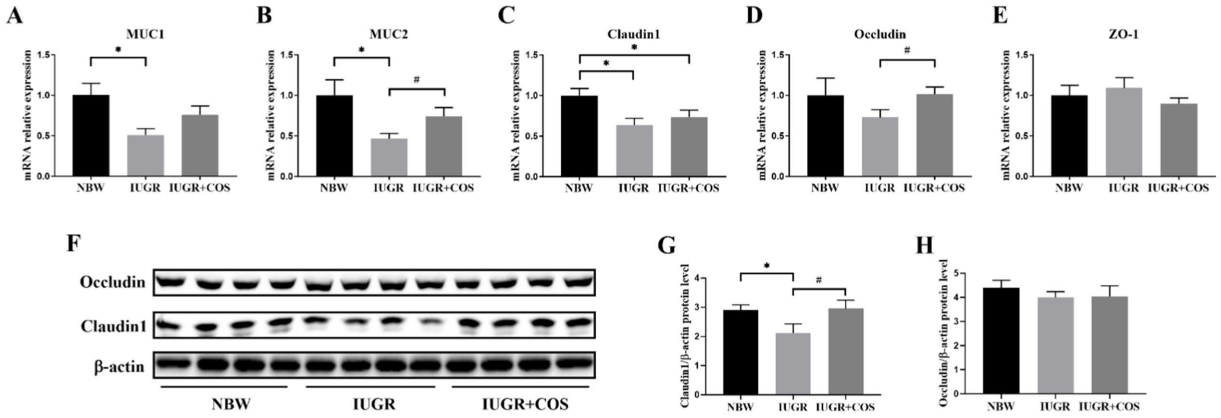

FIGURE 3
Effects of maternal chitosan oligosaccharides supplementation during late gestation and lactation on the jejunal barrier function of 21-day-old suckling piglets. (A–E) Jejunal barrier-related gene expression levels, $n = 8$. (F–H) Jejunal barrier-related protein expression levels, $n = 4$. *Means significant difference between NBW vs. IUGR/IUGR + COS. #Means significant difference between IUGR vs. IUGR + COS, $p < 0.05$.

expression of *IL-10* mRNA in the jejunum of piglets ($p < 0.05$), with a tendency to decrease the expression of *IL-6* ($p = 0.06$) and *IL-8* ($p = 0.08$) mRNA compared to IUGR piglet from control sows.

3.7 Antioxidant status of jejunum in 21-day-old suckling piglets

The results of the antioxidant status of jejunum in suckling piglets were presented in Table 6. Compared to 21-day-old normal body weight piglets, IUGR piglets (from control sows) exhibited a significant increase in MDA and GSSG levels ($p < 0.05$), a significant reduction in the GSH/GSSG ratio ($p < 0.05$), and a tendency to increase CAT level ($p = 0.06$). The addition of COS to the diet of sows during late pregnancy and lactation significantly reduced the MDA content in the jejunum of IUGR piglets ($p < 0.05$). However, GSSG remained significantly elevated ($p < 0.05$) and GSH/GSSG was significantly reduced ($p < 0.05$) after COS supplementation of the sows' diets (IUGR + COS), compared with 21-day-old normal body weight piglets.

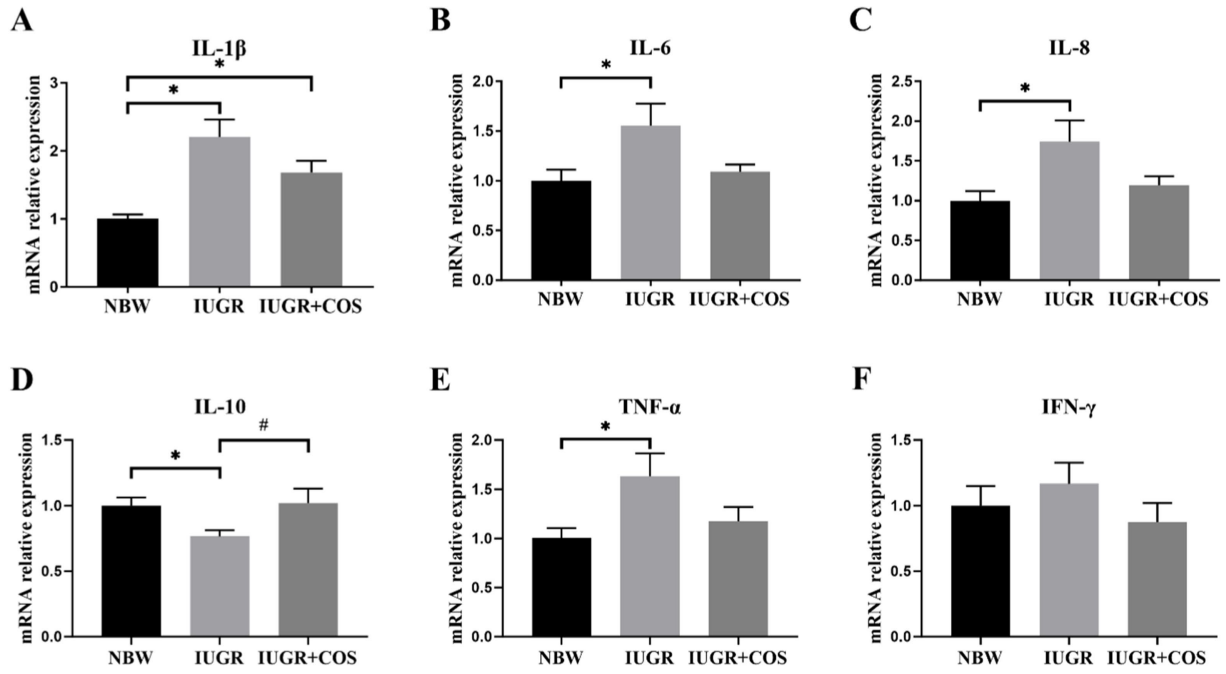

FIGURE 4
Effects of maternal chitosan oligosaccharides supplementation during late gestation and lactation on the jejunal cytokine gene expression levels of 21-day-old suckling piglets. **(A)** IL-1β (Interleukin-1β); **(B)** IL-6 (Interleukin-6); **(C)** IL-8 (Interleukin-8); **(D)** IL-10 (Interleukin-10); **(E)** TNF-α (Tumor necrosis factor-α); **(F)** IFN-γ (Interferon-γ). *Means significant difference between NBW vs IUGR or NBW vs IUGR + COS, #Means significant difference between IUGR vs IUGR + COS (P3), $p < 0.05$, $n = 8$.

TABLE 6 Effects of maternal chitosan oligosaccharides supplementation during late gestation and lactation on the antioxidant status of jejunum in 21-day-old suckling piglets.

Variable	NBW	IUGR	IUGR + COS	*p*1	*p*2	*p*3
MDA, μmol/mg prot	0.98 ± 0.04	1.26 ± 0.05	1.04 ± 0.04	0.01	0.01	0.28
CAT, U/mg prot	6.13 ± 0.47	9.51 ± 1.51	7.16 ± 0.46	0.06	0.17	0.14
T-AOC, U/mg prot	282.00 ± 37.91	255.31 ± 32.64	275.66 ± 48.95	0.60	0.73	0.92
GSH-PX, U/mg prot	12.57 ± 1.28	11.21 ± 1.88	13.04 ± 2.32	0.56	0.55	0.86
GSH, μmol/mg prot	1.07 ± 0.06	1.01 ± 0.04	1.06 ± 0.09	0.43	0.64	0.90
GSSG, μmol/mg prot	9.09 ± 0.72	14.01 ± 1.54	13.47 ± 1.18	0.01	0.78	0.01
GSH/GSSG	0.13 ± 0.02	0.08 ± 0.01	0.08 ± 0.01	0.01	0.54	0.03

p-value means significant difference between two groups, *p*1: NBW vs. IUGR, *p*2: IUGR vs. IUGR + COS, *p*3: NBW vs. IUGR + COS, $n = 8$. MDA, malondialdehyde; CAT, catalase; T-AOC, total antioxidant capacity; GSH-PX, glutathione peroxidase; GSH, glutathione; GSSG, oxidized glutathione.

4 Discussion

The main purpose of this study is to investigate the effect of supplementing COS to the diet of sows during late gestation and lactation on placental function, as well as its effect on intestinal function of IUGR piglets after 21 days of suckling. Previous research has utilized various methods, such as direct nutrient supplementation to piglets post-birth, in order to improve the growth performance and functions of IUGR (19, 20). From another perspective, supplementing nutrients during late gestation and lactation in sows has been demonstrated to improve the reproductive performance of sows and the growth performance of piglets (21, 22). In previous studies, maternal dietary COS supplementation during late gestation and lactation has been shown to significantly increase the average daily gain and weaning weight of piglets, but had no significant effect on the birth weight (16). In addition, it can also improve the born alive (14). Other studies have shown that the supplementation of COS can increase the total number of newborn piglets and live piglets, as well as significantly improve the average live litter weight, however, it has no significant effect on the average daily gain and average weaning weight (18). In this experiment, we found that dietary COS supplementation in sows only reduced the occurrence of stillbirths and mummies, without significant effects on other productive parameters. The results of this study were partially inconsistent with previous findings, possibly due to individual variations among sows and environmental conditions. Additionally, differences in sow management and nutritional control during gestation may also influence reproductive performance. Nevertheless, it is worth noting that our study found a 35.22% reduction in piglets with a birth weight less than 1 kg in the COS group compared to the control group,

indicating potential alleviation of IUGR occurrence through maternal COS supplementation during late gestation.

The placenta serves as a crucial interface between the maternal and fetal compartments. It plays an essential role in ensuring the successful progression of pregnancy and the optimal growth and development of the fetus. During gestation, the placenta is responsible for transporting nutrients. Dysregulated nutrient transport can result in intrauterine growth restriction (23). Our Findings indicating impaired placental nutrient transport as a contributing factor to IUGR, COS supplementation during late gestation effectively ameliorated decreased nutrient transport function following placental injury. Previous studies have emphasized the importance of adequate vascularization within the placenta for normal fetal development (24), however, IUGR placentas exhibited significantly poorer vascular development compared to normal placentas (8). In this study, it was found that impaired vascular growth in sow placenta appeared to be another factor contributing to the emergence of IUGR. However, this impaired state could be restored to a certain extent after COS supplementation. It is important to note that placental nutrient transport and vascular development are interdependent, compromised vasculature limits exchange capacity in IUGR placentae (23). The above results indicated that supplementing sows feed with COS during late gestation effectively enhanced placental nutrient transport capacity, improved angiogenesis within the placenta, and reduced the occurrence of IUGR piglets.

Due to the close connection between the mother and the fetus, facilitated by the placenta as a conduit, maternal well-being significantly influences fetal development. In late pregnancy in sows, activities such as fetal growth, mammary gland development, and milk production resulted in elevated levels of reactive oxygen species (ROS), leading to oxidative stress in sows (25). Maternal oxidative stress is associated with an increased risk of IUGR (25). The placenta, an extremely important organ in this process, is highly sensitive to oxidative stress (26). Our study indicating pronounced oxidation in IUGR sows' placentas and confirming the link between IUGR and oxidative stress. Following COS treatment, effective alleviation of placental oxidative stress. Furthermore, there are reports suggesting that immune cells undergo functional alterations during late gestation, resulting in increased release of inflammatory cytokines which can affect the maternal-fetal health. In this study, we found the same situation in the placenta. This increase in pro-inflammatory cytokines coupled with a decrease in anti-inflammatory cytokines reflects immune damage and inflammation within sow placentas during pregnancy, potentially contributing to adverse outcomes such as IUGR (27). However, following COS supplementation, the inflammatory response was alleviated, to some extent compared to IUGR. Therefore, supplementing COS can effectively alleviate oxidative stress and inflammatory response in the placenta, ultimately reducing the emergence of IUGR piglets.

After parturition, the primary connection between the neonate and the dam is through colostrum and milk, which plays an essential part in neonatal intestinal health. Piglets primarily acquire immunoglobulins by sucking colostrum postnatally, however, IUGR piglets may experience inadequate sucking or exposure to foodborne pathogens due to developmental impairment, resulting in further intestinal damage (6). In this study, changes in jejunal digestive enzyme activity in piglets reflected the addition of COS can effectively improve digestive and absorption function in piglets' intestines.

The intestinal health of piglets can impact their overall growth, and inadequate development in the mother's body and insufficient acquisition of colostrum and milk during lactation can have a detrimental effect on the intestinal health of IUGR piglets. This study observed impaired gut barrier, inflammatory response, and oxidative stress in IUGR piglets. Supplementing COS to the diet of sows during lactation can significantly ameliorates intestinal damage in IUGR piglets. Additionally, maternal COS also alleviated the inflammatory response in IUGR suckling piglets. We found that lipid peroxidation occurred in IUGR piglets' bodies and a change in cellular redox state. However, supplementation of COS to lactating sow diets can inhibit lipid peroxidation. Previous studies have confirmed that supplementing COS to piglet feed can significantly improve the antioxidant defense system function of weaned piglets, protect them from oxidative stress, and improve their immune status (9). In this study, by supplementing COS to lactating sows feed resulted in the transmission of these effect to the piglets, possibly due to some antioxidant and anti-inflammatory components present in mother's milk, thereby enhancing piglet antioxidant and immune status.

This study primarily focuses on improving IUGR in piglets by supplementing sows with COS through the placenta. By enhancing placental angiogenesis and nutrient transport, we aim to promote healthier fetal development and reduce IUGR incidence. Although this study did not examine the composition and changes of colostrum and regular milk, COS supplementation in sows may help alleviate intestinal damage in IUGR piglets via breast milk. However, research on COS's impact on milk is limited, and it remains uncertain whether its anti-inflammatory and antioxidant properties extend to milk.

Furthermore, the role of microorganisms cannot be overlooked. After birth, close contact between piglets and their mothers, particularly their initial exposure to sow feces, facilitates the colonization of microorganisms in the piglets' intestines (28). Consequently, the sow's diet can also influence the microbial composition of piglets (29). Studies indicate that COS supplementation positively affects host intestinal health and gut microbiota (30). We hypothesize that by improving the sow's gut microbiota through COS supplementation, beneficial microorganisms can be transmitted to piglets upon contact, thereby enhancing their intestinal health and mitigating the effects of IUGR.

5 Conclusion

In this study, the results demonstrated that supplementing COS in the late gestation of sows can improve reproductive performance, and placental, alleviate oxidative stress in the placenta, and consequently reduce the incidence of IUGR piglets. Continuing to supplement COS during lactation can improve the intestinal barrier function of 21-day-old suckling IUGR piglets, alleviate inflammatory reactions and oxidative stress, thus effectively alleviate the adverse effects of IUGR piglets. In conclusion, supplementing sow feed with COS during late gestation and lactation can effectively reduce the incidence of IUGR piglets and improve their health. This experiment also provided nutritional strategies for pregnant and lactating women to enhance infant health.

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 approved by Institutional Animal Care and Use Committee of Sichuan Agricultural University. The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

XW: Writing – original draft, Writing – review & editing. TF: Investigation, Software, Writing – review & editing. DC: Supervision, Writing – review & editing. JP: Formal analysis, Writing – review & editing. GT: Writing – review & editing. JH: Writing – review & editing. PZ: Writing – review & editing. XM: Writing – review & editing. AW: Writing – review & editing. BY: Funding acquisition, Project administration, Supervision, Writing – review & editing.

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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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Fermented *Codonopsis pilosula* residue improved milk performance of lactating donkeys by enhancing antioxidant capacity and regulating metabolism

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The fermented *Codonopsis pilosula* residue (FCPR) has a promoting effect on animal health, but it has not been studied in livestock feed. This study investigated the effects of FCPR addition on the milk performance, milk metabolites, and blood biochemical parameters and metabolites of lactating donkeys. Twelve healthy multiparous lactating Dezhou donkeys were randomly divided into two groups (with 6 donkeys in each group): control group (without FCPR, C) and FCPR group (200 g of FCPR per head per day, FF). The experiment lasted for 5 weeks. The milk and blood samples were collected at the end of the experiment. The results showed that addition of FCPR significantly increased the daily milk yield and the milk components (protein, lactose, solids, solids-not-fat, and lactoferrin) yield of lactating donkeys and the weight gain of foals ($p < 0.05$). A total of 568 metabolites were detected in donkey milk, including 21 differential metabolites between group FF and group C. Compared to group C, the significantly up-regulated metabolic pathway in the FF group was renin secretion ($p < 0.05$). In addition, the FCPR significantly increased the concentrations of urea, complement C4 (C4), total antioxidant capacity (T-AOC), and catalase (CAT) in the blood, while reducing the concentration of malondialdehyde (MDA) ($p < 0.05$). A total of 753 metabolites were detected in serum of donkeys, including 86 differential metabolites between group FF and group C. Compared to group C, the significantly up-regulated metabolic pathways in the FF group were renin secretion, cAMP signaling pathway, regulation of lipolysis in adipocytes, and fatty acid biosynthesis ($p < 0.05$). The above results indicated that FCPR addition enhance the milk performance of lactating donkeys by activating the immune system, increasing the antioxidant capacity, and improving the glucose and lipid metabolism. These results provide a foundation for the development and utilization of FCPR additives, which is beneficial for livestock production and improving animal welfare.

KEYWORDS

donkeys, fermented *Codonopsis pilosula* residue, milk performance, metabolites, blood biochemical parameters

1 Introduction

Chinese herbal medicines (CHM) and plant extractive have been used in livestock and aquaculture and play an important role in animals' healthy growth (1, 2). Some CHM or Chinese herbal residues (CHR) are added as feed additives to animal diets. It has been found that addition of CHR improved the gut environment of weaned piglets (2). Samanta et al. (3) studied the effects of ginger residue and inulin on pig fecal microbiota and found that they can improve intestinal microbiota and promote healthy growth of pigs. The results of Du et al. (1) found that dietary supplementation of *Taraxacum mongolicum* flavonoids protected *Channa argus* from lipopolysaccharide-induced inflammatory injury, improved digestion, activated immune responses, and enhanced antioxidant capacity. In study of Samanta et al. (3), the effects of *Lonicera japonica* extract on heat-stressed mid-lactation dairy cows were investigated, and the results found that *Lonicera japonica* extract helped to alleviate heat stress by improving antioxidant status and promoting endocrine and immune functions. The results of Abulaiti et al. (4) also showed that CHM supplementation improved the milk yield, milk composition, and serum metabolites in dairy cows.

In addition, fermented Chinese herbal medicines (FCHM) or herbal residues (FCHR) are also widely used in animal husbandry. *Lactic acid bacteria* fermented herbs can regulate immune responses by activating innate or adaptive immune systems and maintaining intestinal immune homeostasis (5). The results of Fujaya et al. (6) showed that the fermented herbal extract enhanced weight gain, growth and feed efficiency of *Nile Tilapia* by increasing nutrient absorption and improving intestinal immune function. FCHM (3 kg/t) improved the weaned piglets growth performance by improving intestinal health and promoting nutrients digestibility (7). The probiotic-fermented herbal blend prevented death and improved growth performance of *Salmonella pullorum* infected chicks by increasing cecal *lactobacilli*, reducing cecal *Escherichia coli* and *Salmonella*, and enhancing immune function (8). The results of Su et al. (2) also found that addition of FCHR improved the gut environment of weaned piglets. In addition, fermented herbal tea residue (FHTR) increased the concentrations of hydroxyl radicals, urea, and glutathione peroxidase (GSH-Px) in serum of goats (9). Zhuang et al. (10) found that FHTR replacing 30% of the forage significantly increased the immunoglobulin G (IgG), total antioxidant capacity (T-AOC), and superoxide dismutase (SOD) levels, changed the fecal microbiota composition, increased the daily feed intake and daily gain of fattening cattle. It was also found that adding FHTR increased the concentration of immunoglobulin A (IgA) and immunoglobulin G (IgG) in serum, as well as the activity of SOD and GSH-Px, thereby improving the growth of heifers (11).

Codonopsis pilosula, the root of *C. pilosula (Franch.) Nannf.*, which is known as "Dangshen" in Chinese (12) has been used in food (tea, wine, soup and porridge) and prescribed in traditional folk medicine in Asian countries like China for thousands of years (13, 14). *Codonopsis pilosula* has anti-inflammatory, antioxidant, prebiotic, and immune regulation biological activities, playing an important role in the treatment of cardiovascular diseases, digestive diseases, and immune diseases (15). The *Codonopsis pilosula* polysaccharides (CPPs) are the major bioactive substance with biological activities (13). It was found that CPPs significantly enriched the intestinal probiotic *Lactobacillus* and decreased the abundance of *Enterococcus* and *Shigella*, which was beneficial for the invigorating (16). Research has also found that a polysaccharide extracted from *Codonopsis pilosula* residue also has a regulatory effect on glucose metabolism (17). Furthermore, the *Codonopsis pilosula* extract, inulin-type fructan, reduced the mucosa ulcer index of rats by reducing the malondialdehyde (MDA) and nitric oxide (NO) content and promoting the myeloperoxidase (MPO), superoxide dismutase (SOD), and glutathione peroxidase (GSH-Px) activity (18). It was also found that the *A. membranaceus* and *Codonopsis pilosula* extract mixture improved the growth performance and immunity of growing-finishing pigs (19, 20).

It is speculated that fermented *Codonopsis pilosula* residue (FCPR) also has a promoting effect on animal health. Thus, effects of FCPR on milk performance, milk metabolites, and blood biochemical parameters and metabolites of lactating Dezhou donkeys was investigated in this study.

2 Materials and methods

2.1 Samples collection and preparation

Milk and serum samples were from 12 healthy multiparous lactating Dezhou donkeys with an age of 3–5 years and an average bodyweight 278 ± 30 kg at 70 ± 5 days in milk (DIM). The donkeys were housed in a farm in Liaocheng City, China. The donkeys were raised in a semi-closed house. Each donkey was kept in a separate pen. All donkeys drank freely and offered the same diet of grass hay (*ad libitum*) supplemented with 2.5 kg concentrate per head per day (Table 1) (21). The donkeys were randomly divided into two groups, with 6 donkeys in each group. One group did not add FCPR (control group, C), while the other group added 200 g of FCPR per head per day (the FCPR group, FF). The fermentation substrate was the *Codonopsis pilosula* residue. And fermentation was conducted using *Bacillus* and *Lactobacillus plantarum* (0.2%). The nutritional composition of the FCPR was as follows: dry matter (50%), crude protein (8%), nitrogen free extract (18%), crude fiber (15%), and ash (9%). The experiment lasted for 5 weeks. The milk and blood samples were collected at the end of the experiment.

The lactating donkeys were manual-milked twice a day (11 am and 3 pm). The foals were removed from the mother for 4 h before the milking. The milk yield of the donkey was recorded. The milk samples were collected during milking twice a day, frozen quickly in liquid nitrogen and stored at −80°C until analysis. The blood samples were collected from the jugular vein before feeding in the morning. After stratification, the blood samples were centrifuged at 3500 rpm, 4°C for 15 min to separate the serum. Then, the serum was frozen quickly with liquid nitrogen and stored at −80°C.

2.2 Milk composition determination

The milk compositions (fat, protein, lactose, urea, lactoferrin, solids, and solids-not-fat) were determined using the CombiScope FTIR300 (Delta Instruments B.V., Drachten, The Netherlands). There were three replicates for each sample.

TABLE 1 Composition and nutritional level of experimental concentrate (dry-matter basis).

Items	Feed Ingredients (%)
Corn	31.75
Wheat bran	12.00
Wheat flour middling	21.00
DDGS	5.00
Wheat germ	18.00
Soybean meal	6.65
NaCl	0.63
$CaCO_3$	3.72
$CaHPO_4$	0.25
Premix[1]	1.00
Total	100
	Nutrient level (%)
Dry matter	88.53
Crude protein	17.18
Ash	8.37
Crude fiber	4.27
Ether extract	4.59
Calcium	1.27
Phosphorus	0.52
Lysine	0.75

[1]Premix/kg: VA, 600 KIU; VD3, 125 KIU; VE, 3500 IU; Fe, 2 g; Cu, 800 mg; Zn, 6 g; Mn, 13 g; I, 95 mg; Se, 50 mg.

2.3 Measurement of blood biochemical parameters

The concentration or international units (IU) of serum glucose (GLU), total protein (TP), albumin (ALB), triglyceride (TG), urea, aspartate aminotransferase (AST), alanine aminotransferase (ALT), lactate dehydrogenase (LDH), alkaline phosphatase (ALP), complement C3 (C3), complement C4 (C4), IgA, IgG, immunoglobulin M (IgM), SOD, GSH-Px, T-AOC, catalase (CAT), and MDA were measured using the automatic biochemical analyzer (Mindray BS-420, Shenzhen, China), and the test kits were provided by BioSino Bio-Technology and Science Inc. (Beijing, China).

2.4 Detection of milk and serum metabolites

2.4.1 Metabolites extraction

Milk or serum sample (100 μL) was added with prechilled 80% methanol. Then, the sample was placed into the Eppendorf tube and vortexed. Afterwards, the samples were incubated on ice for 5 min. Then the samples were centrifuged for 20 min at 15,000 g, 4°C. Some of supernatant was diluted with LC–MS grade water to the final concentration containing 53% methanol. After that, the samples were transferred to a fresh Eppendorf tube and then were centrifuged for 20 min at 15,000 g, 4°C. Finally, the supernatant was analyzed by liquid chromatography–tandem mass spectrometry (LC–MS/MS).

2.4.2 UHPLC–MS/MS analysis

The Vanquish UHPLC system (Thermo Fisher, Germany) coupled with an Orbitrap Q Exactive TM HF mass spectrometer (Thermo Fisher, Germany) were used in UHPLC–MS/MS analyses. Samples were injected onto a Hypersil Goldcolumn (100 × 2.1 mm, 1.9 μm) at a flow rate of 0.2 mL/min using a 12-min linear gradient. The eluents for the positive and negative polarity modes were eluent A (0.1% formic acid in Water) and eluent B (methanol). The solvent gradient was set as follows: 2% B, 1.5 min; 2–85% B, 3 min; 85–100% B, 10 min; 100–2% B, 10.1 min; 2% B, 12 min. Q Exactive TM HF mass spectrometer was operated in positive/negative polarity mode with spray voltage of 3.5 kV, capillary temperature of 320°C, sheath gas flow rate of 35 psi and aux gas flow rate of 10 L/min, S-lens RF level of 60, Aux gas heater temperature of 350°C.

2.4.3 Data processing and metabolite identification

The Compound Discoverer 3.3 (CD3.3, ThermoFisher) was used to process the raw data files for peak alignment, peak picking, and quantitation for each metabolite. After that, peak intensities were normalized to the total spectral intensity. Then predicted the molecular formula using the normalized data based on additive ions, molecular ion peaks and fragment ions. And then peaks were matched with the mzCloud,[1] mzVault and MassList database to obtain the accurate qualitative and relative quantitative results. The statistical software R (R version R-3.4.3), Python (Python 2.7.6 version) and CentOS (CentOS release 6.6) were used in statistical analyses. Throughout the analytical run, aliquots of all the milk or plasma samples were mixed to create quality control (QC) samples, which were injected at regular intervals to assess the stability of the analytical system.

2.5 Data analysis

These metabolites were annotated using the Kyoto Encyclopedia of Genes and Genomes (KEGG) database,[2] HMDB database[3] and LIPIDMaps database.[4] Principal components analysis (PCA) and Partial least squares discriminant analysis (PLS-DA) was performed at metaX. The statistical significance (p-value) was calculated using univariate analysis (t-test). The metabolites were considered to be differential metabolites when VIP > 1, $p < 0.05$, and fold change (FC) ≥ 2 or FC ≤ 0.5. For clustering heat maps, the data were normalized using z-scores of the intensity areas of differential metabolites and were ploted by Pheatmap package in R language. The functions of these metabolites and metabolic pathways were studied using the KEGG database. The metabolic pathways enrichment of differential metabolites was performed, when ratio was satisfied by x/n > y/N and $p < 0.05$, the metabolic pathway was considered as statistically significant enrichment. The Pearson correlation coefficient R^2 between QC samples was between 0.991 and 0.996, which was very close to 1, indicating good stability and high data quality of the detection process.

1 https://www.mzcloud.org/
2 https://www.genome.jp/kegg/pathway.html
3 https://hmdb.ca/metabolites
4 http://www.lipidmaps.org/

TABLE 2 Effects of fermented herbal residue on milk performance of lactating donkeys.

Items	C	FF	P
Average daily feed intake (kg)	5.54 ± 0.45	5.73 ± 0.44	0.5598
Daily milk yield (mL)	1,103 ± 187[b]	1,452 ± 280[a]	0.0295
Weight gain of foals (kg)	11.75 ± 4.68[b]	19.83 ± 7.50[a]	0.0490
Weight gain of lactating donkeys (kg)	−0.67 ± 9.68	13.33 ± 15.57	0.0909
Milk composition			
Milk fat (%)	0.34 ± 0.42	0.32 ± 0.31	0.9208
Milk protein (%)	1.57 ± 0.18	1.54 ± 0.12	0.6946
Milk lactose (%)	6.53 ± 0.29	6.64 ± 0.08	0.4132
Solids (%)	9.18 ± 0.34	9.23 ± 0.32	0.8009
Solids-not-fat (%)	8.87 ± 0.17	8.96 ± 0.16	0.3636
Urea nitrogen (mg/dL)	13.51 ± 3.31	15.23 ± 2.41	0.3300
Polyunsaturated fatty acid (%)	0.21 ± 0.02	0.20 ± 0.01	0.7976
Lactoferrin (g/L)	0.095 ± 0.0031	0.096 ± 0.0032	0.5593
Daily milk component yield			
Milk fat (g)	3.73 ± 4.61	5.20 ± 5.52	0.6253
Milk protein (g)	17.26 ± 2.93[b]	22.32 ± 4.73[a]	0.0499
Milk lactose (g)	72.01 ± 13.04[b]	96.18 ± 17.62[a]	0.0223
Solids (g)	101.13 ± 16.93[b]	134.44 ± 29.01[a]	0.0355
Solids-not-fat (g)	97.62 ± 15.87[b]	129.84 ± 24.05[a]	0.0208
Polyunsaturated fatty acid (g)	2.28 ± 0.43	2.98 ± 0.67	0.0578
Lactoferrin (mg)	104.69 ± 16.93[b]	140.03 ± 29.86[a]	0.0302

[a,b]Means within a row with different superscripts were significant ($p < 0.05$).

The other data were analyzed by *t*-test in GraphPad Prism 6 software. The results were shown as mean ± SD. The significant difference was defined when $p < 0.05$.

3 Results

3.1 Effects of FCPR on milk performance of lactating donkeys

The FCPR had no effect on the average daily feed intake of lactating donkeys, and had significant impacts on donkey milk yield, foals' weight gain, and milk component yield (Table 2). The donkey daily milk yield and foals' weight gain in FF group were significantly higher than that in the C group ($p < 0.05$). Compared with the control group, the weight gain of lactating donkeys in the FF group showed an increasing trend ($p = 0.0909$). As for milk composition, there was no difference between the two groups. However, except for milk fat and polyunsaturated fatty acid, the yield of other milk components such as protein, lactose, solids, solids-not-fat, and lactoferrin in the FF group was significantly higher than that in the control group ($p < 0.05$).

3.2 Effects of FCPR on milk metabolites

The data of milk metabolites from donkeys with or without FCPR were analyzed using PLS-DA. Figure 1A displayed the PLS-DA score plot of milk metabolites from groups C and FF. Figures 1B,C showed the volcano plots and heatmap of differential metabolites in different groups of donkey milk. A total of 568 metabolites were detected in donkey milk, including 21 differential metabolites, of which 13 were up-regulated and 8 were down-regulated. The differential metabolites were displayed in Table 3 ($p < 0.05$). The main metabolic pathways associated with the differential metabolites and the metabolic pathways classification were shown as Figure 1D. Compared to group C, the significantly up-regulated metabolic pathway in the FF group was renin secretion ($p < 0.05$). And the metabolic pathways of arginine and proline metabolism ($p = 0.0735$), adrenergic signaling in cardiomyocytes ($p = 0.0804$), and regulation of lipolysis in adipocytes ($p = 0.0804$) showed an increasing trend.

3.3 Effects of FCPR on blood biochemical parameters

The changes in blood biochemical parameters of lactating donkeys were shown in Table 4. The addition of FCPR to the diet of lactating donkeys significantly increased the blood urea and C4 concentration and activity of T-AOC, CAT ($p < 0.05$). And the concentration of MDA of group FF was significantly lower than group C ($p < 0.01$). Compared with the control group, the activity of SOD in the FF group showed an increasing trend ($p = 0.0885$). The other detected blood biochemical parameters were not affected by FCPR addition.

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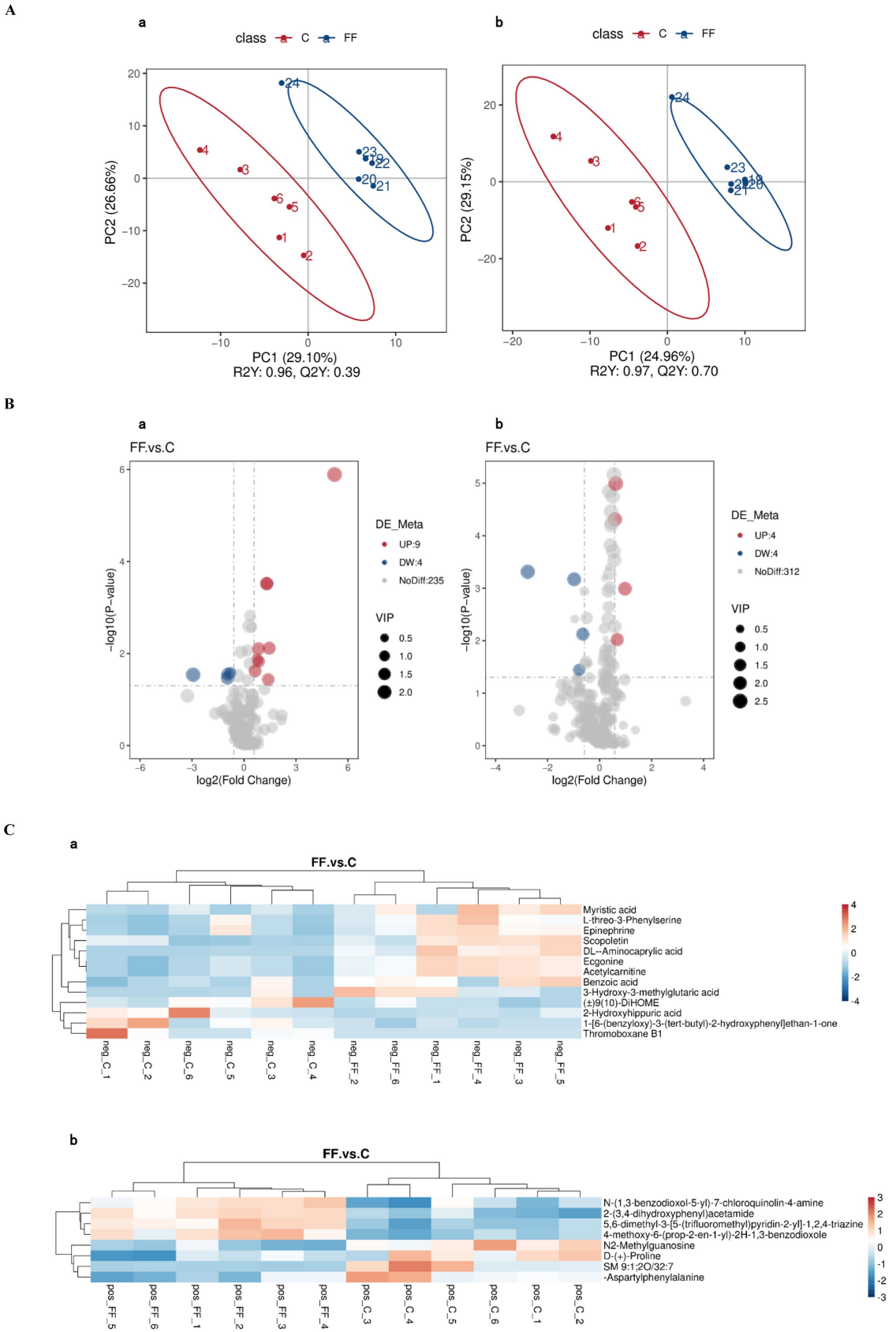

FIGURE 1 (Continued)

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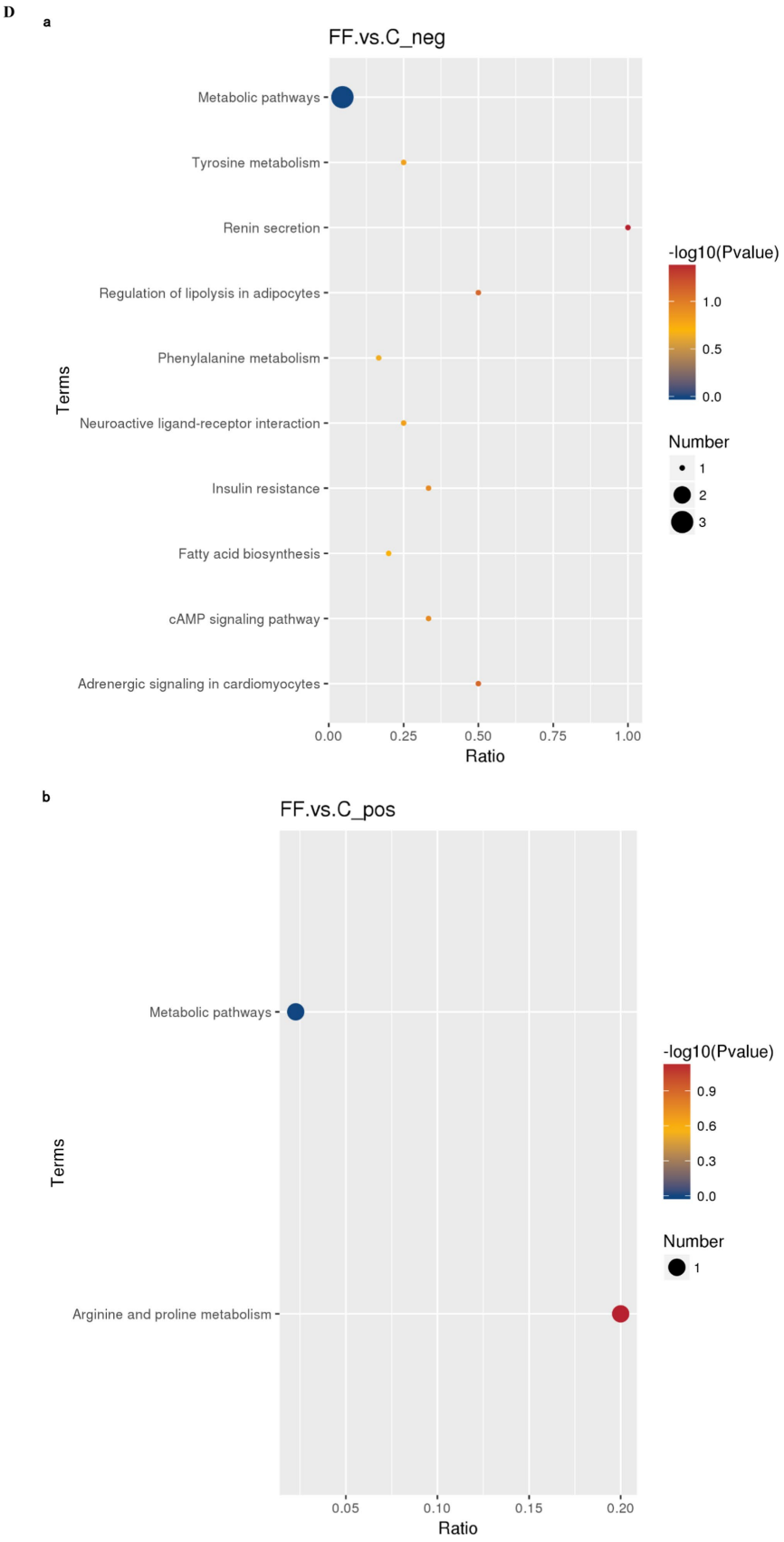

FIGURE 1
The PLS-DA analysis **(A)** of milk metabolites and volcano plots **(B)**, heatmap **(C)**, and the involved metabolic pathways **(D)** of donkey milk differential metabolites between group FF and group C. a (b): the negative (positive) ion mode. **(A)** The horizontal axis PC1 and vertical axis PC2 represent the scores of the principal components ranked first and second, respectively. Different colored dots represent samples from different groups. Ellipses represent a 95% confidence interval. **(B)** FC=fold change. **(C)** Red indicates functions with relatively high values (0–4/3), while blue represents a relatively low value [0-(–4)/(–3)]. The milk samples were taken from 12 lactating donkeys with (FF) or without fermented *Codonopsis pilosula* residue addition (C).

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TABLE 3 Differential metabolites of donkey milk between group FF and group C.

Regulated type	Name	Formula	Molecular weight	RT[a] (min)	FC[b]
Up-regulated	DL-α-Aminocaprylic acid	C8 H17 NO2	159.1264	5.604	37.4205
	Scopoletin	C10 H8 O4	192.0427	5.25	2.74600
	3-Hydroxy-3-methylglutaric acid	C6 H10 O5	162.0531	1.397	2.6382
	Ecgonine	C9 H15 N O3	185.1056	5.754	2.4805
	Acetylcarnitine	C9 H17 N O4	203.1161	5.756	2.4805
	2-(3,4-dihydroxyphenyl)acetamide	C8 H9 N O3	167.0593	1.962	1.9689
	Myristic acid	C14 H28 O2	228.2098	0.228	1.8147
	L-threo-3-Phenylserine	C9 H11 N O3	181.0743	5.519	1.7938
	Epinephrine	C9 H13 N O3	183.0899	5.663	1.7033
	N-(1,3-benzodioxol-5-yl)-7-chloroquinolin-4-amine	C16 H11 Cl N2 O2	298.047	1.284	1.6033
	Benzoic acid	C7 H6 O2	122.0372	5.95	1.5601
	5,6-dimethyl-3-[5-(trifluoromethyl)pyridin-2-yl]-1,2,4-triazine	C11 H9 F3 N4	254.0793	3.137	1.5389
	4-methoxy-6-(prop-2-en-1-yl)-2H-1,3-benzodioxole	C11 H12 O3	192.0805	8.257	1.5104
Down-regulated	Thromoboxane B1	C20 H36 O6	408.2313	9.406	0.1309
	SM 9:1;2O/32:7	C46 H79 N2 O6 P	786.5745	10.126	0.1472
	N2-Methylguanosine	C11 H15 N5 O5	297.1088	4.808	0.5076
	2-Hydroxyhippuric acid	C9 H9 N O4	195.0536	5.008	0.5161
	(±)9(10)-DiHOME	C18 H34 O4	314.2462	10.745	0.5236
	D-(+)-Proline	C5 H9 N O2	115.0641	1.446	0.5793
	1-[6-(benzyloxy)-3-(tert-butyl)-2-hydroxyphenyl]ethan-1-one	C19 H22 O3	298.1611	8.605	0.5780
	α-Aspartylphenylalanine	C13 H16 N2 O5	280.1065	5.348	0.6387

[a]RT, retention time.
[b]FC, fold change, mean value of peak area obtained from group FF/mean value of peak area obtained from group C.

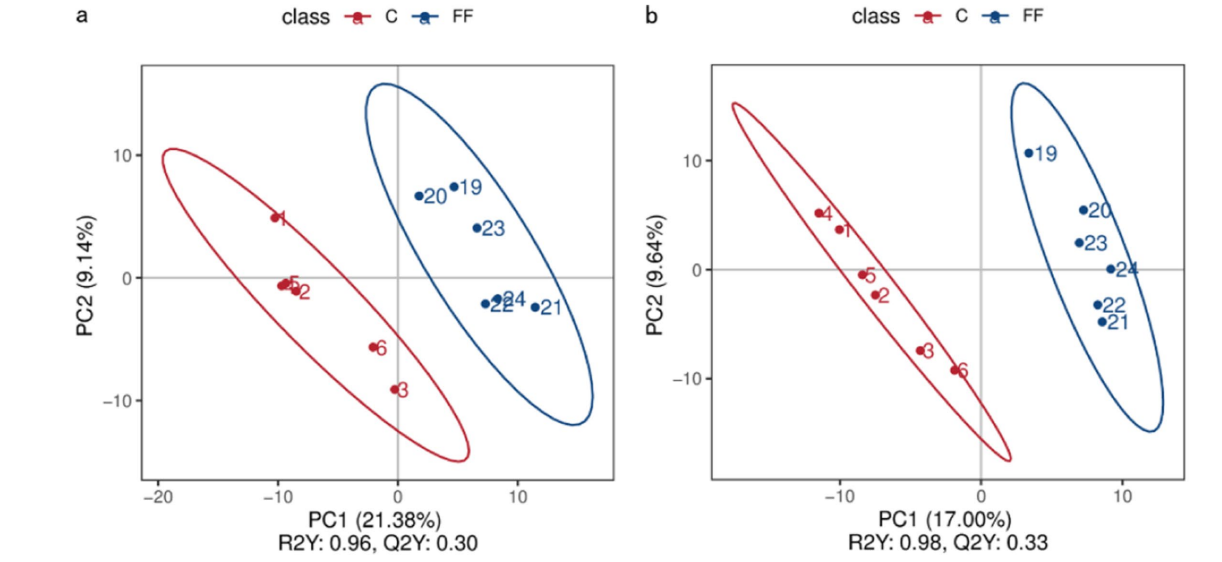
TABLE 4 Blood biochemical parameters analysis between group FF and group C.

Items	C	FF	P
TP (g/L)	62.91 ± 5.94	58.71 ± 3.48	0.1660
ALB (g/L)	23.43 ± 1.76	23.33 ± 1.40	0.9166
TG (mmol/L)	0.17 ± 0.07	0.14 ± 0.05	0.4261
Urea (mmol/L)	7.69 ± 0.32[b]	8.53 ± 0.75[a]	0.0299
GLU (mmol/L)	4.29 ± 0.60	4.37 ± 0.39	0.7783
AST (U/L)	270.55 ± 21.05	298.35 ± 42.88	0.1844
ALT (U/L)	6.41 ± 3.16	9.81 ± 8.03	0.3568
LDH (U/L)	293.02 ± 71.06	284.55 ± 45.94	0.8114
ALP (U/L)	18.64 ± 21.69	13.229 ± 8.71	0.5825
C3 (g/L)	1.34 ± 0.51	1.49 ± 0.65	0.6557
C4 (g/L)	0.39 ± 0.06[b]	0.71 ± 0.19[a]	0.0030
IgA (g/L)	2.03 ± 0.38	2.10 ± 0.19	0.7233
IgG (g/L)	19.19 ± 2.46	19.87 ± 1.95	0.6035
IgM (g/L)	2.90 ± 0.55	2.93 ± 0.49	0.9434
SOD (U/mL)	72.64 ± 12.00	85.45 ± 11.52	0.0885
GSH-Px (U/mL)	182.95 ± 27.00	207.06 ± 45.60	0.2910
T-AOC (U/mL)	8.83 ± 0.33[b]	10.04 ± 0.34[a]	< 0.0001
CAT (U/mL)	33.59 ± 7.22[b]	66.56 ± 12.11[a]	0.0002
MDA (nmol/mL)	4.05 ± 0.67[a]	3.16 ± 0.40[b]	0.0191

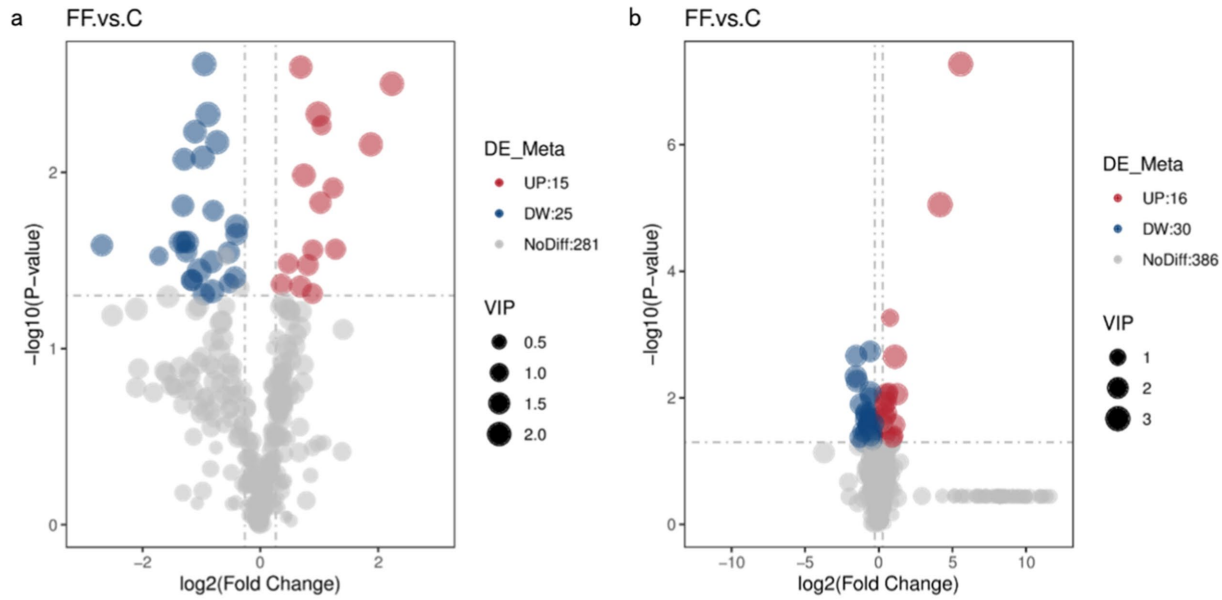
[a,b]Means within a row with different superscripts were significant ($P < 0.05$).

3.4 Effects of FCPR on blood metabolites

The data of serum metabolites from donkeys with or without FCPR were analyzed using PLS-DA. Figure 2A displayed the PLS-DA score plot of serum metabolites from groups C and FF. Figures 2B,C showed the volcano plots and heatmap of differential metabolites in different groups of donkey serum. A total of 753 metabolites were detected in donkey serum, including 86 differential metabolites, of which 31 were up-regulated and 55 were down-regulated. Some differential metabolites were shown in Table 5 (FC > 2, $p < 0.05$). The main metabolic pathways associated with the differential metabolites and the metabolic pathways classification were shown as Figure 2D. Compared to group C, the significantly up-regulated metabolic pathways in the FF group included renin secretion, cAMP signaling pathway, regulation of lipolysis in adipocytes, and fatty acid biosynthesis ($p < 0.05$). And the metabolic pathways of fatty acid elongation, fatty acid degradation, riboflavin metabolism, vitamin B6 metabolism, fatty acid metabolism, and adrenergic signaling in cardiomyocytes showed an increasing trend ($p = 0.0885$).



A



B

FIGURE 2(Continued)

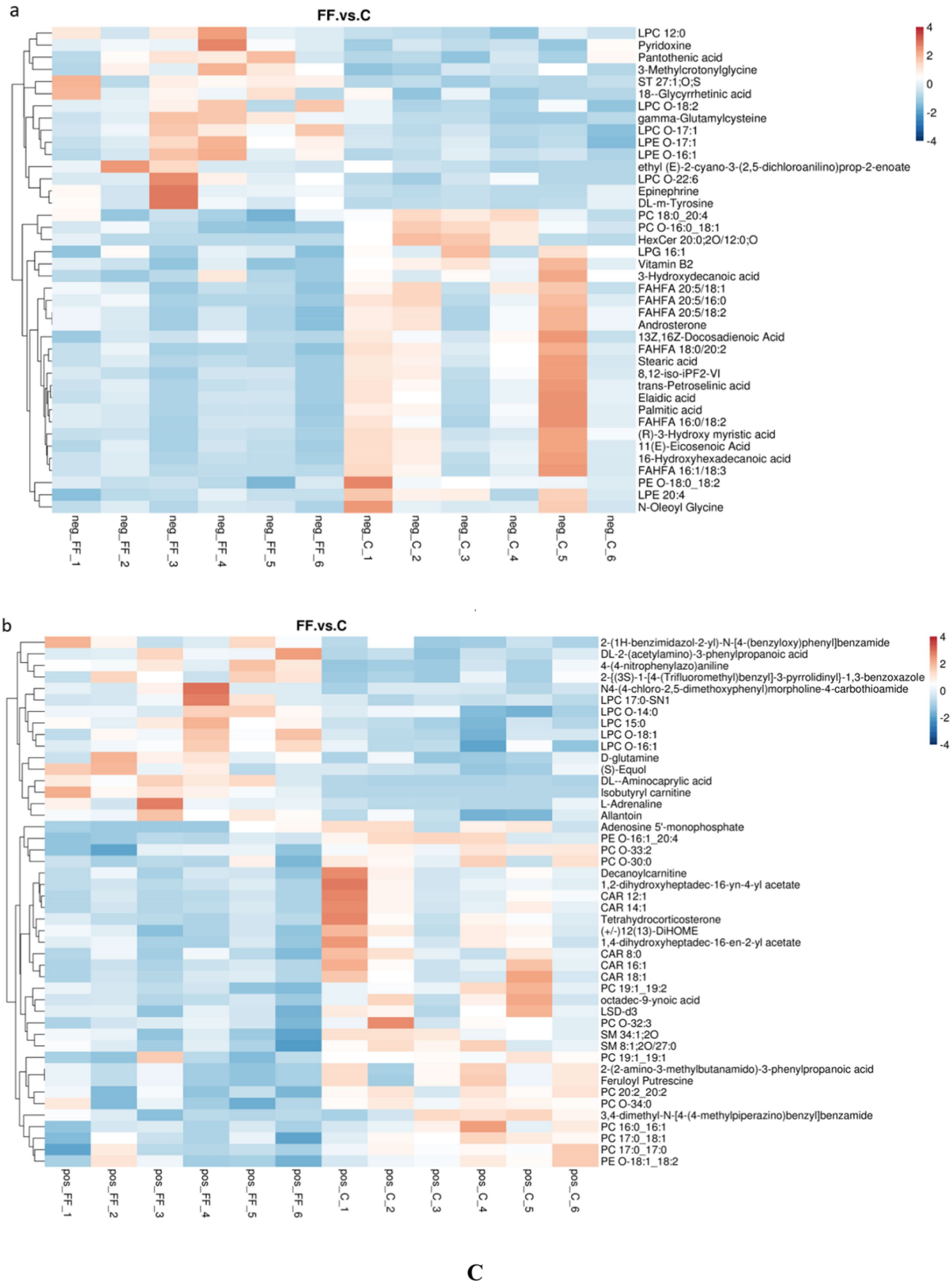


FIGURE 2 (Continued)

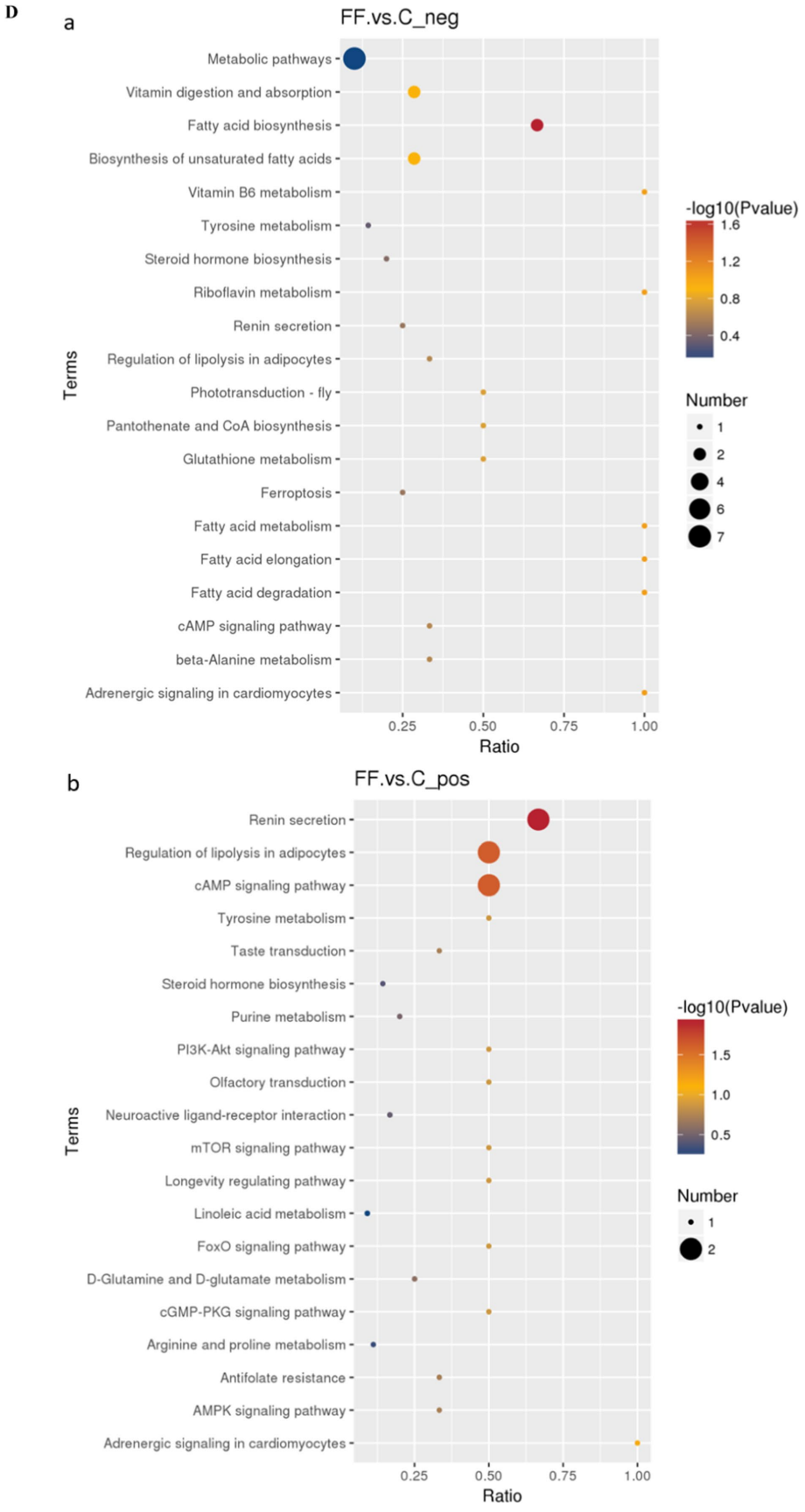


FIGURE 2
The PLS-DA analysis **(A)** of serum metabolites and volcano plots **(B)**, heatmap **(C)**, and the involved metabolic pathways **(D)** of donkey serum differential metabolites between group FF and group C. a (b): the negative (positive) ion mode. **(A)** The horizontal axis PC1 and vertical axis PC2 represent the scores of the principal components ranked first and second, respectively. Different colored dots represent samples from different groups. Ellipses represent a 95% confidence interval. **(B)** FC=fold change. **(C)** Red indicates functions with relatively high values (0–4), while blue represents a relatively low value [0-(−4)]. The serum samples were taken from 12 lactating donkeys with (FF) or without fermented *Codonopsis pilosula* residue addition (C).

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TABLE 5 Differential metabolites of donkey serum between group FF and group C.

Regulated type	Name	Formula	Molecular weight	RT[a] (min)	FC[b]
Up-regulated	DL-α-Aminocaprylic acid	C8 H17 N O2	159.1264	5.621	47.0596
	Isobutyryl carnitine	C11 H21 N O4	231.1477	5.443	17.8705
	Epinephrine	C9 H13 N O3	183.0897	5.662	4.6961
	DL-m-Tyrosine	C9 H11 N O3	181.0738	5.5	3.6673
	Ethyl (E)-2-cyano-3-(2,5-dichloroanilino)prop-2-enoate	C12 H10 Cl2 N2 O2	284.0142	11.158	2.4192
	L-Adrenaline	C9 H13 N O3	183.0901	5.673	2.3813
	LPC O-22:6	C30 H52 N O6 P	599.3502	7.279	2.3527
	4-(4-nitrophenylazo)aniline	C12 H10 N4 O2	242.0774	1.228	2.1387
	2-{(3S)-1-[4-(Trifluoromethyl)benzyl]-3-pyrrolidinyl}-1,3-benzoxazole	C19 H17 F3 N2 O	346.125	1.26	2.0916
	Gamma-Glutamylcysteine	C8 H14 N2 O5 S	250.0631	6.24	2.0527
	LPC 12:0	C20 H42 N O7 P	485.2761	7.845	2.0275
Down-regulated	PC O-33:2	C41 H80 N O7 P	747.5795	10.437	0.6613
	PC O-30:0	C38 H78 N O7 P	691.5531	10.59	0.6588
	PC 16:0_16:1	C40 H78 N O8 P	731.5447	8.884	0.6457
	SM 34:1;2O	C39 H79 N2 O6 P	724.5518	11.165	0.6119
	(+/−)12(13)-DiHOME	C18 H34 O4	296.2358	8.235	0.6057
	Stearic acid	C18 H36 O2	284.2716	11.158	0.6021
	CAR 8:0	C15 H30 N O4	287.2102	6.156	0.5995
	8,12-iso-iPF2α-VI	C20 H34 O5	336.228	10.129	0.5751
	LPG 16:1	C22 H43 O9 P	482.2649	8.81	0.5687
	Palmitic acid	C16 H32 O2	256.2403	10.337	0.5646
	PC 20:2_20:2	C48 H88 N O8 P	837.6267	11.465	0.5503
	Tetrahydrocorticosterone	C21 H34 O4	385.2837	7.16	0.5450
	Vitamin B2	C17 H20 N4 O6	376.1397	9.525	0.5406
	1,4-dihydroxyheptadec-16-en-2-yl acetate	C19 H36 O4	328.2615	8.211	0.5333
	Decanoylcarnitine	C17 H33 N O4	315.2414	6.688	0.5294
	FAHFA 18:0/20:2	C38 H70 O4	590.5259	11.158	0.5177
	FAHFA 16:0/18:2	C34 H62 O4	534.4628	10.337	0.5154
	11(E)-Eicosenoic Acid	C20 H38 O2	310.2875	11.333	0.5086
	PC O-34:0	C42 H86 N O7 P	747.6094	10.301	0.4911
	PE O-18:0_18:2	C41 H80 N O7 P	729.5662	8.866	0.4874
	CAR 18:1	C25 H48 N O4	425.3514	8.546	0.4704
	(R)-3-Hydroxy myristic acid	C14 H28 O3	244.2039	7.45	0.4642
	FAHFA 20:5/18:2	C38 H60 O4	580.45	9.505	0.4472
	Androsterone	C19 H30 O2	290.2249	9.503	0.4472
	1,2-dihydroxyheptadec-16-yn-4-yl acetate	C19 H34 O4	343.273	7.24	0.4283
	Trans-Petroselinic acid	C18 H34 O2	328.2616	10.525	0.4254
	Elaidic acid	C18 H34 O2	282.2558	10.528	0.4192
	Adenosine 5′-monophosphate	C10 H14 N5 O7 P	347.0635	1.397	0.4103
	N-Oleoyl Glycine	C20 H37 N O3	339.2777	9.651	0.4080
	PC O-16:0_18:1	C42 H84 N O7 P	791.6055	9.904	0.4079
	16-Hydroxyhexadecanoic acid	C16 H32 O3	254.2247	9.724	0.4030
	FAHFA 20:5/16:0	C36 H60 O4	556.4495	9.741	0.3890
	CAR 16:1	C23 H44 N O4	397.3199	7.974	0.3588

(Continued)

TABLE 5 (Continued)

Regulated type	Name	Formula	Molecular weight	RT[a] (min)	FC[b]
	CAR 12:1	C19 H36 N O4	341.257	6.931	0.3470
	CAR 14:1	C21 H40 N O4	387.2991	7.471	0.3405
	HexCer 20:0;2O/12:0;O	C38 H75 N O9	749.5789	8.619	0.3035
	FAHFA 16:1/18:3	C34 H58 O4	530.4317	9.722	0.1550

[a]RT, retention time.
[b]FC, fold change, mean value of peak area obtained from group FF/mean value of peak area obtained from group C.

4 Discussion

Some studies have shown that adding FCHM to the diet can improve animal production performance. It was found that addition of FCHM improved the growth performance of weaned piglets (7). Addition of FHTR improved the rumen fermentation, and thus affected serum indicators and meat quality of black goats (22). The results of Zhuang et al. (10) showed that FHTR significantly increased the daily feed intake and daily gain of fattening cattle. In study of Shan et al. (23), the effects of dietary FCHM supplementation on milk performance were investigated, and the results found that FCHM improved the milk yield, milk fat and protein content, and immune function of dairy cows under heat stress conditions. In this study, the addition of FCPR significantly increased the donkeys' milk yield, weight gain of foals, and the milk components (protein, lactose, solids, solids-not-fat, and lactoferrin) yield ($p < 0.05$). And FCPR also changed the metabolites in donkey milk. A total of 21 differential metabolites in donkey milk were detected between FF and C groups. Compared to group C, the significantly up-regulated metabolic pathway in the FF group was renin secretion. The composition of donkey milk is influenced by various factors such as breed, lactation stage, season, and dietary composition (24–26). In this study, the milk was from 12 healthy multiparous lactating Dezhou donkeys with the similar age, weight, and lactation stage. In addition, except for FCPR, lactating donkeys were fed with the same diet. Thus, the above results indicated that FCPR improved the lactation performance of female donkeys and altered the metabolites of donkey milk. Our results were consistent with the previous conclusion.

It has been found that *Codonopsis pilosula* has anti-inflammatory, antioxidant, and immune regulation biological activities (13, 15). Sun and Liu (27) investigated the immunity activity of CPPs, and the results found that it can stimulated lymphocyte proliferation in a dose-dependent manner. The CPPs exerted antitumor activity by enhancing immune system function (28). Results of Fan et al. (29) showed that *Codonopsis pilosula* glucofructan (CPG) improved both the humoral and cellular immunity and effectively inhibited tumor growth in mice. CPG could enhance immunoregulatory function and can be used as a functional food for humans (30). In this study, FCPR exhibited similar immunomodulatory biological activity. Addition of FCPR improved the immune function of lactating donkeys by increasing the level of C4 in the blood, thereby enhancing their milk production performance.

Furthermore, many studies have also shown *Codonopsis pilosula* has antioxidant activity. *Codonopsis pilosula* extract significantly alleviated mitochondrial damage and activated autophagy, thereby improving hippocampal tissue damage and alleviating cognitive impairment in aging mice (31). CPPs significantly reduced serum blood glucose, insulin level and MDA content increased SOD activity, and significantly improved insulin resistance of diabetes mice (32). In study of Liu et al. (33), the polysaccharides increased the activities of total SOD and GSH-Px, and decreased the level of MDA, and thereby increased the BW of red swamp crayfish. The neutral polysaccharide (CPP-1) could protect cells from oxidative damage (increasing the activity of SOD and CAT and reducing the MDA content), reduce the body fat index of nonalcoholic fatty liver disease mice, and improve the liver function (34). Results of other studies also found CPPs exerts a protective effect by enhancing antioxidant activity (increasing GSH-Px, SOD, and T-AOC activity and reducing MDA levels), regulating the dynamic balance of NO and endothelin-1, and significantly improving the survival rate of cells treated with hydrogen peroxide (35–37). The SOD, GSH Px, and CAT are antioxidant enzymes that catalyze the conversion of free superoxide anion radicals into non-toxic compounds, which are crucial for reducing oxidative stress and maintaining animal health (38). In this study, the addition of FCPR to the diet of lactating donkeys significantly increased the concentrations of T-AOC and CAT in the blood, while reducing the concentration of MDA ($p < 0.01$). Our findings were consistent with the conclusions of other studies. Our results indicated that FCPR improved the milk performance of lactating donkeys by enhancing their blood antioxidant capacity.

In addition, the blood metabolites of lactating donkeys were also detected in this study. A total of 753 metabolites were detected in donkey serum, including 86 differential metabolites, of which 31 were up-regulated and 55 were down-regulated. The main up-regulated differential metabolites including DL-α-aminocaprylic acid, isobutyryl carnitine, epinephrine, DL-m-tyrosine, ethyl (E)-2-cyano-3-(2,5-dichloroanilino)prop-2-enoate, L-Adrenaline, LPC O-22:6, 4-(4-nitrophenylazo) aniline, 2-{(3S)-1-[4-(trifluoromethyl) benzyl]-3-pyrrolidinyl}-1,3-benzoxazole, gamma-glutamylcysteine, and LPC 12:0 (FC > 2, $p < 0.05$). The differential metabolic pathways associated with these differential metabolites were renin secretion, cAMP signaling pathway, regulation of lipolysis in adipocytes, and fatty acid biosynthesis ($p < 0.05$). The above metabolic pathways were mainly related to glucose metabolism and lipid metabolism. It has been found that CPPs improved the lipid metabolism and glucose metabolism impairment, and reduce insulin resistance of obesity mice, and has the potential for the treatment of obesity (39). Bai et al. (40) investigated the effects of *Codonopsis pilosula* oligosaccharides (CPO) supplementation on obesity, and the results found that CPO decreased body weight and fat accumulation and improved glucose tolerance in high-fat diet induced obese mice. The results of Hu et al. (41) also found that *Codonopsis pilosula* polyynes participated in lipid

metabolism by inhibiting the expression of squalene monooxygenase gene, which may be used in treatment of hypercholesterolemia and atherosclerosis. Liu et al. (17) also found that the polysaccharide extracted from *Codonopsis pilosula* residue improved the glucose metabolism by alleviating oxidative stress, improving lipid metabolism, and increasing glycolytic enzymes activity. Therefore, in this study, FCPR may enhance the milk performance of lactating donkeys by improving glucose and lipid metabolism and enhancing energy utilization efficiency.

In summary, the addition of FCPR significantly increased the daily milk yield, the yield of milk protein, lactose, solids, solids-not-fat, and lactoferrin, as well as the foals' weight gain. A total of 21 differential metabolites between FF and C groups, which were related to global and overview maps, amino acid metabolism, and endocrine system metabolic pathways. The addition of FCPR also significantly increased the concentrations of urea, C4, T-AOC, and CAT in the blood, while reducing the concentration of MDA. And 86 differential metabolites were detected between FF and C groups, which were associated with renin secretion, cAMP signaling pathway, regulation of lipolysis in adipocytes, and fatty acid biosynthesis. In conclusion, FCPR may enhance the milk performance of lactating donkeys by activating the immune system, increasing the antioxidant capacity, and improving the glucose and lipid metabolism. These results provide a foundation for the development and utilization of FCPR additives, which are beneficial for livestock production and improve animal welfare.

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 approved by the Animal Care and Use Committee of Liaocheng University (Shandong, China) (2022111002). The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

MZ: Conceptualization, Methodology, Writing – original draft. FH: Investigation, Visualization, Writing – review & editing. XD: Investigation, Visualization, Writing – review & editing. GL: Funding acquisition, Methodology, Supervision, Writing – review & editing. CW: Funding acquisition, Supervision, Writing – review & editing.

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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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Role of trefoil factors in maintaining gut health in food animals

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It is imperative to preserve the integrity of the gastrointestinal system in spite of the persistent existence of harmful chemicals and microbial flora in the gut. This is made possible by essential healing initiators called Trefoil factors which helps in mucosal reconstitution and tissue development on the gastrointestinal surface. The trefoil factors are a class of abundant secreted proteins that are essential for epithelial continuity (TFFs). Trefoil factor family (TFF) proteins are biologically active peptides that play significant role in safeguarding, restoring and continuity of the gastrointestinal tract (GIT) epithelium, through collaborative modulations with mucins in the mucosal layer. These peptides are readily produced in reaction to epithelial damage in the digestive tract, thereby contributing to the healing and restituting of the epithelial layers of the intestine. In addition, considerable evidence indicated that TFF peptides trigger proliferation, migration and angiogenesis, all which are crucial processes for wound healing. There is also increasing evidence that TFF peptides modulate the mucosal immune system. These protective properties, suggest that dietary manipulation strategies targeted at enhancing the expression and synthesis of TFF peptides at optimal levels in the GIT epithelium, may constitute a plausible alternative strategy to the use of in-feed antibiotic growth promoters to maintain epithelial integrity and promote resistance to enteric pathogens. This review describes TFF peptides, with importance to their biological functions and involvement in gastrointestinal mucosal protection and repair in food animals.

KEYWORDS

trefoil factor family, peptides, gastrointestinal mucosal injury, mucins, epithelial repair

1 Introduction

The gastrointestinal tract is a complex environment that assembles myriad of components such as peptides, cellular matters, pathogens, microorganisms and nutritional biomaterials. The presence and interaction of these components determines the outcome put up against threat or challenge to the system (1). The GIT harbors immune cells and molecules that grants immunity and biological responses to pathogens and toxins made possible to protect the lubricated epithelial surface of the GIT. Consistent with these functions, it's interesting to note that TFFs are mostly expressed in the healthy gastrointestinal (GI) tract (2), thus it makes sense that they would either positively or negatively correlate with stomach disorders. The peptides known as the trefoil factor family (TFF1, TFF2, and TFF3) are essential for the upkeep, preservation, and repair of the gastrointestinal system.

Intestinal trefoil factor (ITF) is an important member of TFF domain peptides with pivotal roles in the protection of the intestinal epithelium (1, 3, 4). There are three secretory proteins (12-22KD) that make up TFF, and are often produced in adequate quantity in the tissues containing cells that secret mucus (3). TFF proteins are readily produced in reaction to epithelial damage in the digestive tract (1, 4) thereby contributing to the healing of the gastrointestinal epithelium (5). When an injury occurs to the epithelium, TFF are produced close to the injury site and migrate to the site by a process that causes a rapid change in the shape and volume of the cells, thereby allowing free flow of water in and out of the cells (6). There are three known mammalian Trefoil Factors namely TFF1 or the pS2 (7), pancreatic spasmolytic polypeptide TFF2 (8), and TFF3 (or ITF) (1, 9). The distribution of TFF cut across numerous tissues such as Liver, Pancreas, Kidney, brain salivary gland as well as the respiratory tract (10). The isomers of TFF are found in the gastrointestinal tracts with variation in distributions (10). The TFF1 is a 9-KDa mucin-associated secretory protein primarily expressed in the gastrointestinal (GI) tract mucosa, and is characterized by a 38- to 39-amino-acid trefoil domain which contain six cysteine residues that form a cloverleaf disulfide structure (11). This structure renders TFF1 resistant to both acid and enzymatic breakdown, and thus perhaps responsible for the ability of TFF1 to inhibit tumor growth in the gastrointestinal tract (GIT) (12). TFF1 exerts its protective action by stimulating the restitution of the tissues destroyed as a result of inflammation and ulceration (12). Pancreatic spasmolytic polypeptide TFF2 is secreted by the antral and pyloric region of the stomach, and the Brunner's glands of the duodenum (13). In a healthy animal, there is an interaction between TFF2 and mucins that often lead to development and maintenance of the mucus barrier in the gastric and duodenal sites (14). At the site of an injury, TFF2 can be rapidly expressed in the epithelia of the entire GIT and critically contribute to epithelial "restitution" and regenerative processes (9). Furthermore, TFF could modulate immune responses, cell chemotaxis, and cytokine release (15). The gastrointestinal mucosa is the primary site for TFF3 secretion (16). From this site, TFF3 is excreted from the granules with mucins onto the surface of the epithelium, thereby forming a protective mucosal layer. The mechanism of action of TFF3 is such that it stimulates the activation of extracellular signal-regulated kinase/mitogen-activated protein kinase and activates serine phosphorylation of Akt, a kinase associated with apoptotic pathways (17, 18), thereby modulating the E-cadherin/catenin cell adhesion complex in various ways. For instance, TFF3 peptide caused reduction in the level of E-cadherin, β-catenin, α-catenin and the adenomatous polyposis coli (APC) protein in HT-29 cells in, consequently resulting in significant alterations in cell aggregation, detachment from the substratum, and translocation of APC from the cytoplasm to nucleus (19).

Since TFFs are mostly expressed in the healthy gastrointestinal (GI) tract, there exist correlation, either positive or negative, between them and stomach disorders (20). This review aims to examine the significance and therapeutic potentials of TFF in the gastrointestinal tract health, provide current knowledge on the target factors and steer future research toward their perceived mechanisms and clinical applications in gastrointestinal disease management.

1.1 Trefoil factor (ITF): types, structure, and functions

The process of breaking down and absorbing consumed food and liquids is carried out by the gastrointestinal (GI) system. The intricacy of the GI tracts and complex nature of biological processes that occur with the system, preserving the gastrointestinal tract's integrity and homeostasis is crucial even in the face of constant exposure to microbial flora and other detrimental agents. The gut tissues and epithelium are designed to express polypeptide growth factors capable of stimulating cellular growth, proliferation, or differentiation after attachment to surface membrane receptors (21). These factors include epidermal growth factor (EGF), transforming growth factor beta (TGF-β), insulin-like growth factor (IGF), hepatocyte growth factor (HGF), fibroblast growth factor (FGF), trefoil factor, wingless (Wnt) family, and Hedgehog (Hh) family proteins (22). The latter three has been identified over the past few decades and discovered to play mitogenic roles in the development and adult function of the GI tract in animals. TFF peptide is discovered in both serum and luminal fluid of the GIT. TFF has been reported to be expressed in a cell-specific manner on the mucosal surfaces of normal gastrointestinal tissues (21). ITF proteins have been demonstrated to have important functions after mucosal damage by improving superficial cell resistance, lowering epithelial cell permeability, strengthening the cell-to-cell connection in intestinal mucosal injury, suppressing apoptosis and inflammatory signaling (2).

Additionally, some research has shown that ITF reduces the inflammatory response by preventing the digestive tract's production of pro-inflammatory factors (23, 24). These small molecular peptides factors called TFF are produced by the goblet cells scattered across the absorptive cells onto the gastrointestinal tract's surface. It is believed that mechanism of action of TFF is via luminal secretion of the peptide from the epithelial cells lining the GI tract, suggestive that systemic TFF peptides can be secreted into the gastric lumen in tandem with mucus secretion from mucous cells (10). The concept of growth factor started in the 80/s with the identification of spasmolytic protein SP (25) and pS2 (7). The advancement in knowledge and efforts over subsequent decades in the identification of some cell division activities, proteins classified trefoil (TFF) and subsequent of a third ITF in mammals (9). The pS2, spasmolytic peptide, and intestinal trefoil factor which share a distinct motif of six cysteine residues defined as "trefoil" domain, were renamed with the current nomenclature of TFF1, TFF2 and TFF3, respectively, in 1997 at the Philippe Laudat Conference

Abbreviations: TFF, Trefoil factor family; GIT, gastrointestinal tract; ITF, Intestinal trefoil factor; PI3K/Akt, phosphatidylinositol 3′-kinase; TGF-α, transforming growth factor-alpha; GKN2, gastrokine; TFF1, Trefoil Factor 1; TFF2, Trefoil Factor 2; TFF3, Trefoil factor 3; COX2, cyclooxygenase; IL-6, Interleukin-6; TNF-α, tumor necrosis factor-α; NF-κB, Nuclear factor-kappaB; IFABP, intestinal fatty acid binding protein; LFABP, liver fatty acid binding protein; IAP, Intestinal alkaline phosphatase.

(26, 27). The protein data bank (PDB) structure of TFF1, TFF2, and TFF3 is shown in Figure 1.

The TFFs are small secretory peptides with three loop structures that contain a highly conserved motif of cysteine disulfide bonds that maintain the functional stability of the protein. Depending on anatomical location, TFF proteins are both constitutively and inducible expressed (28). The trefoil, or P domain, is a conserved 38–39 amino acid motif containing six cysteine residues held together by three pairs of disulfide bonds configured 1–5, 2–4, and 3–6 (28, 29). It is a collection of secreted peptides with a C-terminal dimerization domain and a trefoil domain (s). The TFF2 has two trefoil motifs as a result of chromosomal duplication, whereas TFF1 and TFF3 only have a single trefoil domain (9, 30, 31).

TFF1 is a monomer that consists of a single trefoil domain, which includes about 40 amino acids forming a stable three-loop structure. This domain is characterized by six conserved cysteine residues that form three disulfide bonds, creating a compact and stable structure (26, 29). TFF1 is glycosylated and this influences its protective function in mucosal tissues. TFF2 is a dimer, composed of two trefoil domains, making it structurally different from TFF1 (Figure 1). These domains are also stabilized by three disulfide bonds in each domain and can form dimers through non-covalent interactions, and this dimerization is critical for its biological activity (26) and it is also similarly glycosylated to TFF1. TFF3 is a dimer, much like TFF2, but can also exist in a monomeric form. It contains a single trefoil domain, similar to TFF1, but typically forms dimers through disulfide bonds between two TFF3 monomers (26). The dimerization of TFF3 enhances its mucosal protective properties and like TFF1 and TFF2, TFF3 is also glycosylated.

The cell-specific patterns of occurrence of TFF expression result in a regional colocalization of specific TFF proteins and secretory mucins, providing a potential molecular basis for the functional mucosa specificity. Trefoil factor family peptides are typically co-secreted together with mucins and interact with mucins in the lumen to enhance protective barriers through mucosal innate immune defense, mucosal repair, and prevention of the infiltration of microorganisms and toxin insults (32, 33). TFF proteins and secretory mucins colocalize in certain regions as a result of cell-specific TFF expression patterns, which may provide a mechanistic explanation for the functional mucosa specificity. It is significant to note that expression patterns of TFF proteins vary greatly among infection specific, species and age-related. Early in the development of the embryo, TFF expression takes place, with the gastrointestinal tract expressing all three TFF proteins in unison with individual expressions becoming more restricted resulting in cell-specific patterns in adult (34, 35).

The expression of TFF is modulated by different factors. Expression of TFFs in the gastrointestinal tract is abundant and is second, in weight of protein, only to the mucins. TFF1 is mostly restricted to the pit cells of the stomach, TFF2 to mucous neck cells of the gastric gland, and TFF3 to goblet cells of the small and large intestine (22).

1.1.1 Interactions and regulations with other immune cells

The immune system of the mucosal of the GIT is crucial because it keeps pathogens from gaining entrance into the body system while simultaneously facilitating the transfer of nutrients from the intestinal lumen to the systemic circulation for proper function (1). TFF peptides are major secretory products of mucous epithelia and play a multifunctional role in cytoprotection, apoptosis, and immune response. TFF influence the activity of immune cells and cytokines, helping to regulate inflammation and maintain immune homeostasis in the gut (Figure 2). Trefoil factors play significant role in the homeostasis of the gastrointestinal tract and are expressed rapidly in response to injury and are up-regulated in inflammation and ulceration (3, 21, 22). This perceived protective is achieved through regulations by pro-inflammatory and anti-inflammatory cytokine expressions (32). Cytokines such as IL-1β and IL-6 down-regulate TFF genes expression by transcriptional repression in GI cells; IL-6 represses TFF1, IL-1β and IL-6 inhibit TFF2 and TFF3 transcription, TNF-α (acting via NFκB transcription factor) decreases trefoil expression during inflammation thereby delaying mucosal restoration, and cytoprotection (36) while IL-4 and IL-13 induce TFF2 (37).

Pattern recognition Toll-like receptors (TLRs) is a key player in the initiation of an inflammatory process, TLR2 regulates TFF3 expression in the gut to control terminal GC differentiation, which protects the intestinal mucosa against apoptosis (38). The gut epithelial activating transcription factor 4 (ATF4) plays an important role, as its deficiency results in decreased peptide trefoil factor 3 levels (39). The identification of TFF-interacting proteins presents a potential regulatory mechanism for TFF function in the GI tract.

1.2 Trefoil factor family peptides (TFF1, TFF2, and TFF3)

1.2.1 Trefoil factor 1

TFF1 is mainly expressed in the fundus and antrum of gastric mucous cells but with low expressions in ileum, colon, salivary glands, pancreas and respiratory tract (2). The major site of expression of TFF1 is primarily in the gastric foveolar cells and surface epithelial cells across the entire stomach, and it is also present in the upper ducts of brunner's glands localized in the proximal duodenum, salivary glands and gastric juice (10). TFF1 plays an important role in the number of differentiated cells in the epithelium of the stomach. TFF1 is induced by hypoxia-inducible factor-1 (HIF-1α) pathway resulting in the inhibition of activation of IL6-STAT3 pro-inflammatory signaling axis that stimulate gastric lesions (40). The Tff1 and the gastrokine genes (Gkn1, Gkn2, and Gkn3) are expressed selectively in the duodenum and but at much lower levels when compared with the stomach (41). The subcutaneous administration of TFF1 has been recorded to suppress tumor growth *in vivo*, affirming that TFF1 enters the bloodstream to reach tumor cells, where it functions as a tumor suppressor.

TFF1 help cells to counteract bacteria colonization and the development of a chronic inflammation. TFF 1 induces the following function with respective interactions; MUC2/MUC5AC (protection of the mucosal) (42), Fragment of IgG Binding Protein (FCGBP) (binding of microorganisms) (43) and Gastrokine 2 (GKN2) (antiproliferative and pro-apoptotic and homeostasis) (44).

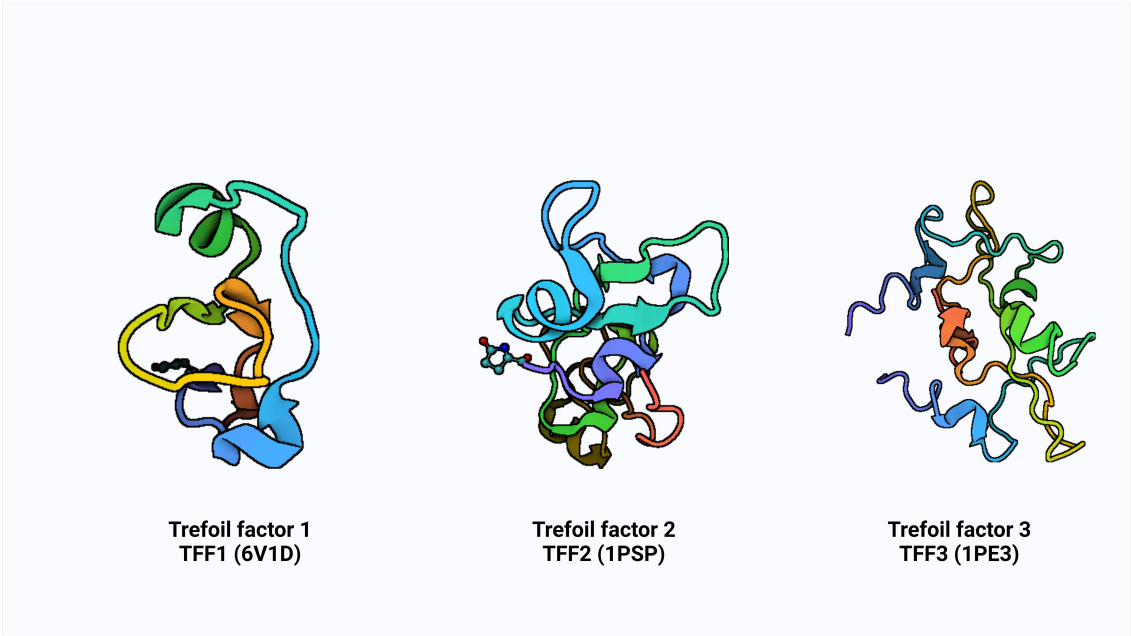

FIGURE 1
The PDB structure of TFF1, TFF2, and TFF3. Created with Biorender.com.

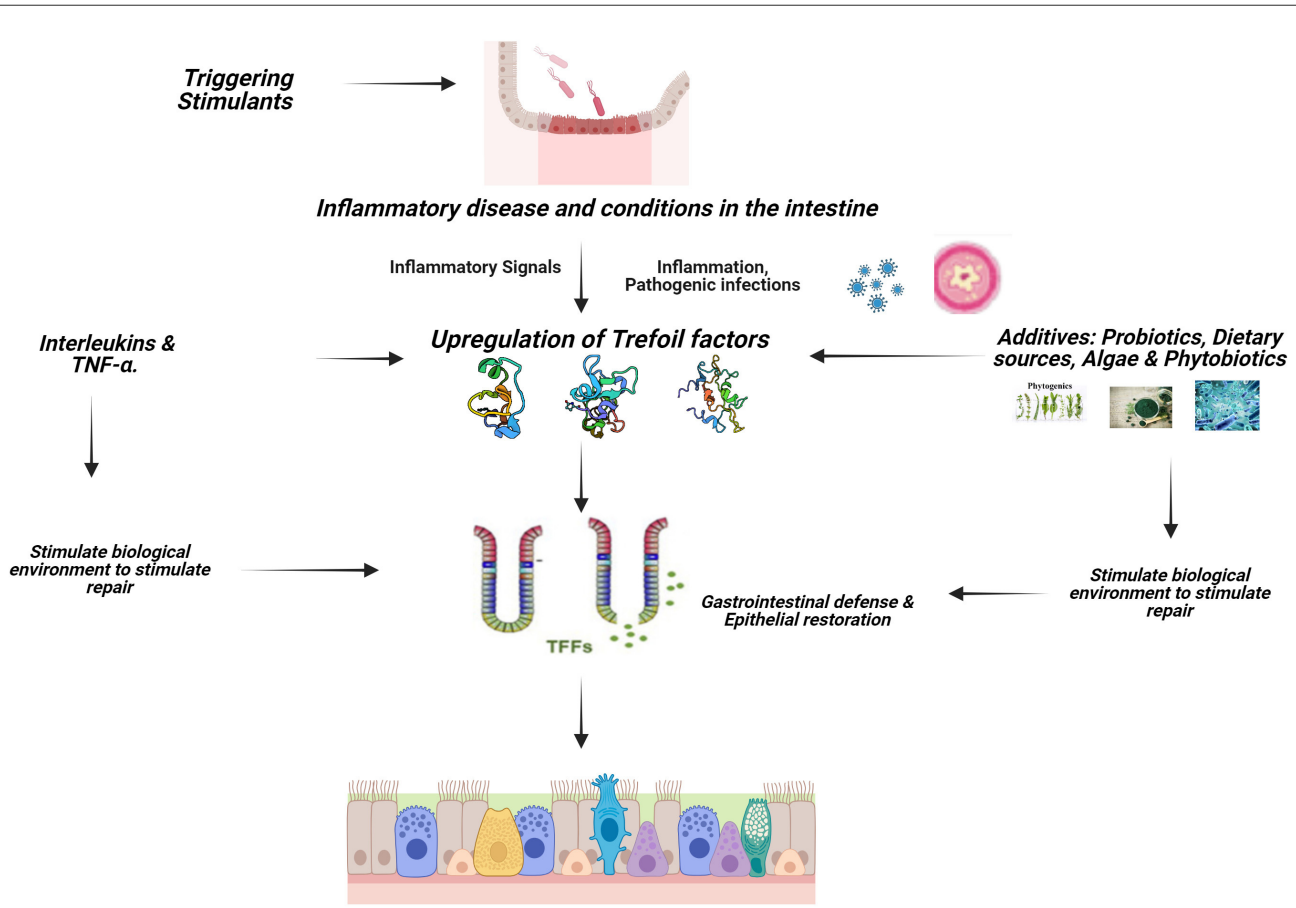

FIGURE 2
Signaling mechanism of TFFs in animals. Created with Biorender.com.

1.2.2 Trefoil factor 2

The spatial localization of TFF2 in the gastrointestinal tract varies among different species and this offers highlights on species-specific expression patterns and the potential functional diversity of TFF genes across species (13). TFF2 peptides expressions have been found in acinar cells of the pancreas, gastric, pyloric and Brunner's glands (Humans and rodents), mucous cells of stomach and throughout the intestine (13, 18). The functional diversity can be increased by the interactions of each trefoil loops with different substrates such as the amino acids. Expressions shift suggests a spatial transition of expression abundance from upper intestine to lower intestine as the gut matures as illustrated in the study in chickens (45). It has been particularly observed that TFF2, when added to a mucin solution, greatly improves the viscosity and flexibility of the mucus, indicating that ITF plays a vital role in repair responses to maintain mucosal surface integrity during pathological processes (46). Trefoil factor-2 is a stable secretory protein expressed in gastrointestinal mucosa responsible for protecting the epithelial layer from insults, stabilizing the mucus layer and promoting healing of the epithelium Two trefoil motifs identified and present in TFF2 is believed to be essential for the protein's proper function as *in vitro* recombinants with a single trefoil domain was discovered to lose its anti-apoptotic properties but continue to stimulate cell migration (45). TFF2 and Fragment of IgG Binding Protein (FCGBP) interaction has been proven to participate in intestinal immunity (mucosal protection). TFF2 binds to the mucin MUC6 thereby stabilizing the inner insoluble gastric mucus barrier layer (47). The interaction of TFF2 and MUC6 also alters the viscoelastic properties of gastric mucous gels thereby contributing to the gastric mucosal innate immune defense.

1.2.3 Trefoil factor 3

Trefoil factor family protein 3 (Tff3) is a small peptide (59 amino acids; 7 kDa) that is a member of the trefoil factor family proteins (Tffs) (48), and it is specifically distributed in the surface of the intestinal mucosa (49). It is known as intestinal trefoil factor and was identified from a rat cDNA sequence in 1991 (50, 51). It is a stable secretory protein expressed in gastrointestinal mucosa, selectively dispersed on the intestinal mucosa's surface (49) and has been discovered to be predominantly expressed by goblet cells of the large and small intestines (50, 52), and also interact with mucins on the apical cell surface (10). TFF3 plays a significant role in mucosal regeneration and repair in intestinal goblet cells, where it is primarily co-secreted or co-expressed alongside MUC2, while it's expression is termed "intestinal". By controlling tight junctions, TFF3 improves the function of the intestinal barrier by reducing the intestinal epithelium's paracellular permeability via regulation of tight junctions. This finding provide insight into the protective functions of TFF3 in epithelial cells and demonstrate its potential for treatment of inflammatory diseases in the GIT (10).

TFF3 suppresses the proliferation and differentiation of activated T cell subsets early during the process of CD4+ T cell differentiation. In recent times TFF3 has been recognized to affect liver metabolism and possible involvement in metabolic pathways through its action in improvement of glucose tolerance in diet-induced obesity model (53). Intestinal trefoil factor 3 (TFF3) protects and repairs the gastrointestinal mucosa and restores normal intestinal permeability, which is dependent on the integrity of the tight junction (TJ) barrier and the TJ associated proteins claudin-1, zona occludens-1 (ZO-1) and occludin (54). Mucus is essential to the gastrointestinal tract's integrity and for protecting epithelial cells from outside stimuli, infections, and mechanical damage (55). TFF3–FCGBP and MUC2 are the major components of intestinal mucus, and they both colocalize in the gut and play a key role in the innate immune defense of mucous epithelia (22). The combination of TFFs and mucin was effective in protecting the barrier function of epithelial cells. TFF3 promotes immunity and wound healing by de-repressing inhibitory LINGO2-EGFR complexes through interactions with LINGO2. TFF3 binds leucine rich repeat receptor and nogo-interacting protein 2 (LINGO2) to de-repress and enhance epidermal growth factor receptor protein (EGFR) signaling that drives wound healing and immunity against helminths (56).

2 Role of trefoil factors in protecting the gastrointestinal epithelium

Trefoil factors represent group of proteins with a crucial involvement in mucosal protection and repair processes. The complex interplay within the mucosal environment resulted in keen interest in understanding their functions in advancing treatments in gastrointestinal disease (57). This section unravels their biological significance and potential therapeutic implications in various animal species with a summary of actions in Table 1.

2.1 Trefoil factors on the gut health of pigs/piglets

Intestinal epithelial cells form the basic unit of the gastrointestinal tract, and therefore play a unique role of maintaining the integrity of the mucosal barrier, nutrient absorption, and disease prevention (57). In the GIT of pigs, TFFs functions in the establishment of mucosal protection and repair. Although TFF1 is mainly expressed in the pyloric gland and the neck cells of the stomach, all TFFs are abundantly produced in the goblet cells—a mucin-producing epithelial cell in the small and large intestines (2). In general, the intestinal epithelial cells in the gut of pigs contribute to maintaining the defense and integrity of the mucosal layer (58). In general, TFF peptides maintain intestinal epithelial integrity in various organisms through restitution, wound healing, apoptosis, cell motility, and as well as establishing the protective effects of the intestinal barrier (59, 60). During mucosal repair, both pro and anti-inflammatory cytokines regulate the actions of TFFs (61). For instance, during intestinal development in pigs, transforming growth factor-alpha (TGF-α, a cytokine) modulates TFF2 and TFF3 to exhibit different regulation patterns (36, 62). Accordingly, TFF2 expression was significantly upregulated after weaning phase, and this in-turn enhanced mucosal integrity. During the pre-weaning phase in piglets, TFFs such as TFF3 may facilitate intestinal repair of injury induced by inflammation, thereby enhancing intestinal

TABLE 1 Summary of expressions of intestinal trefoil factors in animals.

Species	Treatment	Pre-condition	Action	References
Rat	Wheat peptides (0.1, 0.2, 0.4 g/kgbw) and omeprazole (20 mg/kgbw)	–	Increasing trefoil factor 1 (TFF1) levels	(84)
Rat	Low dose administration of mature silkworm powder	Ethanol-induced gastric damage	Promoting genes related gastric mucosal protection and biosynthesis including mucin 5AC and trefoil factors. Induces gastric mucosal defense factors in ethanol-induced gastric injury rat model	(85)
Rodents	Astragalus polysaccharides and matrine treatment	Ulcerative colitis (UC)	Upregulating trefoil factor 3 expression	(49)
Rodents	Oral administration of TFF3	Hemotherapy- and radiotherapy-induced mucositis	Decreased the intestinal damage	(86)
Rodents	[1]Lingo2 mice	[1]200 *Trichuris muris* eggs/mouse by oral gavage	ITF(TFF3) restrains T_H1 cell proliferation, promotes Type 2 immunity against infection	(87)
Rodents	Oxyresveratrol, an active ingredient of Artocarpus lakoocha administered to five treatment groups	Induction of gastric ulcer in mice	Elevated expression levels of cytoprotective TFF-2 levels, and attenuated expression levels of IL-6, TNF-α, NF-κB, and COX-2	(88)
Pigs	Control diet (supplemented 25 mg/kg quinocetone and 11.25 mg/kg aureomycin in the basal diet), three treatment diets supplemented with 200, 400, or 600 mg/kg bacteriophage in the basal diet	Healthy weaned piglets	ITF, and tumor growth factor-alpha (TGF-α) was enhanced	(89)
Pigs	Weaned piglets (28 D old)	Diarrhea occurrence (Enterotoxigenic *E. col*) Five levels of Wheat bran fiber or pea fiber.	Increased colonic peptide trefoil factors in piglets on the WBF and PF diets	(90)
Pigs	Weaned Piglets	Post-weaning (7 and 14 days) Dietary treatment [0, 200, or 400 μg/kg epidermal growth factor (EGF) supplementation].	Enhanced abundance of mucin 2 and intestinal trefoil factor 3 at 200 μg/kg EGF	(91)
Calves	Calves with coccidiosis received a single dose of toltrazuril (15 mg/kg) and supportive care	Control group (healthy calves) vs. Treatment group (calves with coccidiosis at 0th and 72nd hours infection)	Calves with coccidiosis had higher TFF-3 levels and ACTG2 after treatment (72nd hours) compared to the control group.	(91)
Cattle	Control group (negative controls) Positive control Orally infected with 10,000 Ostertagia ostertagi L3s (third-stage larva) and killed at 6, 9, and 24 days post infection (dpi)	Infection with nematode *Ostertagia ostertagi*	TFF1 and TFF3, co-expressed with mucins in the GI tract was upregulated in infected animals.	(46)
Cattle	Healthy cow, right displacement of the abomasum (RDA) and left displacement of the abomasum (LDA)	Displacement of abomasum	TFF-3 concentrations lower in RDA. Negative correlation between lactate and TFF-3	(92)
New-born Calves	Normal (NG) and test group (TG): *E. coli* O1(2.5×10^{11} CFU/mL, 100 mL)	Early pathogenic *Escherichia coli* infection	Lower ITF levels in the colons of TG calves than NG. Concentration of ITF in both the TG and NG decreased over experimental time	(23)
Sheep	[1]Group One (Chiswick Avermectin Resistant CAVRS) and Group two (ivermectin-sensitive McMaster)	*Haemonchus contortus* infection (two isolates: 500 McMaster L3 and 500 CAVR L3)	TFF3 was the second most highly upregulated gene after Intelectin 2 (IL-2) in the comparisons of day 22 vs. 3.	(93)
	Genetically Resistant group and Susceptible group	10,000 *H. contortus* L3s	TFF 2 was higher in resistant sheep	(94)
Sheep	Control group two (nfected with 30,000 T. *colubriformis* larvae L3) Group three: (infected with 10,000 H. *contortus* L3	Sheep infected with *Haemonchus contortus* and *Trichostrongylus columbriformis*	TFF3 was also found to be significantly upregulated in the small intestine (proximal jejunum), TFF2 was expressed in the abomasum.	(95)
Poultry	Arbor acre broilers	Supplementing paraformic acid (PFA)	Elevated intestinal mucosal factors (mucin 2, trefoil factor family, and zonula occludens-1) concentrations	(33)

(Continued)

TABLE 1 (Continued)

Species	Treatment	Pre-condition	Action	References
	Arbor Acre broilers: basal diet [control (CON) group] and Tannic Acid (TA) group	Tannic acid extracted from *Galla chinensis* (300 mg/Kg TA in basal diet)	Intestinal mucosal ZO-1 and TFF expression was similar in both groups.	(96)
	Control (PBS-treated), AKK (orally administered 1×10^6 CFU Akkermansia muciniphila for 10 days), SAL (orally administered 1×10^9 CFU Salmonella pullorum-treated on the 5th day) & AKK+SAL (orally administered 1×10^6 CFU Akkermansia muciniphila for 10 days and orally administered 1×10^9 CFU Salmonella pullorum-treated on the 5th day).	*S. pullorum* (1×10^9 CFU) and *Akkermansia muciniphila* (1×10^6 CFU) gavaging of birds.	Increased number of goblet cells in the intestine and up-regulation of Mucin 2 and trefoil factor 2 (TFF2)	(97)
	Male Cobb 500	live *Eimeria* vaccination or salinomycin and combinations of in-feed inclusion of gelatin and vitamin E	TFF2, cytokines, and MUC2 was increased by *Eimeria* vaccination.	(98)
	SPF chickens (control group and infection group)	Avian Influenza Virus Subtype H9N2 (i noculated with 3 doses of A/Chicken/Henan/SH01/2015 at 10^6 $TCID_{50}$/0.1 mL through the respiratory tract	TFF2, MUC2, ZO-1, and caudin-3 expressions was downregulated by H9N2 AIV infection	(99)
	d-1 Cherry Valley ducks	0, 1, 2, 4, 8, and 16 mg/kg iodine (dietary ethylenediamine dihydroiodide) in diet	Similar expression level of TFF2 in treatment groups	(100)
	Arbor acre broilers CON (basal diet group), Group two (AB) Group three (BP)	Basal diet supplemented with 50 mg/kg aureomycin (AB group) and basal diet supplemented with 40 mg/kg Bopu powder (BP group)	Dietary Bopu powder supplementation increased concentration of trefoil factor family member and mRNA expressions of superoxide dismutase	(101)
	Fertile Cobb 500 eggs	Eggs Injected in the amnion with 100 μL of either sterile water (sham), 1×10^5, 1×10^6, or 1×10^7 (P1, P2, and P3) probiotic product (Primalac W/S containing *Lactobacillus acidophilus*, *Lactobacillus casei*, *Enterococcus faecium*, and *Bifidobacterium bifidum*)	In ovo probiotic supplementation was associated with downregulated expression of Toll-like receptors-2 and−4, inducible nitric oxide synthase, trefoil factor 2, mucin-2, interferon-γ, and interleukins-4 and−13 in both the ileum and cecal tonsils	(102)

development and growth performance (50). The ability of TFFs to rebuild (or repair) the intestinal epithelium has been attributed to their anti-apoptotic mechanisms that drive the migration of epithelial cells over the damaged section of the intestinal mucosa, where the apoptosis is induced by anchorage-dependent cells detaching from the surrounding extracellular matrix (52) in pigs. In addition, TFF3 has also been reported to suppress factors that downregulate tight junction proteins, thereby decreasing mucosal permeability, promoting the integrity of tight junctions, and consequently enhancing intestinal barrier function (63).

2.2 Trefoil factors on the gut health in chicken

Although the molecular mechanisms underlying the functions of TFFs has been well-explored in mammalian species like rodents, humans and other amphibians, information is scanty regarding avian species (64). It has been established that the protective effect of TFFs result from their interactions with mucins to generate signaling mechanisms that result in their overexpression during mucosal damage, consequently culminating in intestinal restitution of the mucosa and enhanced barrier function (65). A similar mechanism of action has been reported in the chicken, and this was characterized by migration of the epithelial cells to the epithelial layers of the intestinal villi during injury (50). TFF2 bearing one single trefoil domain promote cell migration (45), since a damaged intestinal mucosa encourages the migration of pathogens into the body thereby causing systemic infection, trefoil factors mediate as a host defense mechanism by keeping the gastrointestinal mucosal layer impermissible to pathogenic attack (66). Accordingly, it can be proposed that the activities of TFFs inhibit pathogenic infection in concert with the actions of other antimicrobial peptides. This notion is corroborated by the finding that chickens infected with avian influenza (H9N2) showed significant decrease in the expression of TFFs and other antimicrobial peptides, and consequently, this resulted in infection with E. coli bacteria (67).

2.3 Trefoil factors on the gut health in rodents

The fact that most of the studies investigating the role of TFFs in gastrointestinal health were done in rodents warrant the exploration of literature in this regard in mice. Several studies have demonstrated the protective and healing effects of TFFs following mucosal damage in rodents (68). Conversely, anomalies

TABLE 2 Feed additives and their impact on trefoil factor (TFF) activation in animal models.

Additive	Treatment	Action	References
Lactic acid bacteria (LAB)	LAB-fermented formula milk supplementation given to weaned piglets	Increased the average daily gain, UP-regulated TFF2, selective enrichment of lactate-producing and short-chain fatty acid (SCFA)-producing bacteria in the ileum	(103)
Lactobacillus rhamnosus	Newborn piglets were orally administered with Wild-type (WT) and mutant (ΔluxS) *Lactobacillus rhamnosus,* Control (CON)	ITF levels were increased in the WT and ΔluxS group than in the CON group	(104)
Epidermal growth factor	Dietary treatment (0, 200, or 400 μg/kg EGF supplementation)	Dietary supplementation with 200 μg/kg enhanced abundances of mucin 2 and intestinal TFF3 compared with the control	(91)
Dietary Fiber	Control diet without fiber source (CT), and diets: maize was replaced by 10 % maize fiber (MF), 10 % soyabean fiber (SF), 10 % wheat bran fiber (WBF) or 10 % pea fiber (PF).	WB and PF resulted in increased expression of transforming growth factor-alpha (TGF-α), trefoil factors.	(90)
Dietary crude protein	Weanling pigs fed Three (3) diets containing 22, 19, or 16% CP	A reduction in occludin, trefoil factor-2, trefoil factor-3, and mucin 2 was observed at 16% CP	(105)
Zearalenone (ZEA)	Diet supplemented with ZEA at 0, 0.15, 1.5, and 3.0 mg/kg fed to weaned piglets	ZEA at 0.15 upregulated TFF3	(60)
Probiotic mixture	Probiotic mixture VSL containing four species of *Lactobacilli*: three species of *Bifidobacteria* and *Streptococcus salivarius* subsp. *thermophilus* sp.	Increased production of mucus, TFF1 and TFF2	(106)
Taurine	Lipopolysaccharide (LPS) and dietary taurine	Taurine increased the expression of TFF-3 mRNA. LPS-challenged animals lowered expressions of trefoil factor-3, transforming growth factor β-1 expression and number of goblet cells	(107)
Palygorskite	Basal diet supplemented with 0, 5, and 10 g/kg palygorskite for 21 days in chicks	Supplementation of 5 g/kg palygorskite increased jejunal trefoil factor 2 (TFF2) mRNA abundance	(108)
Lactiplantibacillus plantarum	Negative control (NC): basal diet only; OTC: basal diet + 0.01% oxytetracycline; RG11: basal diet + 0.1% Postbiotic RG11; RI11: basal diet + 0.1% postbiotics RI11 and RS5: basal diet + 0.1% postbiotic RS5	The concentrations of acidic mucin, sulfated mucin, and intestinal trefoil factor were higher in the birds fed with RI11 and RS5.	(109)
Paraformic acid	Basal diet (CON) or a basal diet supplemented with 1,000 mg/kg PFA	PFA supplementation elevated intestinal mucosal factors (mucin 2, trefoil factor family, and zonula occludens-1)	(33)
Yeast nucleotides	Control (CON = basal diet), groups 2–4 were fed the basal diet supplemented with 0.1, 0.3, and 0.5% yeast nucleotides	0.1 and 0.3% yeast nucleotides exhibited higher expression of Mucin 2 (MUC2) and trefoil factor 2 (TFF2) gene	(110)
Probiotics (Primalac W/S)	Probiotics (Primalac: 1×10^5, 1×10^6, or 1×10^7 (P1, P2, and P3 probiotic bacteria.) and Sham *in ovo* injection of Cobb500 eggs	Probiotic supplementation downregulated expression of Toll-like receptors-2 and−4, inducible nitric oxide synthase, TFF2, mucin-2, interferon-γ, and interleukins-4 and−13 in both the ileum and cecal tonsils.	(102)
Macleaya Cordata (Bopu powder)	Basal diet (CON group), basal diet supplemented with 50 mg/kg aureomycin (AB group), or a basal diet supplemented with 40 mg/kg Bopu powder (BP group)	Dietary Bopu powder supplementation significantly increased the concentration of trefoil factor family member	(101)
Algae	Seaweed extract (SWE; 10.0 g/d) and fish oil (FO; 100 g/d) inclusion	SWE supplementation increase colonic TFF3 mRNA expression	(111)
Bifidobacterium bifidum	Milk formula containing 5×10^6 CFU per day of *Bifidobacterium bifidum* OLB6378 and dam-fed littermates fed by surrogate mothers as a baseline control	*B. bifidum* treatment of neonatal necrotizing enterocolitis (NEC) markedly reduced number of Tff3-positive cells to values seen in normal healthy controls.	(112)
Cinnamaldehyde	Treatment with cinnamaldehyde (100 or 200 mg/kg bodyweight/day)	Cinnamaldehyde upregulated the expression of TFF3	(113)

develop in mucosal layer when there is deficiency of TFFs in mice. Studies have shown that TFF-2 deficient mice showed high susceptibility to gastric injury, and TFF-3 deficient mice showed a reduced resistance to colonic injury (69, 70). Mode of action of TFFs involve the formation of mucous defense and barriers, modulation of the mucosal immune response as well as enhancing a rapid mucosal repair via cell migration; a process known as restitution (71). The restitution process is a pivotal mechanism

following a mucosal damage as it helps in mucosal repair by migrating neighboring cells to the injury sites (71). This is evident in previous research reporting that TFF3 restitutes epithelial cells by stimulating the migration of cells surrounding the injured area to the damaged site (73). In addition, TFFs also exhibit antiapoptotic effects by rapidly preventing cell death through maintaining cell migration and cell survival (71). A research study conducted by Sturm and Dignass (72) showed that TFF3 promotes migration of epithelial cells by influencing localization and expression of catenin in epithelial cells, and by stimulating phosphorylation of catenin. The expression of TFF2 and TFF3 have also been observed to simultaneously induce the migration of monocytes in the bone marrow, lymph nodes, thymus and other lymphoid tissues (71). It has been established that the inflammatory responses of immune systems of mice is dysfunctional when TFF2 is mostly deficient (15), thus confirming that TFF2 is closely associated with immune response in addition to its involvement in gastric repair of epithelial cells. The phosphatidylinositol 3′-kinase (PI3K/Akt) signaling pathway which is activated by ITF, serves as a regulatory intracellular avenue that restores the epithelial mucosal integrity following an injury (74).

Many studies have confirmed that the PI3K/Akt signaling pathway actively participates in key physiological and pathological processes such as regulating cell proliferation, migration and apoptosis, as well as exhibiting inflammatory responses in the epithelial mucosa (75, 76). For instance, it was observed that higher concentration of ITF increased the proliferation and migration of gut esterase-1 (GES-1) cells (74), thereby corroborating the findings of previous studies (77, 78). In the large intestine, the absence of TFF3 expression was found to increase sensitivity to colonic injury by stabilizing the mucosal layer and inducing repair at injury sites, and rapidly upregulating and enhancing the restorative process (6). This protective effect may be attributed to the collaborative action of TFF3 and Muc2 in the intestinal mucosa as the first line of defense against epithelial injury (79). It has also been reported that recombinant human TFF3 (rhTFF3) can stimulate the expression of tight junction proteins which are directly connected with intestinal barrier functions, thereby reducing intestinal mucosal permeability (74). This was evident in an rhTFF3 expression study on intestinal wall injury in the rat (80). In this study, Wang et al. (80) observed a significant reduction in damage to ileal mucosa, and a reduced intestinal permeability.

2.4 Trefoil factors on the gut health in calves

The intestinal mucosa is crucial for regulating the interaction between bacteria and host cells, as well as affecting nutrient absorption. Calves are mostly susceptible to numerous mechanical obstructions as a result of abnormalities in the bowel lumen, intestinal wall, or outside the intestinal tract (81). Calves, like other young animals, experience gastrointestinal injuries or insults due to factors such as weaning stress, infections, or dietary imbalances. Trefoil factors, particularly TFF2 and TFF3 has been established to play a significant role in maintaining and repairing the intestinal mucosa in calves (23). Studies have shown that trefoil factors like TFF3 can help prevent intestinal epithelial damage and promote repair of the intestinal mucosa. In calves, this protection is particularly crucial during the early stages of life when gastrointestinal system is still developing and vulnerable to various stressors, including dietary changes and pathogens. The immunological barrier function mediated by intestinal microbes is destroyed by changes in the makeup of the intestinal flora in calves, making the intestinal tract more vulnerable to harmful germs and putting the animals' health at risk (82). The abomasal infection by nematodes in calves such as *Ostertagia ostertagi* larvae causes substantial tissue injury to the abomasal mucosa, and these injuries include acute epithelial cytolysis, hyperplasia of gastric glands, and reduction in acid-producing cells, all culminating in increased gastric pH concentration and inhibition of protein metabolism (83). In another study, the mucin glucosaminyl (N-acetyl) transferase 3 which catalyzes one of the key rate-limiting steps in mucin biosynthesis was up-regulated in the bovine abomasum during nematode infections (46). Together, these results suggest TFF-induced enhanced tissue repair and mucin secretion may contribute directly to mucosal protective immunity. It can be suggested that TFFs enhance mucosal defense by inducing tissue repair mechanisms in the GIT through stimulating cell movement in a manner that reestablishes a healthy mucosa and inhibit apoptosis (84). Intestinal biomarkers have been found to be elevated in inflammatory bowel damage and acute intestinal ischemia (blood flow to the intestine), and intestinal mucosal injury has been linked to elevated expression levels of IFABP, LFABP, IAP, and TFF3 (81).

3 Feed additives: TFFs activation

TFFs play critical roles in maintaining the integrity and repair of mucosal barriers, particularly in the gastrointestinal tract (1). Certain feed additives and nutrients have been studied for their ability to stimulate or enhance the expression of TFFs, potentially improving gut health and promoting healing. Some feed additives that can stimulate or activate trefoil factors are listed in Table 2.

4 Conclusion

The GIT tracts of animals is often exposed to injuries, proliferation of pathogenic bacterial, stress caused by chemicals, physical stress and also adverse effects related to side effects of medical drugs used as growth promoters, consequently necessitating the need for tissue repairs. Various studies have indicated that TFFs enhanced protection and restitution of the mucosal surfaces of various vital organs, including the GIT. The beneficial effects of TFFs are achieved through molecular interactions with mucins to improve cellular migration. The findings from this literature exploration confirms that TFF peptides play significant beneficial roles in intestinal epithelial healing and amalgamation of the mucus layer. Such protection of the intestinal epithelial barrier is prone to culminate in improved animal health, growth performance, and production. Accordingly, future research

should be conducted to identify biogenic feed additives that have potential to induce optimum synthesis of TFFs in the GIT of food animals.

Author contributions

YF: Conceptualization, Funding acquisition, Investigation, Supervision, Writing – original draft, Writing – review & editing. TO: Conceptualization, Investigation, Supervision, Writing – original draft, Writing – review & editing. DE: Investigation, Writing – original draft, Writing – review & editing, Conceptualization, Supervision. GD: Investigation, Writing – original draft, Writing – review & editing. JR: Investigation, Writing – original draft, Writing – review & editing. OA: Investigation, Writing – original draft, Writing – review & editing.

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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 rumen-protected glucose level and taurine supplementation on weight change and oxidative stress state of yaks after transport

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Rumen-protected glucose (RPG) and taurine (TAU) are crucial for the nutrition and physiology of ruminants, enhancing production performance and mitigating negative energy balance. This study aimed to assess the impact of RPG levels and TAU supplementation on the body weight, antioxidant capacity, immune function and stress responses in yaks before and after transport. Thirty-two healthy male yaks, aged 3 years and weighing 172.5 ± 10.2 kg, were randomly assigned to one of four dietary treatments: (1) low RPG and low TAU (LRLT), with 1% RPG and 5 g/d TAU; (2) low RPG and high TAU (LRHT), with 1% RPG and 15 g/d TAU; (3) high RPG and low TAU (HRLT), with 3% RPG and 5 g/d TAU; and (4) high RPG and high TAU (HRHT), with 3% RPG and 15 g/d TAU. The yaks were treated with the corresponding diet for 7 days, then received 9 h of transportation, and finally fed the same diet at their destination for 30 days. The weight was measured before and on days 0 and 30 after transport, and the plasma was collected before and on day 0, 10, and 30 after transport for analysis of biochemical, antioxidant, immune, and stress response indicators. We observed that transport increased plasma concentrations of total cholesterol, total protein, lactate dehydrogenase, creatine kinase, malondialdehyde, cortisol and lipopolysaccharides of yaks among treatments, while decreased their BW and plasma IL-10 concentration. Increasing TAU supplementation reduced weight loss (8.42 vs. 11.9 kg) and weight loss percent (4.83% vs. 6.87%) in yaks after transport. The concentration of MDA in plasma was lower in HRHT than in LRLT at day 0 after transport ($p = 0.03$). The activity of GSH-PX was higher in HRHT than in LRLT at day 10 after transport ($p = 0.04$). Concentrations of IL-10 at day 0 and 10 after transport was higher in HRHT than in LRLT and HRLT ($p = 0.02$, $p = 0.01$, respectively). With the increase of TAU supplementation, concentrations of IL-1β at day 30 after transport ($p = 0.02$), TNF-α at day 0 after transport ($p = 0.02$), COR at day 10 ($p = 0.03$) and 30 ($p = 0.05$) after transport as well as LPS at day 0 after transport ($p = 0.04$) decreased. In addition, concentrations of COR at day 0 after transport was lower in LRHT and HRHT than in LRLT ($p = 0.03$). Based on all the results, we demonstrated that increasing TAU supplementation levels reduced post-transport weight loss in yaks, improved their antioxidant capacity and immune function, and alleviated stress responses. Considering the effect of resistance to transport stress and costs, the optimal treatment identified in this study involved a diet containing 1% RPG and supplemented with 15 g/d TAU.

KEYWORDS
rumen-protected glucose, taurine, yak, transport, stress response

1 Introduction

Yak is a vital component of animal husbandry in China's high-altitude grazing regions, providing herders with high-quality milk, meat, and fur, thus holding significant economic value. Grazing on native pasture is the traditional way of raising yaks. Yet, these pastures experience substantial seasonal fluctuations in forage availability, often leading to considerable stress on the pastures, particularly in winter (1). Typically, due to the intake of nutrients falling below the necessary maintenance levels, the weight of grazing yaks will be reduced by about 25% after a cold season, which extends the feeding cycle of yaks, reduces the slaughter yield, and seriously hinders the sustainable development of the yak industry (2, 3). Off-site fattening has increasingly become the primary feeding method for yaks during the cold season, mitigating the traditional contradiction between the forage availability and livestock demand in grazing areas, while enhancing the growth efficiency and meat quality of yaks, resulting in higher economic returns.

Some herders relocate yaks from high-altitude grazing areas to low-altitude farms for breeding. However, live transportation means yaks are exposed to multiple stressors, including noise, crowding, starvation and high temperature, which can exacerbate the adaptive and defensive responses, causing transport stress and compromising health and welfare in animals (4, 5). It has been reported that feed and water deprivation during transport caused dehydration in the body, weight loss and negative energy balance, thereby damaging organismal metabolism and reducing slaughter value (6). Furthermore, prolonged crowding and standing can increase muscle tension and fatigue, and may also promote the development of respiratory diseases (7, 8). Currently, nutritional regulation is a commonly used measure to alleviate transport stress in animals.

Rumen-protected glucose (RPG) can be released directly from the small intestine into the blood circulation, because its surface is covered with special materials like fatty acids, preventing it from being fermented and degraded in the rumen. Unlike monogastric animals, ruminants have a strong dependency on the endogenous glucose produced by gluconeogenesis, so exogenous glucose intake is relatively low. The RPG supplement can change energy supply path, improve energy utilization efficiency, and alleviate negative energy balance (9–11). Zhang et al. (12) also reported that RPG enhanced the large intestine fermentation function and mucosal immunity of transition dairy cows. Taurine (TAU) is a sulfur-containing amino acid that plays an important role in muscle growth, immune response and energy metabolism (13, 14). However, the TAU synthesized in the liver of most mammals has a limited ability to remove free radicals, while supplementation with exogenous TAU improved the antioxidant enzyme activity of animals and their liver tissues, thereby enhancing antioxidant capacity (15, 16).

Our previous study uncovered that supplementing with RPG and TAU reduced weight loss in yaks during transportation. However, the optimal addition of both supplements to effectively alleviate transport stress in yaks remains unclear. Our study aimed to investigate: (1) the effects of four supplemental levels on the weight of yaks before and after transportation; (2) the changes of plasma antioxidant, immune and stress response indicators before and after transportation; and (3) the impacts of continuous supplementation of RPG and TAU on the above indicators in yaks. We hypothesized that one of the four additive combinations of RPG and TAU could minimize the adverse effects of transportation on growth performance in yaks, and that sustained treatment could improve their antioxidant capacity, immunity and oxidative stress status. This study provides evidence for the use of nutritional intervention to alleviate the stress of yaks during transportation.

2 Materials and methods

2.1 Animals and experimental design

All animal procedures adhered to the Chinese Guidelines for Animal Welfare, and the study protocols were approved by the Experimental Animal Committee of the Animal Nutrition Institute at Sichuan Agricultural University (Approval no.: SCAUAC2019-36).

The study was conducted from January to March 2023. Thirty-two healthy male yaks, aged 3-years; and weighting 172.5 ± 10.2 kg, were assigned randomly into 4 different treatments: (1) low RPG and low TAU (LRLT), the level of RPG in the diet was 1%, and the addition of TAU was 5 g/d; (2) low RPG and high TAU (LRHT), the level of RPG in the diet was 1%, and the addition of TAU was 15 g/d; (3) high RPG and low TAU (HRLT), the level of RPG in the diet was 3%, and the addition of TAU was 5 g/d; and (4) high RPG and high TAU (HRHT), the level of RPG in the diet was 3%, and the addition of TAU was 15 g/d. Ingredients and chemical composition of diets offered to yaks are shown in Table 1. The RPG and TAU used in our study were purchased from Shanghai Meinong Biotechnology Co., Shanghai, China, and Qingdao Kailide Biotechnology Co., Ltd., Qingdao, China, respectively. Both had active ingredients at 50% and a small intestine release rate of 90%. Each treatment involved eight replicates of one yak each replicate. Yaks were housed individually in pens within a pasture in Hongyuan County, Sichuan Province, and were fed a total mixed ration (TMR) twice a day (0700 and 1900 h) during the trial, with a guaranteed daily feed intake of 105–110%. The feed was pushed at least 10 times daily, and yaks had unrestricted access to fresh water throughout the study period. After 7 days on different diets, the yaks were treated for 9 h of long-distance transport. The yaks were fasted for 12 h prior to transportation and loaded into a semi-trailer measuring (9 m × 2.55 m × 4 m) at 10:00 AM on the day of transport, and then transported for 9 h on expressways until they arrived at the Research Farm of Animal Nutrition Institute, Sichuan Agricultural University (Yaan, China). Fasting and water prohibition during transportation, and no feed and fresh water are also provided to yaks within 1 h after transportation. The yaks were eventually raised here for 30 days during which they were fed the same diet as prior to their transportation.

2.2 Determination of growth performance

Yaks were weighed both before transport and on days 0 and 30 post-transport to analyze changes in the body weight (BW) of the

TABLE 1 Ingredients and chemical composition of diets offered to yaks.

Items	Treatments[1]	
	LR	HR
Ingredient composition, % of DM		
Leymus chinensis	27.0	27.0
Straw	35.0	35.0
Corn	15.5	14.7
Wheat bran	5.60	5.00
Soybean meal	9.00	9.40
Rapeseed meal	3.00	3.00
Palm oil	2.00	1.00
Sodium chloride	0.20	0.20
Premix[2]	1.70	1.70
RPG	1.00	3.00
Chemical composition, % of DM		
NEg[3] (MJ/kg)	5.97	5.97
CP	11.6	11.9
NDF	46.1	45.8
ADF	30.7	30.4
Calcium	0.52	0.50
Available phosphorus	0.36	0.33

[1]LR diet (Low RPG): Both LRLT and LRHT treated yaks were provided with this diet, which contains 1% RPG but has TAU content of 5 g/d (LRLT) and 15 g/d (LRHT) respectively; HR diet (High RPG): Both HRLT and HRHT treated yaks were provided with this diet, which contains 3% RPG but has TAU content of 5 g/d (HRLT) and 15 g/d (HRHT) respectively. [2]The premix provided the following per kilogram of the diet: vitamin A 4500 IU, vitamin D 1500 IU, vitamin E 200 IU, Co 12 mg, Cu 800 mg, Cl 29.5 g, Zn 1,500 g, Fe 3,000 mg, Mn 1,200 mg, I 25 mg, Se 15 mg. [3]The net weight gain energy (NEg) is calculated, and the rest is measured.

experimental yaks. The BW loss percent of yaks during transportation was calculated using the formula: BW loss percent (%) = [(BW before transport – BW after transport)/ BW before transport] × 100%. From day 0 post-transport to day 30, daily feed intake was measured to determine dry matter intake (DMI). The yaks were weighed again on d 30, and the average daily gain (ADG) and feed conversion ratio (FCR) were calculated.

2.3 Sample collection

Blood samples were collected using vacuum blood collection tubes from the jugular vein of yaks 3 h after morning feeding before transport and on d 0, 10 and 30 after transport. Blood samples were allowed to stand for 30 min and then centrifuged at 4°C and 3,000 × g for 15 min to prepare plasma. The plasma was stored at-20°C for subsequent analysis of biochemical, antioxidant, immune, and stress response indicators.

2.4 Determination of biochemical, antioxidant, immune and stress response indicators in plasma

Plasma biochemical, including total cholesterol (TC), total protein (TP), alanine aminotransferase (ALT), lactate (LAC), triglyceride (TG), glucose (GLU), lactate dehydrogenase (LDH) and creatine kinase (CK) were determined using a fully automatic bio-chemical analyzer (Hitachi 3,100, Olympus Optical, Tokyo, Japan). Plasma antioxidant indexes, including activities of catalase (CAT, A007-1-1), superoxide dismutase (SOD, A001-1-2), and glutathione peroxidase (GSH-PX, A005-1-2), as well as total antioxidant capacity (T-AOC, A015-1-2) and malondialdehyde (MDA, A003-1-2) concentrations, were evaluated with an antioxidant assay kit supplied by the Nanjing Jiancheng Bioengineering Institute (Nanjing, China). Plasma immune indexes, like IL-1β (MB-9650A), IL-4 (MB-4904A), IL-6 (MB-4905A), IL-10 (MB-4931A), TNF-α (MB-4838A) concentrations, were determined using an ELISA kit from Jiangsu Enzymebiao Bio-technology Co., Ltd. (Nanjing, China), following the manufacturer's instructions. For plasma stress response indicators, concentrations of cortisol (COR, EB0062) and lipopolysaccharides (LPS, EU3126) were tested by ELISA kit from FineTest, Wuhan Fine Biotech Co. (Wuhan, China).

2.5 Statistical analysis

Before comparing treatments, the normality and variance homogeneity of all data were verified. Growth performance, plasma antioxidant, immune and stress response indicators of yaks were subjected to two-way ANOVA by the SPSS statistical software version 22.0 (IBM SPSS, Chicago, IL, USA) to determine the effects of RPG and TAU supplemental levels on the above indexes. When the interaction between RPG and TAU levels in diets was significant, one-way ANOVA was conducted to compare two levels of TAU

supplementation within the same RPG levels and two levels of RPG in the diet within the same TAU supplementation. In addition, we also compared the changes of BW, plasma biochemistry, antioxidant, immune and stress response indicators of yaks before and after transport using ANOVA by the SPSS statistical software version 22.0 (IBM SPSS, Chicago, IL, USA). All results are reported as least squares means ± SEM. The Tukey multiple comparison test was employed to identify statistically significant differences at $p \leq 0.05$.

3 Results

3.1 Weight changes in yaks before and after transportation

The effects of dietary RPG level and TAU supplementation on the changes of yak BW before and after transportation are shown in Table 2. There was no significant interaction between RPG and TAU regarding the weight of yaks pre-and post-transport, weight loss as well as weight loss percent ($P > 0.05$). Compared with before transportation, the weight of yaks decreased after transportation ($P < 0.01$). Increasing TAU supplementation reduced weight loss ($p = 0.05$, 11.9 and 8.42 kg in LT and HT, respectively). The weight loss percent also decreased ($p = 0.04$) with the increase of TAU supplementation.

3.2 Growth performance in yaks after transportation

The effects of dietary RPG level and TAU supplementation on yak growth performance from days 0 to 30 post-transportation are presented in Table 3. The level of RPG in the diet and TAU supplementation did not affect final BW, ADG, DMI and FCR in yaks ($P > 0.05$).

3.3 Changes of biochemical, antioxidant, immune and stress response indicators in plasma before and after transportation

We did not observe any changes in the concentrations ($P > 0.05$) of ALT, LAC, TG, and GLU in plasma before and after transport or among different treatments (Table 4). Compared with before transport, the concentration of TC, TP, LDH and CK in plasma of yaks with different treatments increased after transport ($P < 0.01$). The plasma TP concentration before transport was higher in HRHT than in LRLT ($p = 0.04$). The changes of antioxidant, immune and stress response indicators in plasma before and after transportation are provided in Tables 5–7. Transport increased plasma concentrations of MDA ($p = 0.05$), COR ($P < 0.01$) and LPS ($P < 0.01$), while decreased plasma IL-10

TABLE 2 Changes in BW of yaks[1] before and after transport under various treatments.

Items	Treatment[2]				SEM	R		T		*P*-value[3]			
	LRLT	LRHT	HRLT	HRHT		LR	HR	LT	HT	S	R	T	R*T
BW before transportation (kg)	170.7	171.8	174.7	173.1	5.34	171.2	173.9	172.7	172.4	< 0.01	0.47	0.93	0.89
BW after transportation (kg)	158.5	162.6	163.2	165.3	5.16	160.6	164.3	160.8	163.9		0.31	0.39	0.62
Weight loss (kg)	12.2	9.06	11.5	7.78	2.44	10.6	9.64	11.9	8.42	–	0.57	0.05	0.25
Weight loss percent (%)	7.20	5.27	6.53	4.39	1.38	6.23	5.47	6.87	4.83	–	0.45	0.04	0.20

[1]Values are least squares means of eight replicate yaks ($n = 8$). [2]LRLT, the dietary addition of RPG was 1%, and the addition of TAU was 5 g/d; LRHT, the dietary addition of RPG was 1%, and the addition of TAU was 15 g/d; HRLT, the dietary addition of RPG was 3%, and the addition of TAU was 5 g/d; and HRHT, the dietary addition of RPG was 3%, and the addition of TAU was 15 g/d. In the "R" to the right of this line, "LR" refers to LRLT and LRHT, while "HR" refers to HRLT and HRHT; In the "T," "LT" refers to LRLT and HRLT, while HT refers to LRHT and HRHT. [3]"S" means the transportation; "R" means the RPG; "T" means the TAU; and "R*T" means the interaction between RPG and TAU.

TABLE 3 Effects of RPG and TAU levels in the diet on the growth performance of yaks[1] 0–30 d after transportation.

Items	Treatment[2]				SEM	R		T		*P*-value[3]		
	LRLT	LRHT	HRLT	HRHT		LR	HR	LT	HT	R	T	R*T
Final BW (kg)	187.9	189.4	193.5	193.9	5.39	188.7	193.7	190.7	191.8	0.18	0.80	0.62
ADG (kg)	0.57	0.59	0.63	0.69	0.08	0.58	0.66	0.60	0.64	0.19	0.48	0.51
DMI (kg)	4.34	4.41	4.32	4.45	0.08	4.38	4.39	4.33	4.43	0.86	0.08	0.35
FCR	7.93	8.26	7.51	6.85	0.77	8.10	7.18	7.73	7.55	0.29	0.16	0.69

[1]Values are least squares means of eight replicate yaks ($n = 8$). [2]LRLT, the dietary addition of RPG was 1%, and the addition of TAU was 5 g/d; LRHT, the dietary addition of RPG was 1%, and the addition of TAU was 15 g/d; HRLT, the dietary addition of RPG was 3%, and the addition of TAU was 5 g/d; and HRHT, the dietary addition of RPG was 3%, and the addition of TAU was 15 g/d. In the "R" to the right of this line, "LR" refers to LRLT and LRHT, while "HR" refers to HRLT and HRHT; In the "T," "LT" refers to LRLT and HRLT, while HT refers to LRHT and HRHT. [3]"R" means the RPG; "T" means the TAU; and "R*T" means the interaction between RPG and TAU.

TABLE 4 Changes in plasma biochemical of yaks[1] before and after transport under various treatments.

Items	Transport	Treatment[2]				SEM	*P*-value[3]			
		LRLT	LRHT	HRLT	HRHT		S	R	T	R*T
TC (mmol/L)	Before	1.84	2.04	1.94	1.77	0.12	< 0.01	0.36	0.88	0.17
	After	2.92	2.88	3.25	2.83	0.27		0.46	0.22	0.38
TP (g/L)	Before	47.8^{b}	53.7^{ab}	51.7^{ab}	55.5^{a}	2.58	< 0.01	0.16	0.02	0.04
	After	73.6	71.2	74.2	73.5	2.23		0.38	0.32	0.57
ALT (U/L)	Before	54.5	57.3	57.9	58.8	3.62	0.92	0.34	0.47	0.68
	After	56.2	54.6	57.8	60.2	3.74		0.17	0.88	0.49
LAC (U/L)	Before	63.9	62.4	61.1	62.7	2.59	0.36	0.49	0.99	0.75
	After	62.2	60.4	62.5	61.3	1.47		0.60	0.15	0.50
TG (mmol/L)	Before	0.13	0.16	0.14	0.13	0.02	0.87	0.45	0.39	0.35
	After	0.14	0.12	0.16	0.13	0.02		0.34	0.07	0.19
GLU (mmol/L)	Before	2.95	2.49	2.53	2.67	0.24	0.80	0.47	0.36	0.22
	After	2.86	2.61	2.51	2.77	0.17		0.44	0.96	0.17
LDH (U/L)	Before	875.2	924.7	894.3	853.1	42.8	< 0.01	0.39	0.89	0.41
	After	1305.8	1236.7	1275.3	1228.3	41.9		0.53	0.08	0.25
CK (U/L)	Before	129.5	132.0	126.9	123.2	12.0	< 0.01	0.50	0.95	0.90
	After	392.9	374.2	362.7	320.3	35.1		0.10	0.24	0.23

[1]Values are least squares means of eight replicate yaks ($n = 8$). [2]LRLT, the dietary addition of RPG was 1%, and the addition of TAU was 5 g/d; LRHT, the dietary addition of RPG was 1%, and the addition of TAU was 15 g/d; HRLT, the dietary addition of RPG was 3%, and the addition of TAU was 5 g/d; and HRHT, the dietary addition of RPG was 3%, and the addition of TAU was 15 g/d. [3]"S" means the transportation; "R" means the RPG; "T" means the TAU; and "R*T" means the interaction between RPG and TAU. [a, b]Means within a row with different superscripts differ significantly ($p \leq 0.05$).

TABLE 5 Changes in plasma antioxidant capacity of yaks[1] before and after transport under various treatments.

Items	Transport	Treatment[2]				SEM	*P*-value[3]			
		LRLT	LRHT	HRLT	HRHT		S	R	T	R*T
CAT (U/mL)	Before	17.5	17.1	18.0	17.8	0.76	0.26	0.27	0.56	0.67
	After	18.2	18.0	17.8	18.3	0.92		0.91	0.77	0.96
SOD (U/mL)	Before	39.6	40.3	40.5	40.7	1.43	0.27	0.46	0.66	0.86
	After	39.8	41.1	41.3	43.3	2.48		0.30	0.35	0.59
GSH-PX (U/mL)	Before	434.4	469.0	447.9	472.4	16.8	0.19	0.53	0.02	0.12
	After	426.2	454.2	440.0	461.6	15.2		0.38	0.03	0.14
T-AOC (μmol/mL)	Before	2.81	2.83	2.90	2.84	0.18	0.35	0.67	0.89	0.96
	After	2.64	2.77	2.84	2.74	0.25		0.62	0.93	0.88
MDA (nmol/mL)	Before	16.0	14.9	15.6	14.7	0.53	0.05	0.75	0.03	0.16
	After	16.7^{a}	15.8^{ab}	16.3^{ab}	15.1^{b}	0.48		0.17	0.01	0.03

[1]Values are least squares means of eight replicate yaks ($n = 8$). [2]LRLT, the dietary addition of RPG was 1%, and the addition of TAU was 5 g/d; LRHT, the dietary addition of RPG was 1%, and the addition of TAU was 15 g/d; HRLT, the dietary addition of RPG was 3%, and the addition of TAU was 5 g/d; and HRHT, the dietary addition of RPG was 3%, and the addition of TAU was 15 g/d. [3]"S" means the transportation; "R" means the RPG; "T" means the TAU; and "R*T" means the interaction between RPG and TAU. [a,b]Means within a row with different superscripts differ significantly ($p \leq 0.05$).

concentration ($P < 0.01$). Increasing TAU supplementation increased plasma GSH-PX activity before ($p = 0.02$) and after transportation ($p = 0.03$). The plasma MDA concentrations after transport was lower in HRHT than in LRLT ($p = 0.03$, 15.1 vs. 16.7 nmol/mL). The plasma IL-4 concentration after transport was higher in HRHT than in LRLT ($p = 0.03$). The plasma IL-10 concentration after transport was higher in LRHT and HRHT than in LRLT and HRLT ($p = 0.02$). In addition, increasing TAU supplementation decreased the plasma concentration of COR before transportation ($p = 0.02$) as well as LPS before ($p = 0.05$) and after transportation ($p = 0.04$). The plasma COR concentration after transport was lower in LRHT and HRHT than in LRLT ($p = 0.03$).

3.4 Plasma antioxidant in yaks after transportation

We found no significant changes ($p > 0.05$) in the activities of CAT and SOD, T-AOC as well as MDA concentrations in plasma on days 10, and 30 post-transport across treatments (Table 8). The TAU

TABLE 6 Changes in plasma immune index of yaks[1] before and after transport under various treatments.

Items	Transport	Treatment[2]				SEM	*P*-value[3]			
		LRLT	LRHT	HRLT	HRHT		S	R	T	R*T
IL-1β (ng/L)	Before	54.9	55.9	56.1	56.1	1.59	0.08	0.50	0.64	0.85
	After	55.7	57.4	57.7	56.9	0.99		0.34	0.58	0.25
IL-4 (ng/L)	Before	89.4	94.9	94.3	95.7	2.05	0.11	0.22	0.13	0.20
	After	90.5^{b}	91.6^{ab}	91.0^{ab}	93.5^{a}	0.94		0.14	0.02	0.03
IL-6 (ng/L)	Before	79.8	81.9	81.7	80.1	1.99	0.23	0.96	0.81	0.63
	After	81.9	82.5	82.1	81.7	1.97		0.81	0.96	0.98
IL-10 (ng/L)	Before	44.1	44.6	43.6	45.8	1.32	< 0.01	0.72	0.15	0.41
	After	41.5^{b}	43.0^{a}	41.2^{b}	43.3^{a}	0.72		0.99	0.01	0.02
TNF-α (ng/L)	Before	271.3	262.4	274.7	258.2	11.2	0.28	0.97	0.10	0.45
	After	284.8	266.7	280.9	259.9	11.4		0.56	0.02	0.14

[1]Values are least squares means of eight replicate yaks ($n = 8$). [2]LRLT, the dietary addition of RPG was 1%, and the addition of TAU was 5 g/d; LRHT, the dietary addition of RPG was 1%, and the addition of TAU was 15 g/d; HRLT, the dietary addition of RPG was 3%, and the addition of TAU was 5 g/d; and HRHT, the dietary addition of RPG was 3%, and the addition of TAU was 15 g/d. [3]"S" means the transportation; "R" means the RPG; "T" means the TAU; and "R*T" means the interaction between RPG and TAU. [a,b]Means within a row with different superscripts differ significantly ($p \leq 0.05$).

TABLE 7 Changes in plasma stress response indicators of yaks[1] before and after transport under various treatments.

Items	Transport	Treatment[2]				SEM	*P*-value[3]			
		LRLT	LRHT	HRLT	HRHT		S	R	T	R*T
COR (ng/mL)	Before	185.3	175.9	183.8	168.2	7.30	< 0.01	0.43	0.02	0.11
	After	266.8^{a}	237.8^{b}	248.4^{ab}	234.9^{b}	10.1		0.23	0.01	0.03
LPS (EU/L)	Before	385.7	366.1	380.7	352.5	15.8	< 0.01	0.45	0.05	0.18
	After	473.2	448.3	470.3	431.4	19.6		0.31	0.04	0.19

[1]Values are least squares means of eight replicate yaks ($n = 8$). [2]LRLT, the dietary addition of RPG was 1%, and the addition of TAU was 5 g/d; LRHT, the dietary addition of RPG was 1%, and the addition of TAU was 15 g/d; HRLT, the dietary addition of RPG was 3%, and the addition of TAU was 5 g/d; and HRHT, the dietary addition of RPG was 3%, and the addition of TAU was 15 g/d. [3]"S" means the transportation; "R" means the RPG; "T" means the TAU; and "R*T" means the interaction between RPG and TAU. [a,b]Means within a row with different superscripts differ significantly ($p \leq 0.05$).

TABLE 8 Effects of RPG and TAU levels in the diet on the plasma antioxidant capacity of yaks[1] after transportation.

Items	Treatment[2]				SEM	R		T		*P*-value[3]		
	LRLT	LRHT	HRLT	HRHT		LR	HR	LT	HT	R	T	R*T
CAT (U/mL)												
10 d	17.0	17.6	17.1	17.1	1.15	17.3	17.1	17.1	17.3	0.75	0.72	0.94
30 d	16.9	17.5	16.8	17.0	0.64	17.2	16.9	16.8	17.3	0.52	0.35	0.71
SOD (U/mL)												
10 d	39.6	42.6	40.2	41.8	2.03	41.1	41.0	39.9	42.2	0.98	0.11	0.44
30 d	40.9	42.2	42.3	43.2	1.85	41.6	42.7	41.6	42.7	0.37	0.41	0.69
GSH-PX (IU/L)												
10 d	422.5^{b}	452.2^{ab}	445.8^{ab}	463.1^{a}	13.1	437.5	454.5	434.1	457.8	0.12	0.03	0.04
30 d	423.9	427.1	441.8	450.1	16.1	425.0	445.9	432.4	438.6	0.07	0.60	0.33
T-AOC (μmol/mL)												
10 d	2.68	2.82	2.65	2.57	0.24	2.75	2.61	2.66	2.70	0.39	0.83	0.76
30 d	2.67	2.77	2.67	2.53	0.11	2.72	2.60	2.67	2.65	0.15	0.83	0.25
MDA (nmol/mL)												
10 d	16.0	16.5	16.3	15.6	0.56	16.3	15.9	16.2	16.0	0.44	0.67	0.42
30 d	15.4	15.9	16.0	15.6	0.64	15.6	15.8	15.7	15.7	0.72	0.90	0.81

[1]Values are least squares means of eight replicate yaks ($n = 8$). [2]LRLT, the dietary addition of RPG was 1%, and the addition of TAU was 5 g/d; LRHT, the dietary addition of RPG was 1%, and the addition of TAU was 15 g/d; HRLT, the dietary addition of RPG was 3%, and the addition of TAU was 5 g/d; and HRHT, the dietary addition of RPG was 3%, and the addition of TAU was 15 g/d. In the "R" to the right of this line, "LR" refers to LRLT and LRHT, while "HR" refers to HRLT and HRHT; In the "T," "LT" refers to LRLT and HRLT, while HT refers to LRHT and HRHT. [3]"R" means the RPG; "T" means the TAU; and "R*T" means the interaction between RPG and TAU. [a,b]Means within a row with different superscripts differ significantly ($p \leq 0.05$).

TABLE 9 Effects of RPG and TAU levels in the diet on the plasma immune index of yaks[1] after transportation.

Items	Treatment[2]				SEM	R		T		*P*-value[3]		
	LRLT	LRHT	HRLT	HRHT		LR	HR	LT	HT	R	T	R*T
IL-1β (ng/L)												
10 d	55.5	55.9	57.0	56.4	1.04	55.7	56.7	56.3	56.1	0.16	0.88	0.52
30 d	56.4	54.5	56.3	55.3	0.77	55.5	55.8	56.3	54.9	0.62	0.02	0.09
IL-4 (ng/L)												
10 d	90.7	91.5	89.2	92.7	1.13	91.1	91.0	90.0	92.1	0.88	0.02	0.07
30 d	92.7	93.8	90.9	92.7	1.71	93.2	91.8	91.8	93.3	0.23	0.22	0.41
IL-6 (ng/L)												
10 d	82.4	82.0	82.7	81.0	2.13	82.2	81.9	82.6	81.5	0.91	0.71	0.88
30 d	76.5	75.5	76.4	75.3	2.67	76.0	75.8	76.4	75.4	0.96	0.74	0.79
IL-10 (ng/L)												
10 d	41.7^{b}	43.2^{ab}	42.3^{b}	44.9^{a}	0.80	42.4	43.6	41.9	44.0	0.13	< 0.01	0.01
30 d	42.8	44.1	43.3	44.3	1.17	43.4	43.8	43.1	44.2	0.76	0.35	0.82
TNF-α (ng/L)												
10 d	282.1	279.6	277.2	273.9	13.7	280.8	275.6	279.7	276.7	0.57	0.76	0.94
30 d	281.2	272.1	272.7	265.2	8.78	276.6	269.0	276.9	268.7	0.23	0.20	0.38

[1]Values are least squares means of eight replicate yaks ($n = 8$). [2]LRLT, the dietary addition of RPG was 1%, and the addition of TAU was 5 g/d; LRHT, the dietary addition of RPG was 1%, and the addition of TAU was 15 g/d; HRLT, the dietary addition of RPG was 3%, and the addition of TAU was 5 g/d; and HRHT, the dietary addition of RPG was 3%, and the addition of TAU was 15 g/d. In the "R" to the right of this line, "LR" refers to LRLT and LRHT, while "HR" refers to HRLT and HRHT; In the "T," "LT" refers to LRLT and HRLT, while HT refers to LRHT and HRHT. [3]"R" means the RPG; "T" means the TAU; and "R*T" means the interaction between RPG and TAU. [a,b]Means within a row with different superscripts differ significantly ($p \leq 0.05$).

TABLE 10 Effects of RPG and TAU levels in the diet on the plasma stress response indicators of yaks[1] after transportation.

Items	Treatment[2]				SEM	R		T		*P*-value[3]		
	LRLT	LRHT	HRLT	HRHT		LR	HR	LT	HT	R	T	R*T
COR (ng/mL)												
10 d	218.9	208.7	216.0	200.8	7.96	213.8	208.4	217.4	204.7	0.39	0.03	0.15
30 d	191.5	182.1	189.9	181.3	6.35	186.8	185.7	190.7	181.7	0.82	0.05	0.29
LPS (EU/L)												
10 d	408.7	367.9	384.9	357.1	21.8	388.3	371.0	396.8	362.5	0.31	0.09	0.14
30 d	361.7	346.7	364.3	330.7	19.4	354.2	347.5	363.0	338.7	0.65	0.08	0.32

[1]Values are least squares means of eight replicate yaks ($n = 8$). [2]LRLT, the dietary addition of RPG was 1%, and the addition of TAU was 5 g/d; LRHT, the dietary addition of RPG was 1%, and the addition of TAU was 15 g/d; HRLT, the dietary addition of RPG was 3%, and the addition of TAU was 5 g/d; and HRHT, the dietary addition of RPG was 3%, and the addition of TAU was 15 g/d. In the "R" to the right of this line, "LR" refers to LRLT and LRHT, while "HR" refers to HRLT and HRHT; In the "T," "LT" refers to LRLT and HRLT, while HT refers to LRHT and HRHT. [3]"R" means the RPG; "T" means the TAU; and "R*T" means the interaction between RPG and TAU.

($p = 0.03$) and the interaction between RPG and TAU ($p = 0.04$) had significant effects on plasma GSH-PX activities at day 10 d after transport, which was lower in HRHT than in LRLT.

3.5 Plasma immune in yaks after transportation

Table 9 shows the effects of dietary RPG level and TAU supplementation on the plasma immune index of yak after transportation. Increasing TAU supplementation decreased plasma IL-1β concentrations at day 30 after transport ($p = 0.02$), while increased plasma IL-4 concentrations at day 10 after transport ($p = 0.02$). Besides that, plasma immune index did not differ at all treatments ($P > 0.05$).

3.6 Plasma stress response indicators in yaks after transportation

As shown in Table 10, the RPG and TAU did not change the plasma LPS concentration of yaks after transport ($P > 0.05$). However, the plasma COR concentration in yaks decreased at 10 ($p = 0.03$) and 30 ($p = 0.05$) days after transport with the increase of TAU addition.

4 Discussion

Harsh conditions during transportation, like noise and overcrowding, can induce oxidative stress in animals, resulting in weight loss. Lendrawati et al. (17) reported that transport reduces the weight of sheep, and the weight loss increases with the longer the

transport time. González et al. (18) reported that while rearing patterns, age, transportation time, and truck facilities all affect the weight of cattle transported long distances, these tests all show a decrease in weight. In our study, all yaks experienced weight loss after 9 h of long-distance transport, and even supplementation with high levels of RPG and TAU could not reverse this outcome. However, yaks fed high supplemental TAU had lower weight loss (8.42 vs. 11.9 kg) and lower weight loss percent (4.83 vs. 6.87%), which was closely related to the biological function of TAU. Mice with inadequate TAU intake reportedly exhibited low body weight, slower responses to stimuli, and exercise intolerance, indicating that TAU was crucial for maintaining body weight and muscle endurance (19). Kim et al. (20) demonstrated the fatigue resistance of TAU following acute exercise, which were attributed to increased oxygen uptake and enhanced lipid oxidation in a mouse model. Taurine is also a promoter of energy metabolic balance (13) and may help increase energy supply of yak during transport. In addition, feeding TAU can improve the performance of animals (16, 21). However, we found that the growth performance of yaks within 30 days after transport did not change due to the increase in TAU and RPG. One possible reason is that this study focused more on the dosage of RPG and TAU rather than whether they were added, which may limit the comparison of this work with other studies and will be refined in future work.

Plasma TP is clinically significant for assessing liver synthesis function, nutritional status, and water balance. Mufarrej et al. (22) observed that transport increased the levels of TP and albumin in plasma of lambs. In our study, long-term transport may promote the fluid loss in yaks, causing blood to become concentrated and plasma TP levels to rise. Plasma TC concentration is commonly used to evaluate lipid metabolism and diagnose certain diseases (23, 24). We observed an increase in plasma TC concentration post-transport, likely due to the damage and dysfunction of cell membranes caused by lipid peroxidation, which affects the metabolism of lipoproteins and thus indirectly affects the TC in plasma. Plasma levels of LDH and CK are considered biomarkers of muscle injury (25, 26). Fang et al. (27) reported that transport induced an increase in plasma LDH concentration and changes in other blood indicators, suggesting the occurrence of oxidative stress. Alcalde et al. (28) also reported that road transportation, irrespective of the duration, consistently increased plasma CK levels in suckling goat kids, suggesting significant stress and muscle damage. Similarly, we observed elevated levels of LDH and CK in yak plasma post-transport, indicating potential health risks for yaks.

Stress during transport negatively affects the antioxidant system in animals, resulting in reduced antioxidant enzyme activity, which hinders the effective removal of free radicals and peroxide products from the body (29, 30). MDA is the end product of lipid peroxidation, shows a positive correlation between its plasma concentration and the severity of oxidative stress (31). Transport leads to increased plasma MDA concentration, indicating the onset and progression of oxidative stress (32, 33), which is also supported by our study findings. We observed that increasing TAU levels reduced plasma MDA concentration in yaks after transport, mitigating transport stress. Han et al. (16) found that TAU alleviates oxidative damage by increasing gene expression of NRF2, GPX and HO-1. Seidel et al. (34) also found that TAU reduces cellular stress levels, possibly by upregulating iron-related proteins like iron storage protein, thereby inhibiting lipid peroxidation. Therefore, we speculated that increasing TAU levels alleviated transport stress in yaks by inhibiting lipid peroxidation, and the specific mechanism needs to be further explored. GSH-PX is crucial for cellular antioxidant defense as it eliminates ROS from the body. Han et al. (35) indicated that TAU supplementation increased the glutathione contents and the GSH-PX activity, alleviating the oxidative stress induced by LPS. In our study, at 0 and 10 days after transport, increased TAU supplementation elevated plasma GSH-PX activity, implying an enhanced antioxidant capacity in yaks. It is to be noted that the plasma antioxidant capacity differences among treatments diminished over time after transport, possibly because the antioxidant function of TAU was activated in majority cases when exposed to oxidative stress risk.

During oxidative stress, the body generates a surplus of ROS, which activate the inflammasome and trigger the release of numerous pro-inflammatory cytokines, leading to cellular damage (36). Transport increased the concentration of proinflammatory cytokines like TNF-α and IL-1β in animal plasma (37, 38). However, in our study, transport did not change the concentration of IL-1β, IL-6 and TNF-α, while decreased the concentration of anti-inflammatory factors like IL-10, which also means that the health status of yaks may be potentially damaged. In addition, we found that increasing TAU supplementation decreased plasma IL-1β concentrations at 30 day after transport and TNF-α concentrations at 0 day after transport. TAU has been demonstrated to mitigates the adverse effects of LPS by dampening pro-inflammatory responses and oxidative stress (39). Zheng et al. (40) reported that TAU deficiency aggravated the DSS induced inflammatory model in mice, while TAU supplementation reduced proinflammatory factors levels like TNF-α and IL-6 by inhibiting the TLR4/NF-κB pathway. Han et al. (35) also indicated that TAU supplementation decreased the expression of IL-1β, TNF-α, and IL-6 genes, thereby resisting the LPS-induced inflammatory response in broilers. The release of pro-inflammatory cytokines accompanies the onset of inflammation, and over time, the pathogen is cleared by the body, and anti-inflammatory cytokines then come into play to repair the damaged tissue (41). TAU has a positive effect on the formation of the immune system of body (42). Zhou et al. (43) reported that TAU increases IL-10 concentration in weaned piglets and enhances immunity against weaning stress and inflammation. Elevated plasma concentrations of IL-4 and IL-10 in yaks were observed following transport when RPG and TAU were combined at high levels, indicating enhanced immunity, yet these levels stabilized after 30 days of feeding. It is likely that TAU exerts an anti-inflammatory effect during transport stress occurs, but the influence wanes when transport stress diminishes or even disappears.

The COR is an important glucocorticoid, and its concentrations serve as a crucial index for assessing stress responses, with their dynamic changes being closely tied to stress progression (44). Lee et al. (45) reported that road transport increased the plasma COR concentration of dairy cows. In our study, the concentrations of COR and LPS in yak plasma increased after transport, indicating the occurrence of transport stress. We also observed that the increase of TAU supplementation decreased the COR concentrations in yaks throughout the experiment, which suggested that TAU could mitigate transport stress in yaks, with a lasting beneficial effect (30 d). LPS stimulates the immune system, thereby triggering inflammation. He et al. (46) reported that transport impaired morphology and tissue in

jejunum, induced stress responses, and elevated LPS concentrations. In our study, the LPS concentration of yaks decreased before and after transport with the increase of TAU addition, which was beneficial to the health of yaks after transport.

5 Conclusion

Transport induces oxidative stress in yaks, which can lead to reduced body weight and health impairment. TAU mitigates the adverse effects of transport on yak weight. Increasing TAU levels enhanced antioxidant capacity and immunity of yaks after transport, alleviating their oxidative stress, but this beneficial effect was weakened with time after transport. RPG had less effect on transport stress than TAU. Considering both cost and efficacy, the recommended dietary inclusion of RPG for yaks is 1%, with a TAU supplement of 15 g/d to mitigate transport stress. This work presents an effective strategy for mitigating transport stress, which is of great significance for off-site fattening of grazing yaks in the cold season.

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 approved by the Experimental Animal Committee of the Animal Nutrition Institute at Sichuan Agricultural University. The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

XW: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Supervision, Writing – original draft, Writing – review & editing. KZ: Formal analysis, Investigation, Methodology, Supervision, Writing – review & editing. SZ: Supervision, Writing – review & editing. JZ: Supervision, Writing – review & editing. MC: Supervision, Writing – review & editing. LL: Supervision, Writing – review & editing. YC: Supervision, Writing – review & editing. HY: Supervision, Writing – review & editing. BZ: Supervision, Writing – review & editing. CS: Supervision, Writing – review & editing. YZ: Supervision, Writing – review & editing. RT: Supervision, Writing – review & editing. BX: Methodology, Project administration, Resources, Supervision, Validation, Writing – review & editing.

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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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The roles of phytogenic feed additives, trees, shrubs, and forages on mitigating ruminant methane emission

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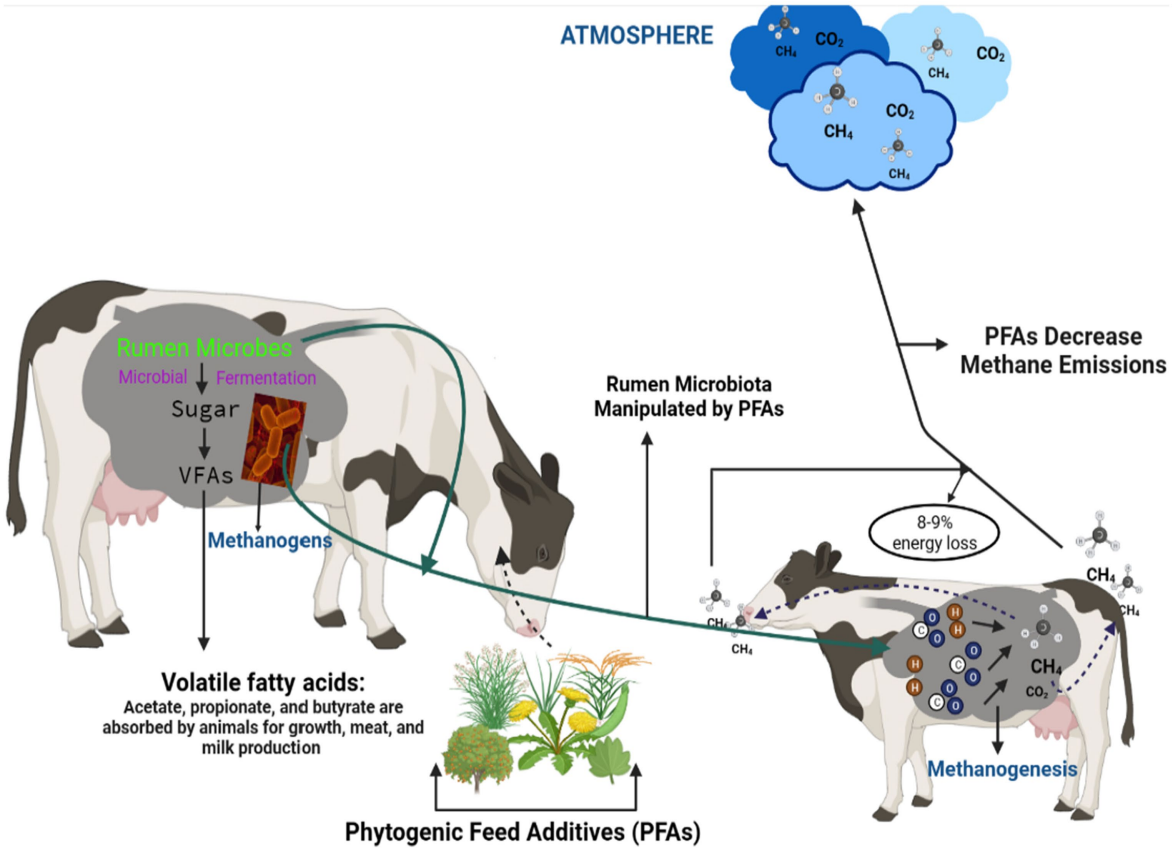
Ruminant animals naturally emit methane gas owing to anaerobic microbial fermentation in the rumen, and these gases are considered major contributors to global warming. Scientists worldwide are attempting to minimize methane emissions from ruminant animals. Some of these attempts include the manipulation of rumen microbes using antibiotics, synthetic chemicals, dietary interventions, probiotics, propionate enhancers, stimulation of acetogens, manipulation of rumination time, vaccination, and genetic selection of animals that produce low methane (CH_4). The majority of synthetic additives are harmful to both beneficial rumen microbes and the host or only temporarily affect methanogenesis. Phytogenic feed additives (PFAs) have recently emerged as the best alternatives to antibiotics and synthetic chemicals because of growing public concerns regarding drug resistance and the negative impacts of antibiotics and synthetic chemicals on humans, livestock, and the environment. These additives reduce methane production and improve the volatile fatty acid profile. In this review, we provide an overview of PFA sources and how their bioactive components affect the rumen microbiome to reduce methane emissions. Additionally, we highlight the mechanisms of action of PFAs as a whole, as well as some of their bioactive components. We also review some selected trees, herbs, shrubs, and forages and their roles in reducing methane emissions.

KEYWORDS

global warming, microbial fermentation, phytogenic feed additives, rumen microbiome, rumen manipulation, rumen microbe, bioactive material



GRAPHICAL ABSTRACT

1 Introduction

The world's human population is anticipated to reach almost 10 billion people by the year 2050; therefore, an increase in ruminant animal production is necessary to meet the demand for animal protein needs of humans by supplying daily meat and dairy products worldwide (1). This has resulted in the intensification of agriculture, especially livestock production, and consequently inflated the global index of methane (CH_4) produced by livestock by almost 2.5-fold (2). Methane accounts for 16% of the global greenhouse gas emissions. It is estimated that ruminant animals contribute to 33% of the global methane emissions index (3). Approximately 81 million tons of enteric methane is produced annually by livestock worldwide. It is primarily emitted from the rumen and lower digestive tract when carbohydrates are fermented by microbes (4). These animals are among the largest producers of enteric methane, and they contribute to global warming by adding greenhouse gases to the ozone layer. This process is gaining attention worldwide for identifying rumen microbes that are important for methane production to develop the best methane mitigation strategy (5). Rumen fermentation produces a variety of beneficial products, including methane. Cattle alone contribute 15–20% of the global methane production every year (6). Methane is the most abundant hydrogen sink synthesized by methanogens in the rumen. In addition to contributing to global warming, enteric methane emissions contribute 8–9% of the total energy lost by ruminants, which, if not lost, can be used by animals for growth, meat, and milk production (7).

Most work done to reduce methane emissions in the 1950s focused on reducing feed energy loss, whereas recent efforts have focused on both energy savings and their effects on climate change. Despite the success of manipulating rumen fermentation using antibiotics and ionophores, their use has been limited by environmental and human health concerns (8). Because phytogenic feed additives (PFAs; additives derived from plants) contain many bioactive compounds, unlike antibiotics and ionophores, the global scenario has shifted toward the use of phytogenic feed additives rather than antibiotics or ionophores. This compound is capable of manipulating the microbiota in the rumen through more potent mechanisms of action, including inhibition of the activities of protozoa, methanogenic archaea, and some fiber degraders through its antimicrobial potential and decreasing hydrogen availability (9). PFAs have been reported to manipulate ruminal fermentation and to successfully reduce methane emissions from ruminants (10).

PFAs are increasingly being used in animal nutrition because of the negative effects of antibiotics and synthetic chemicals (11). These additives have sparked interest because of their potential to improve nutrient utilization and promote health (12, 13). PFA comprises various phytochemicals that are biologically active during fermentation. Various metabolic pathways are believed to mediate their antimicrobial, metabolic, immune, and antioxidant effects (14).

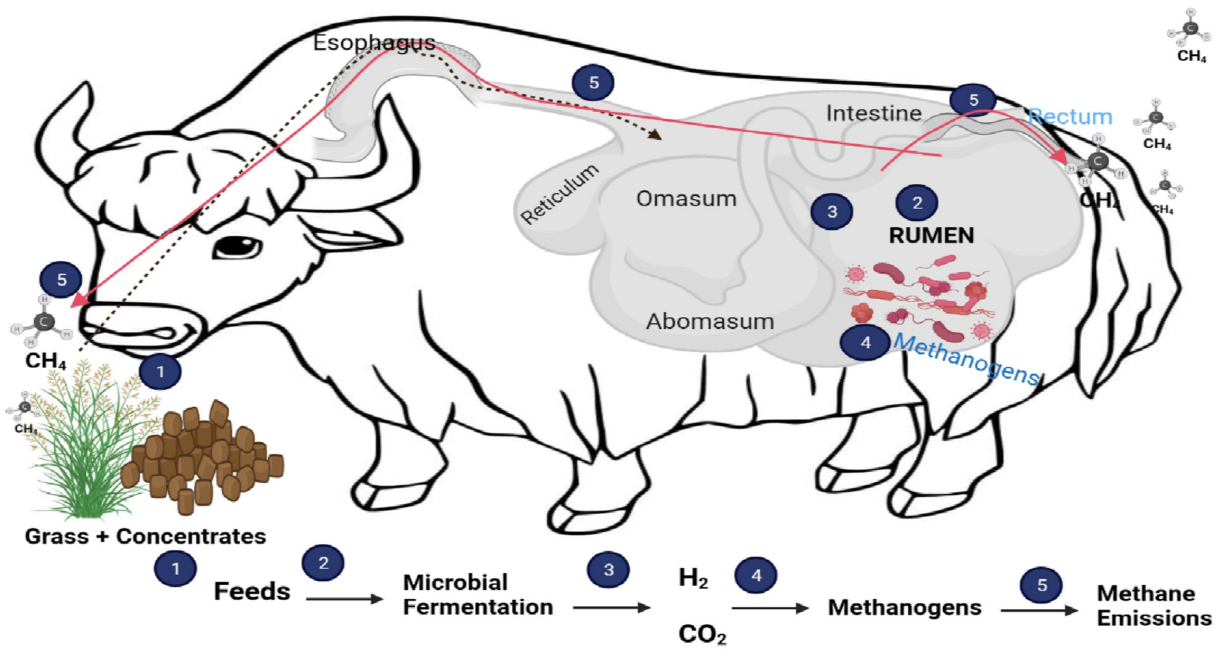


FIGURE 1
Process of methane production and role of rumen microbes.

PFAs have been tested in various ruminant models to manipulate enteric fermentation (15). The use of plant bioactive compounds (PBC) such as tannins, saponins, and essential oils for methane mitigation has been reviewed; however, most studies have focused on PBC rather than providing insight into the sources of these compounds. However, given the current trend and importance of research on climate change and global warming, more research and review are required. In this review, we explore the impact of PFAs on reducing methane emissions, with an emphasis on their effects on rumen ecology as well as the possible underlying mechanisms and factors affecting these effects.

2 Insight into the role of rumen microbial ecology on methanogenesis

Rumen microbes and ruminant animals have a symbiotic relationship. These microbes obtain their substrate when ruminant animals ingest feed and, in return, ferment the feed and supply valuable nutrients to the host, producing methane as a byproduct (Figure 1) (16). The microbial community in the rumen is one of the most diverse gut ecosystems hitherto described in the animal kingdom. It consists of anaerobic bacteria (10^{10}–10^{11} organisms/mL), archaea (10^{8}–10^{9} organisms/mL), ciliated protozoa (10^{5}–10^{6} organisms/mL), anaerobic fungi (10^{3}–10^{4} organisms/mL), and viral community that is largely uncharacterized (17). To date, only a few of these microbes in microbial ecologies have been cultured and characterized (18). The use of culture-based approaches to study ruminal content has decreased in recent years. However, the introduction of high-throughput sequencing techniques has allowed us to gain a better understanding of the rumen microbiome in different diets, species, and geographical locations (19). These advancements can provide a deeper understanding of the diverse microbial species in the rumen ecosystem. Using metagenomics, it will be much easier to determine which rumen microbial community is responsible for methane production. This information will enable scientists to develop the best methane mitigation strategy, which in turn will reduce the negative impacts of ruminant animals on the environment.

3 Phytogenic tools for reducing methane emissions and their effects on ruminal microbial ecology

Since the 18th century, the loss of energy in the rumen as CH_4 has been well-documented in a journal titled "Zeitschrift für Biologie" (Journal of Biology), written by German Scientist Tappeiner in 1884 (20). Since then, scientists worldwide have been working to reduce CH_4 emissions without affecting livestock growth and productivity. Owing to the greenhouse gas potential of CH_4 and the importance of ruminant contributions, policymakers worldwide are currently seeking effective mitigation strategies. In recent years, numerous studies have been conducted to reduce ruminant CH_4 emissions (21).

Recently, PFAs have attracted the attention of researchers worldwide. These additives have been reported to increase feed conversion efficiency; enhance growth, productivity, and animal health; and reduce CH_4 emissions (22). PFAs have been tested by scientists and found to significantly reduce CH_4 emissions, manipulate rumen microbial ecology, and change the fermentation dynamics of ruminants (Figure 2) (23). These additives include; plants, part of plants, plant oil extracts, trees, shrubs, grasses, and legumes. These PFAs are rich in plant bioactive compounds (PBC) such as saponins, tannins, organosulfur compounds, essential oils, flavonoids, propolis, terpenes, and glycosides.

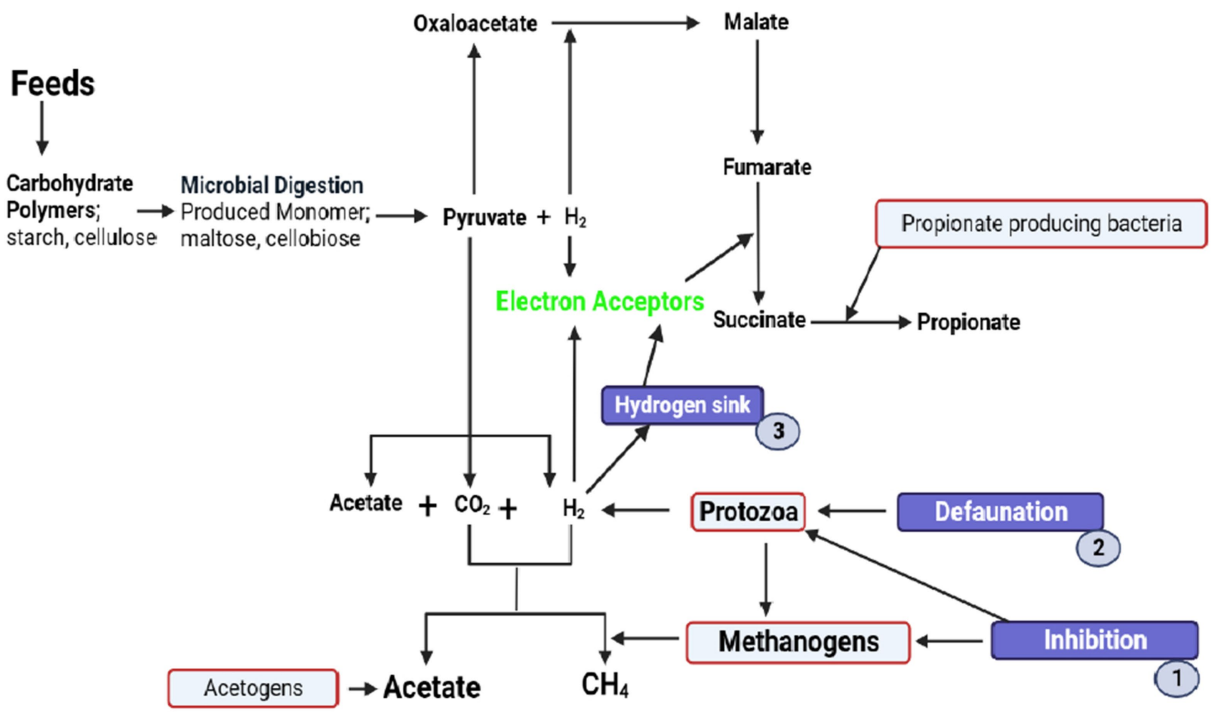


FIGURE 2
Biochemical pathways of methanogenesis affected by plant bioactive compounds to decrease methane production in the rumen. These bioactive compounds inhibit the activity of methanogens and protozoa (1). This bioactive compound decreases the number of protozoa (defaunation) (2). They also induce the rechanneling of metabolic hydrogen from CH_4 to propionate (3).

3.1 Mechanism of action of phytogenic feed additives on rumen microbial cells

Compared to antibiotics, PFAs have a greater potential to modify the ruminal microbiome and reduce methane emissions by disrupting cell membranes, modulating signal transduction and gene expression pathways, inhibiting enzyme activity, and inhibiting bacterial colonization (24). Generally, PFAs increase the permeability and fluidity of cellular membranes, resulting in the efflux of metabolites and ions and leading to cell leakage and microbial death (Figure 3). Moreover, they can manipulate the rumen metabolism by increasing the permeability of a specific group of rumen bacteria (25). There are several possible mechanisms of action, including disruption of the cytoplasmic membrane, disruption of the proton motive force, electron flow, active transport mechanisms, and coagulation of the cell composition (26).

3.2 Phytogenic feed additives: sources and effects

3.2.1 Trees, shrubs, and forages

Several trees, shrubs, and forages are used for methane mitigation because they are rich in bioactive compounds that can suppress methanogenic activity. Trees, shrubs, and forages contain many bioactive compounds including terpenes, saponins, cyanogenic glycosides, flavones, isoflavones, tannins, coumarins, and other phenolics (27). In addition to these, trees, shrubs, and forages provide an excellent source of protein. Therefore, feeding trees, shrubs, and forage can be beneficial protein sources and methane reducers (28). Bioactive compounds in these plants can manipulate the rumen microbial ecosystem, thereby reducing methane production (29). The mechanism of action of these bioactive compounds could have direct effects on methanogens, anti-protozoal effects (defaunation), or inhibition of fiber digestion, followed by a lower H_2 supply to the methanogens (Table 1).

3.2.2 Trees and shrubs

3.2.2.1 Gliricidia (*Gliricidia sepium*)

Gliricidia is a member of the family *Fabaceae* (legume family), subfamily *Faboideae* (*Papilionoideae*), and tribe *Robinieae*. Medium-sized semi-deciduous trees with broad canopies, native to Central America and perhaps northern South America, typically grow to a height of 10 m (occasionally 15 m) (30). *Gliricidia* foliage is rich in tannins and saponins, making it useful for mitigating methane emissions (31). The effect of long-term supplementation with *G. sepium* foliage reduced CH_4 production in heifers, and this response persisted over time, without affecting the microbial population and VFA concentration and a slight reduction in CPD digestibility (32). Zain et al. (33) reported that 30% supplementation with *Gliricidia sepium* decreased methane gas production (from 27.22 mM to 13.13 mM) and the number of protozoa (from 6.3 × 105 cell/ml rumen fluid to 4.7 × 105 cell/mL rumen fluid) while increasing digestibility and rumen fermentation parameters. A diet supplemented with 20% *Gliricidia sepium* leaf meal has the potential to modify rumen fermentation, resulting in improved post-ruminal nutrient utilization (34).

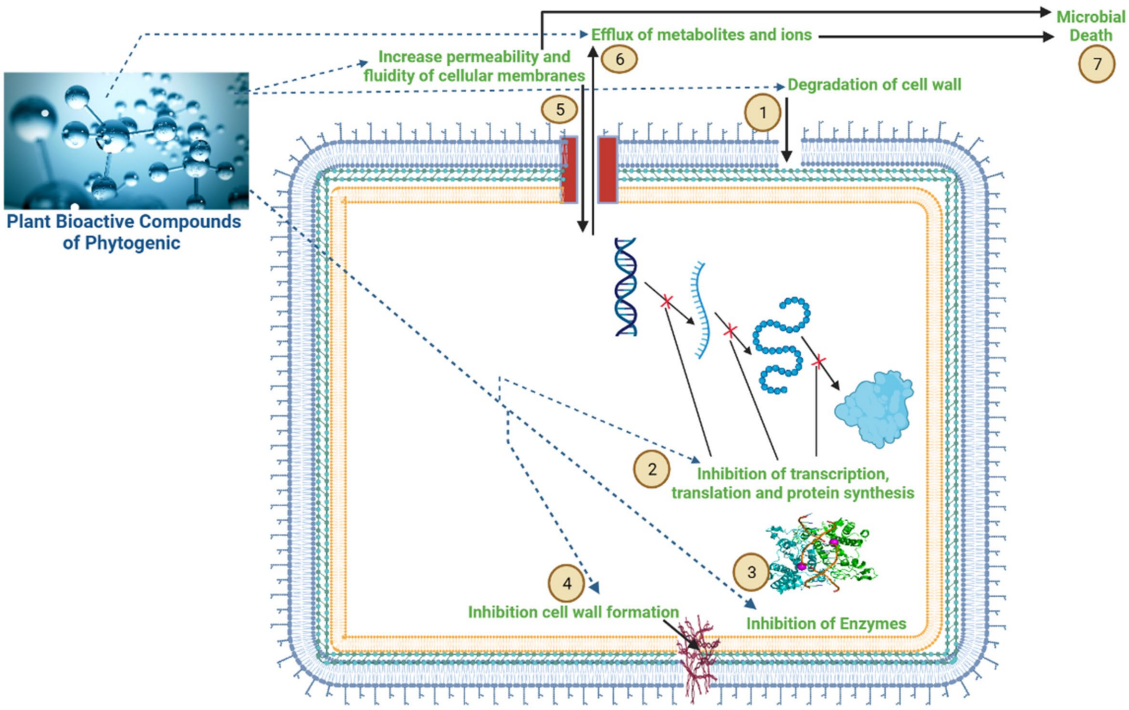


FIGURE 3
Mechanism of action of the bioactive compounds of phytogenic feed additives (PFAs) on rumen microbial cells. The bioactive compounds of phytogenic feed additives have a greater potential to modulate the ruminal microbiome and reduce CH_4 emissions, as indicated in figure: (1) disruption of cell membranes; (2) inhibition of gene transcription, translation, and protein synthesis; (3) inhibition of enzyme activity; (4) inhibition of cell wall formation; (5) increasing permeability and fluidity of cellular membranes; (6) resulting in an influx of metabolites and ions, leading to cell leakage; and (7) microbial death.

The incorporation of *Gliricidia sepium* into animal diets reduces *in vitro* methane production and the population of ruminal protozoa (35).

3.2.2.2 Calliandra (*Calliandra calothyrsus*)

Calliandra are shrubs native to the American continent that belong to the *Mimosoideae* family. These shrubs are rich in tannins (36). *Calliandra calothyrsus* is notable for its high tannin content, making it a significant candidate for mitigating methane emissions (37). Tiemann et al. (38) reported that adding tannin-rich *Calliandra* plants reduced methane emissions by 24% per day as well as per unit of feed and energy intake. They believed that the mechanism of this reduction was a reduction in the available H_2 required for methane production by donating electrons to H_2 to form a stable radical. *In vitro*, supplementation with *C. calothyrsus* reduces methane production without any negative effects on rumen fermentation parameters (37). According to Ridwan et al. (39), 50% silage containing *C. calothyrsus* decreased enteric CH_4 production by reducing the total number of methanogens and goats supplemented *Methanobacteriales* but decreased bacterial diversity and organic matter digestibility. PE dairy with *C. calothyrsus* had reduced enteric methane emissions and improved milk production (40). Mwangi et al. (41) reported that replacing 40% of a protein-deficient basal diet with *Calliandra calothyrsus* reduces enteric methane emissions in both absolute terms and intensity. *Calliandra calothyrsus*, when used as a two-thirds replacement for protein in lamb diets, partially reduces methane emissions due to associated reductions in N and energy retention (38).

3.2.2.3 Mulberry (*Morus alba*)

Mulberry is a fast-growing deciduous tree of the *Moraceae* family that is native to India and China's Himalayan foothills (42). The leaves of mulberry trees are widely used as livestock feed because of their high crude protein content and metabolizable energy. In addition, they are rich in flavonoids, a plant bioactive compound known to reduce enteric methane emissions (43). *Morus alba* is significant in reducing rumen methanogenesis because it contains long-chain unsaturated fatty acids that can effectively decrease methane production in the rumen (44). The *in vitro* supplementation of mulberry leaf flavonoids at a concentration of 15 mg/100 g decreased methane emission, improved dry matter digestibility, and improved the Total Volatile Fatty Acids (TVFA) profile of sheep (45). Adding 300 g of *Morus alba* to a dairy cow's diet changed the microbial community and fermentation process in the rumen, which increased propionate production and reduced methane emissions (46). *Morus alba* is a promising candidate for reducing enteric methane emissions while providing an optimal level of nitrogen when used as a supplement to low-quality forages (47). Considering its potential, further research is needed to test its effect on reducing methane emissions while improving the TVFA profile of ruminants.

TABLE 1 Mechanism of actions of some phytogenic feed additives sources on methane emissions.

Sources of PFAs	Plant bioactive compounds (PBC)	Mechanism of mitigation	References
Carduus pycnocephalus	Essential oil, Flavonoids, Terpenes	Inhibit methanogens	Bodas et al. (53)
Paeonia lactiflora	Total glucosides of peony	Inhibition of Gram-positive bacteria	Castillo-González et al. (58)
Leucaena leucocephala	Tannins	Reduction in the total number of methanogens and protozoa	Tan et al. (64)
Brassica	Glucosinolates	Alter the mean retention time of digestion in the rumen	Sun (66)
Rapeseed oil	Sterols and tocopherols	Decrease protozoal population in the rumen	Villar et al. (96).
Camelina sativa oil	Unsaturated fatty acids and antioxidant	Inhibiting rumen protozoa and methanogens. Hydrogen sink	Hassan et al. (15)
Garlic oil	Organosulfur	Reducing the abundance of protozoa	Kongmun et al. (108).
Palm oil	Fatty acids	Decrease the number of ciliate protozoa	Yilmaz and Kara (111)

3.2.2.4 Italian plumeless thistle (*Carduus pycnocephalus*)

This plant belongs to the *Astraceae* family and genus *Carduus* (48). This plant is also popularly used in Traditional Chinese Medicine to treat various human diseases, such as colds, rheumatism, and stomachache (49). *Carduus* contains numerous classes of phytochemicals, including lignans, flavonoids, alkaloids, sterols, triterpenes, coumarins, essential oils, hexadecanoic acid, sterols, and triterpenes (50). The leaves of *C. pycnocephalus* contain tannins and saponins (51, 52). *C. pycnocephalus* decreases methane production in a hay-based diet while improving microbial protein synthesis in dairy cattle (51, 52). An *in vitro* screening of 450 plants for their potential anti-methanogenic effects concluded that *C. pycnocephalus* was the first among the six selected species, and had the potential to reduce methane emissions by more than 25% without adverse effects on digestibility, total volatile fatty acids, and gas and production (53). Owing to the antimicrobial properties of *C. pycnocephalus,* its mode of action in reducing methanogenesis may be its effect in reducing the number of rumen methanogens.

3.2.2.5 Chinese peony (*Paeonia lactiflora*)

Chinese peony (*Paeonia lactiflora*) is commonly known as chishao (赤芍) in China. More than 1,200 years ago, *P. lactiflora* root was used in Traditional Chinese Medicine (54). Glucosides of Peony, or Total Glucosides of Peony (TGP), are extracted from *P. lactiflora* and contain almost 15 components, including albiflorin, benzoyl paeoniflorin, galloylpaeoniflorin, lactoferrin, oxybenzone-paeoniflorin, oxypaeoniflorin, paeony, phenol, phonolite, paeoniflorin, paeoniflorin, paeoniflorin, paeoniflorin, and paeoniflorin (55). The structures of most of these extracts are monoterpene glucosides, among which paeoniflorin is a water-soluble compound, the most abundant (>90%) has a molecular weight of 480.45 and has the highest pharmacological effects among all TGP in both *in vitro* and *in vivo* studies (54). *P. lactiflora* extracts have anti-methanogenic effect (56). *P. lactiflora* reduces methane emissions by 8–53% in cattle (57). Methane reduction is caused by the inhibition of gram-positive bacteria (58). Considering its potential antimicrobial and anti-methanogenic effects, this plant requires further investigation.

3.2.2.6 Leucaena (*Leucaena leucocephala*)

The Leucaena tree belongs to the family Mimosaceae, genus; *Leucaena* and the best-known species is *Leucocephala* it has many common names Worldwide, in China, it is called "Yin ho huan" (59). Phytochemical analysis of *Leucaena* leaves revealed the presence of almost 30 compounds including tannins, squalene, phytol, phylobatanins, alkaloids, cardiac glycosides, flavonoids, saponins, and glycosides (60). Phytochemicals in Leucaena have been shown to have several anti-methanogenic effects (60). *Leucaena* decreased methane production in crossbred cows housed in an open-circuit respiration chamber (61). However, this treatment did not affect the microbial community. Supplementation with *Leucaena* decreased methane emissions by up to 20% in Colombian Lucerna heifers (62). Another 20% decrease in methane emissions has been reported in grazing cows consuming leucaena pastures in Australia (63). Leucaena is a major source of condensed tannins (CT), and *in vitro* studies of CT extracts from Leucaena resulted in 99 and 83% reductions in the total number of methanogens and protozoa, respectively (64).

3.2.3 Forages

3.2.3.1 Brassica forages

In temperate countries, Brassica is an annual plant that has been traditionally used in grazing systems to cover periods of feed deficits for ruminants. Brassica forage crops have four main types that are usually used worldwide to provide food for ruminant livestock feeds during shortage, this includes; kale (*Brassica oleracea* spp. *acephala*), turnips (*Brassica rapa* spp. *rapa*), swedes (*Brassica napus* spp. *napobrassica*), and forage rape (*Brassica napus* spp. *biennis*) (65). Brassica leaves, stems, bulbs, and roots are used as phytogenic additives (66). They contain bioactive compounds such as S-methyl-cysteine sulfoxide (SMCO) and glucosinolates (65). Both SMCO and glucosinolates reduce the available H_2 for methane production through hydrogen sulfide scavenging mechanisms (67). Brassica forages were reported to reduce CH_4 emissions in sheep by 37%; however, the experiment did not examine the effect on rumen microbial ecology (68). However, dairy cows fed Brassica forage did not show any methane mitigation effects, and the protozoal count did

not significantly differ from those fed 250 g/kg DM grains as a control diet (69). This may be due to species variation or the methane measurement method used. However, Sun (66) reported that *Brassica* is rich in glucosinolates (GSLs) when ruminants consume Brassica forages, which are broken down in the rumen, resulting in absorption into the blood, which stimulates the secretion of thyroid hormone FT3 in ruminants, and the altered thyroid hormone concentration changes rumen physiology. This would alter the mean retention time of digestion in the rumen, resulting in a reduction in methane emissions.

3.2.3.2 Alfalfa (*Medicago sativa* L)

Alfalfa (*Medicago sativa*), also known as lucerne, is a perennial flowering legume belonging to the *Fabaceae* family. It is the most important legume forage species in the world (70). Alfalfa is rich in saponins, which are known to reduce methane emissions. Kozłowska et al. (71) reported that ensiled Verko and Kometa alfalfa varieties (rich in saponins) reduced methane production without adversely affecting fermentation parameters. Dietary inclusion of alfalfa hay in crossbred Simmental cattle feed improves nitrogen utilization efficiency and reduces methane emissions (72). Sheep-fed alfalfa hay as a substitute for concentrate decreases CH_4 emissions, digestibility, and urinary N and NH_4+-N outputs (73). A study conducted by Hironaka et al. (74) indicated that cattle fed pelleted alfalfa hay produced less methane than those fed chopped alfalfa hay.

3.2.3.3 Clover (*Trifolium species*)

Clover belongs to the *Fabaceae* family, genus *Trifolium*, and has approximately 240 species distributed over the temperate and subtropical regions of the Mediterranean Basin, western North America, and eastern Africa (75). Several flavonoids, saponins, chloramines, and phenolic acids have been found in *Trifolium* plants (76). Methane yield (g/kg DM) was significantly lower in cattle-fed red clover silage (17.8 ± 3.17) than in those fed grass silage (77). A linear increase in dry matter intake (DMI) and reduced methane output per kilogram of DM consumed were observed when white clover was increased in dairy cattle diets (78). The methane yield per kilogram of dry matter and digestible organic matter intake was lower for heifers fed red and white clover silage (79). Based on *in vitro* experiments, white clover leaves containing soluble CTs of 1.6–2.4% DM reduced methane production by 19% ($p \leq 0.01$) and ammonia production by 60% (80). Dairy cattle fed white clover pastures produce less CH_4 than those fed ryegrass pastures in small-scale dairy systems (81). Navarro-Villa et al. (82) reported that red clover showed reduced *in vitro* rumen methane output compared to that of perennial ryegrass. In a study using portable accumulation chambers, sheep that graze subterranean clover produced lower daily CH_4 emissions (23.5 g/day) compared with sheep grazing lucerne (27.3 g/day) and perennial ryegrass (32.3 g/day) (83).

3.2.3.4 Chinese Lespedeza (*Sericea lespedeza*)

Lespedeza is a perennial herb in the family *Leguminosae* (*Fabaceae*), which is native to Japan, the Korean Peninsula, China, the Himalayas, Afghanistan, and Malaysia. Lespedeza is rich in condensed tannins and other phenolics (84). Regardless of the feeding level, goats fed CT-containing Lespedeza forage showed decreased CH_4 emissions (85). Substituting *Eragrostis curvula* hay with 60% *S. lespedeza* on a DM basis resulted in the greatest reduction in CH_4 yield (21.4%) compared to a diet of 100% *Eragrostis curvula* (86). Dietary inclusion of *S. lespedeza* increases propionate production and reduces CH_4 production in the rumen (87). Liu et al. (88) reported that Alpine doelings fed on *S. lespedeza* forage emit less methane compared to the control. A study was conducted to assess nutrient digestibility, volatile fatty acid (VFA) concentrations, microbial protein synthesis, bacterial nitrogen (N) efficiency, and enteric methane (CH_4) production in four grass-legume diets rich in condensed tannins (CT) (alfalfa, birdsfoot trefoil, crown vetch, and *S. lespedeza*). The results indicated that the lowest total CH_4 production was observed in the *S. lespedeza* diet (89).

3.2.4 Plant oil extracts

Plant oil extracts are high in lipids, making them an excellent option for mitigating methane emissions (Table 1). Numerous studies have indicated that the addition of oils to ruminant diets reduces methane production (90). This supplementation decreases the number of protozoa and methanogens in the rumen and bio-hydrogenates unsaturated fatty acids, thereby reducing methane production (91). The shift from carbohydrates to lipids in ruminant diets modifies gas production in the rumen, reducing CH_4 emissions (92, 93).

3.2.4.1 Rapeseed oil

Rapeseed is the third most popular vegetable oil in the world and is extracted from rape (canola) brassica forage. Rapeseed oil is low in erucic acid and glucosinolates (94). The inclusion of rapeseed oil in the diet of dairy cows decreased enteric CH_4 emissions and modified the microbial community structure without affecting the total counts of bacteria, archaea, or ciliate protozoa (95). Cattle supplemented with canola oil (rapeseed oil) and nitrate reduce enteric methane emissions and protozoal populations in the rumen (96). Supplementation of nursing dairy cows with 5% rapeseed oil to nursing dairy cows reduced 23% of CH_4 emissions with no effect on archaea and bacterial abundance (97). Dietary supplementation of rapeseed (41 g oil/kg DM) decreased daily CH_4 emissions from lactating dairy cows by up to 22.5%, which increased the relative abundance of *Methanosphaera* and *Succinivibrionaceae* in the rumen and decreased the abundance of *Bifidobacteriaceae* (98). An *in vitro* experiment reported a decrease in the population of Thermoplasmata archaea (a methylotrophic methanogen) in the rumen after adding rapeseed oil to silage (99). Growing cattle supplemented with a diet containing 46 g of rapeseed oil/kg of diet DM decreased CH_4 emissions, but reduced feed intake (100).

3.2.4.2 *Camelina sativa* oil

Camelina sativa oil is a rich source of unsaturated fatty acids, particularly linoleic, linolenic, and oleic acids (101). The rich unsaturated fatty acid profile of *Camelina sativa* oil makes it a very good supplement for ruminants because it has high-quality fat (102). In addition to being rich in unsaturated fatty acids, it also has a rich antioxidant profile (102). Researchers have established that oilseeds are generally one of the most effective ways to reduce enteric CH_4 production from ruminants, as they can mitigate CH_4 emissions by directly inhibiting rumen protozoa and methanogens while increasing the bio-hydrogenation of polyunsaturated fatty acids to serve as a sink for hydrogen produced by rumen microbes. Supplementation with *C. sativa* oil at different levels in an *in vitro* study significantly decreased CH_4 production (15). Camelina oil at all levels significantly affected ammonia nitrogen and microbial protein

TABLE 2 The effects of plant bioactive compounds (PBCs) on rumen ecology and potential mechanisms.

Plant bioactive compound	Effect on rumen ecology	Potential mechanism	References
Tannin	Bacteriostatic in rumen	Inhibit the activities of rumen microbes	McSweeney et al. (118); Jayanegara et al. (119)
	Reduce fiber digestion in the rumen.	Reduce methanogenesis by decreasing the level of available H_2 needed for the production of methane	Patra (120); Bodas et al. (24)
	Increase in the abundance of butyrate-producing bacteria and other probiotic bacteria, such as *Bifidobacterium* and *Lactobacillusamino*	Decreased the production of short-chain fatty acids like acetate and reduced methane production	Buccioni et al. (121); Correa et al. (122)
	Suppressing the archaea communities and increasing total rumen bacteria populations	Lower methane production	Fagundes et al. (123)
	Suppressing the growth of methanogens directly	Reduce CH_4 production	Aboagye and Beauchemin (124)
	Decreased organic matter digestion in the rumen	Reduce methanogenesis	Grainger et al. (125)
	Decreased the relative abundance of protozoa, methanogens, and *Ruminococcus albus*	Reduce methanogenesis by inhibiting methanogen and protozoal growth	Yang et al. (126); Volpe et al. (127); Witzig et al. (128)
Saponins	Inhibition of protozoal ecology in rumen and other methanogens associated with protozoa	Reduce protozoal population by interaction with sterol moiety present in the protozoa membrane thereby reducing methanogenesis	Patra and Saxena (129); Bodas and Prieto (24); Jayanegara et al. (130); Ramírez-Restrepo et al. (131); Guyader et al. (132); Liu et al. (133); Tan et al. (134)
Essential oil	Alteration of rumen microbial ecology. Inhibit the growth of methanogenic Archaea in the rumen	Inhibit the HMG-CoA reductase, which will lead to membrane instability and ultimately, death of methanogenic archaea cells. Reduce methanogenesis	Patra and Yu (135); Ye et al. (136); Lei et al. (137); Belanche et al. (138)
	Inhibition activity of gram-positive (+ve) and gram-negative (−ve) bacteria	Antimicrobial capabilities are mainly due to their interface with the cell membrane of rumen microbes by disrupting membrane stability of lips bilayers of bacterial cells. This inhibition in the rumen may lead to an increase in propionate levels in the rumen, thereby reducing the rate of methane production	Zengin and Baysal (139); Cobellis et al. (140); Schären et al. (141); Poudel et al. (142)
	Increased the abundance of *Succinivibrio species*, *Bacteroides species,* and *Succinivibrio species* in rumen.	Shift in rumen fermentation pattern, favoring propionate production over acetate. This may reduce methane production	Evans and Martin (143); Lei et al. (137)
Flavonoids	Antimicrobial properties	Their interaction with rumen microbes can decrease the population of methanogenic archaea	Purba et al. (144)
	Increase the abundance of *Fibrobacter succinogenes* diversity and decrease *Ruminoccocus albus* and *Ruminoccocus flavefaciens population*	Create a competition for hydrogen between rumen microbes and other methanogens for VFA production and methanogenesis.	Kim et al. (145)
	Reduce ciliate protozoa and hydrogenotrophic methanogens population	Inhibit methanogenesis	Oskoueian et al. (146); Seradj et al. (147)
Propolis	Reduce the population of methanogenic Archaea	Inhibit methanogenesis	Morsy et al. (148)

TABLE 3 Results from recent research on the effect of plant bioactive compounds (PBC) on methane emission in the rumen.

Sources of PBC	Type of experiment	Dosage	Diet	Methane emission	References
Tannins					
Acacia mimosa Extracts – CT	*In vivo* (6 Cannulated Nellore cattle)	1.25 and 2.25%	Grazing	28%	Fagundes et al. (123)
Extracts of Lipid encapsulated-Acacia Tannin	*In vivo* (4 rumen-cannulated Merino withers)	50 g/kg feed	Eragrotis Lucerne hay	19%	Adejoro et al. (149)
Extracts of Crude-Acacia Tannin	*In vivo* (4 rumen-cannulated Merino withers)	40 g/kg feed	Eragrotis Lucerne hay	30%	Adejoro et al. (149)
Extracts of *Acacia nilotica* Leaves and Pods	*In vitro* (Sheep rumen fluid)	Leaves (187 g/kg DM HT) Pods (350 g/kg/DM HT)	*Acacia nilotica* Leaves and Pods	64%	Rira et al. (150)
Tannin-containing – Birdsfoot trefoil, Sainfoin, and Small burnet	*In vitro* (Heifer)	2.5% CT 4.5% HT	Hay	21–34%	Stewart et al. (151)
Tannic acid	*In vivo* (Beef Cattle)	6.5, 13.0, or 26.0 g/kg DM	Corn silage and concentrate mixture	11.1, 14.7 and 33.6%	Yang et al. (126)
Purified hydrolyzable (chestnut and sumach) and Condensed tannins (mimosa and quebracho)	*In vitro (Cattle)*	0.5, 0.75 and 1.0 mg/mL	70% Hay 30% Concentrate	22–37%	Jayanegara et al. (119)
Saponins					
Tea saponin	*In vivo* (Sheep)	2.0 g/Day	Basal diet	8.8%	Liu et al. (133)
Tea saponin	*In vitro* (Bovine)	0.50 g/L	54% Corn silage 6% Hay 40% Concentrate	29%	Guyader et al. (132)
Extracts of *Yucca schidigera*	*In vivo* (Sheep)	170 mg per day	75% Hay 35% Concentrate	16%	Wang et al. (152)
Extracts of *Knautia arvensis* leaves	*In vitro* (Holstein Cow)	10.2 and 20.4 g/kg	50% Hay 50% Concentrate	5.5 and 6.4%	Goel et al. (51, 52)
Leaves of *Sesbania sesban*	*In vitro* (Holstein Cow)	174 g/kg	32% Hay: 68% Concentrate	12%	Goel et al. (51, 52)
Seeds of *Trigonella foenum-graecum*	*In vitro* (Holstein Cow)	30.4 g/kg	50% Hay 50% Concentrate	2%	Goel et al. (51, 52)
Essential oil					
Essential oil blend	*In vivo* (Dairy cow)	1 g/d/cow	Total Mixed Ration	8.8%	Belanche et al. (138)
Essential oil blend (Coriander, geranyl acetate, and eugenol)	*In vivo* (Dairy cow)	1 g/d/cow	Total Mixed Ration	6%	Hart et al. (153)
Anise oil	*In vivo* (Sheep)	0, 50, 100, 200, 400 mg/L	40% Hay 60% corn-based concentrate	47%	Wang et al. (154)
Garlic oil Eucalyptus oil Origanum oil Clove oil Peppermint oil	*In vitro* (Lactating Jersey Cow)	0.25, 0.50 and 1.0 g/L Fermentation medium	Ground alfalfa hay and concentrate 50% each	22–42% 17–26% 12–86% 11–34% 8–16%	Patra and Yu (135)

CT, Condense Tannins; HT, Hydrolysable Tannins; DM, Dry Matter Intake.

in all rations because it altered the proportions of individual ruminal volatile fatty acids and decreased CH_4 production by altering the total number of bacteria, protozoa, and methanogens (103). Dietary supplements of camelina oil in Finnish Ayrshire cows *in vivo* decreased ruminal CH_4 and CO_2 production, but there was no change in the total number of bacteria, methanogens, protozoa, and fungi in the rumen (104).

3.2.4.3 Garlic oil

Garlic oil was produced from ground garlic cloves and collected as a distillate from the vapor when the cloves were heated at a temperature of 100°C. Garlic oil is a mixture of various bioactive organosulfur compounds; including diallyl disulfide ($C_6H_{10}S_2$), diallyl sulfide ($C_6H_{10}S$), allicin ($C_6H_{10}S_2O$), and allyl mercaptan (C_3H_6S) and others (105). This metabolic profile makes it difficult to determine the exact mechanism of fermentation in the rumen. Both gram-positive and gram-negative bacteria are sensitive to the antibacterial effects of garlic oil (106). In an *in vitro* experiment, garlic oil was reported to decrease methane emissions by 74%, increase propionate and butyrate proportions, and decrease acetate and branch-chain VFA proportions (107). Garlic oils and their components have been found to modify fermentation parameters in the rumen and inhibit methane production by reducing the abundance of protozoa (108).

3.2.4.4 Palm oil

Palm oil is an edible vegetable oil extracted from the fruit of palm oil trees by squeezing or crushing fleshy fruits or kernels. It contains saturated fatty acids (palmitate, 44.3%; stearate, 4.6%; myristate, 1%), monounsaturated fatty acids (oleic acid, 38.7%), and polyunsaturated fatty acids (linoleic acid, α-linoleic acid 10.5%, and others 0.9%) (109). Recent research on supplementation of three levels of palm oil (20, 40, and 60 g/kg) to heifers fed low-quality grass on enteric CH_4 emissions were reported, the results show that total daily CH_4 production decreased by 4% for every 10 g/kg palm oil added while feed conversion efficiency, apparent digestibility, intake of nutrients were not affected by the inclusion (110). It has also been reported that the addition of 4% palm oil decreases *in vitro* methane production and the number of ciliate protozoa (111).

3.3 Mechanism of actions of plant bioactive compounds (PBC) and their role in reducing methane emissions

PBCs are bioactive compounds that have various effects on plants and other living organisms. Many PBCs exhibit anti-methanogenic, antioxidant, antimicrobial, anti-inflammatory, anti-helminthic, anticoagulant, antidiabetic, and lipid-lowering properties (112). They are biologically active metabolites that can exert beneficial effects on methane emission, feed digestion, rumen fermentation productivity, and the health of livestock animals (113).

These compounds were extracted from the plants. These include tannins, saponins, and essential oils that affect methanogenesis by inhibiting the growth, development, and activities of the methanogen population, both directly and indirectly, by reducing the number of protozoa associated with methanogens (Table 2). They may also result in a shift toward propionate production, which reduces hydrogen competition, thereby affecting methanogenesis (23). PBC additives can be used instead of antibiotics in ruminants owing to their antibacterial properties against ruminal bacteria, protozoa, and methanogens (114). These metabolites are believed to have beneficial effects on livestock end products by altering fermentation in the rumen without causing microbial resistance (115).

These phytochemicals can modify the rumen microbiome to alter its physiology because of their excellent antimicrobial activity (116). Numerous experiments on the potential effects of these phytochemicals on fermentation have been conducted both *in vitro* and *in vivo*, and have been found to significantly improve feed digestibility and decrease methanogenesis in the rumen (21). Despite various strategies to modify the microbiome of the rumen, PBC has significant potential to replace antibiotics in modifying rumen ecology and decreasing methane production through various mechanisms used by antimicrobial compounds (117). Some of the recognized mechanisms of action include disruption of proton motive force, disruption of cytoplasmic membranes, active transport mechanisms, coagulation of cell composition, and electron flow (26). PBC also significantly affects rumen microflora, resulting in the modification of fermentation and improved productivity of livestock (117).

Several PBCs, including tannins, saponins, essential oils (EO), flavonoids, and propolis have been found to have a significant impact on methanogens, protozoal population, feed conversion efficiency, absorption, and fermentation parameters as well as reducing CH_4 emissions from animals (15) (see Table 3).

4 Conclusion

This review highlights various phytogenic feed additives capable of changing the rumen microbial ecology and reducing methane production. Trees, shrubs, and legumes are the most effective sources of phytogenic substances that reduce methane while improving the volatile fatty acid profile of ruminants because they contain numerous bioactive compounds. Most of the results in this review are *from in vitro* experiments; however, to understand the efficiency of phytogenic substances and their effects on methanogenesis, animal performance, animal health and welfare, rumen ecology, safety of phytogenic substances, environmental influence, quantity and quality of animal products, and applicability of phytogenic additives, *in vivo* studies over a longer period and across various parts of the world are needed. These are paramount to providing livestock farmers, policymakers, and climate change agencies with reliable information on the precise effect of phytogenic feed additives in reducing methane emissions while improving animal production.

Author contributions

IB: Conceptualization, Methodology, Software, Writing – original draft. WX: Writing – review & editing. XD: Funding

acquisition, Project administration, Supervision, Writing – review & editing.

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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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Evaluation the protective role of baicalin against H_2O_2-driven oxidation, inflammation and apoptosis in bovine mammary epithelial cells

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Mastitis is one of the most common diseases in dairy farms. During the perinatal period, the bovine mammary epithelial cells (BMECs) of High-yielding dairy cows accelerate metabolism and produce large amounts of reactive oxygen species (ROS). It is one of the primary causes of mastitis and will lead to the breakdown of redox balance, which will induce oxidative stress, inflammation, and apoptosis. Baicalin is a flavonoid substance extracted from the root of natural plant *Scutellaria baicalensis*, which has anti-inflammatory, anti-oxidant, anti-viral and other biological functions. In this research, hydrogen peroxide (H_2O_2) was used to construct a mastitis oxidative stress model, and relevant mechanisms were analyzed by immunofluorescence techniques, qRT-PCR and Western Blot to explore how baicalin affects BMECs' oxidative stress and inflammation caused by H_2O_2, as well as to provide new perspectives on the combined application of baicalin in the prevention and treatment of mastitis. The results demonstrated that baicalin treatment could reduce the accumulation of H_2O_2-induced intracellular ROS and decrease the expression of inflammatory cytokines Tumor Necrosis Factor-α (TNF-α), interleukin 6 (IL-6), interleukin-1β (IL-1β) and the apoptosis rate. The inhibitory effect of baicalin on H_2O_2-induced intracellular ROS accumulation and the expression of inflammatory cytokines and apoptotic factors in BMECs was blocked by pretreatment with the Nuclear factor erythroid 2-related factor 2 (Nrf2) inhibitor retinoic acid (RA) prior to H_2O_2 and/or baicalin treatment. In summary, baicalin could served as a natural antioxidant agent to regulate cell apoptosis through its anti-inflammatory, antioxidant and anti-apoptotic effects to combat BMECs damage caused by H_2O_2.

KEYWORDS

baicalin, bovine mammary epithelial cells (BMECs), nuclear factor erythroid 2-related factor 2 (Nrf2), inflammation, oxidative stress, apoptosis

Introduction

Mastitis is one of the main diseases in dairy cows, which not only affects their health but also reduces milk production and quality. Especially during the perinatal period and peak lactation period, the extensive synthesis of breast milk raises the energy requirements of breast tissue. When the synthesis rate of reactive oxygen species (ROS) is greater than

the removal rate of antioxidants, this will cause excessive accumulation of ROS in the body, destroying cell membrane structure and resulting in oxidative stress (1, 2). The excessive accumulation of ROS could also destroy the dynamic balance of the antioxidant defense system, induce cell apoptosis, and increase the incidence of mastitis (3). Hydrogen peroxide (H_2O_2) is an inorganic oxide. Numerous studies have demonstrated that H_2O_2 could induce the overproduction of ROS and initiate oxidative stress through a feedback system involving intricate physiological processes, such as apoptosis and autophagy, and H_2O_2-treated BMECs could more accurately simulate the actual state of oxidative damage and mastitis in dairy cows, with stable properties (4–6). Therefore, the H_2O_2 was used in the current study to construct an oxidative stress model, providing a basis for investigating the potential mechanism and effective intervention measures of cell apoptosis.

The bovine mammary gland epithelial cells (BMECs) are the primary cellular components of the cow mammary gland, not only synthesizing and secreting milk but also playing an important role in the innate immunity of the gland (7). Metabolic activity is enhanced during lactation, and BMECs are highly susceptible to oxidative stress in breast tissue (8). Therefore, it is becoming increasingly important to take measures to reduce oxidative stress damage to BMECs in dairy cows. The nuclear factor erythroid 2-related factor 2 (Nrf2) is a major transcriptional protein regulating oxidative damage. When cells are under oxidative stress, Nrf2 dissociates from Keap1, transported to the nucleus to bind to ARE, activating the expression of downstream antioxidant genes, such as quinone oxidoreductase 1 (NQO1) and heme oxygenase 1 (HO-1) (9, 10). An essential inflammatory signaling pathway, activation of nuclear factor kappa-B (NF-κB), could increase the synthesis of pro-inflammatory proteins such as Tumor Necrosis Factor-α (TNF-α), interleukin 6 (IL-6), and interleukin-1β (IL-β). It has been reported that Nrf2 activation reduces the incidence of inflammatory reactions by inhibiting the expression of IκB (inhibitor of NF-κB) phosphorylation and the NF-κB pathway. However, this effect is eliminated when Nrf2 and HO-1 are inhibited, indicating that Nrf2 signaling plays an indispensable role in NF-κB-mediated inflammation (11, 12). In addition, excessive ROS can cause irreversible cell damage, such as degradation of cell structure and components, as well as DNA damage, leading to cell apoptosis. In addition, excessive ROS could cause irreversible cellular damage, such as degradation of cell structure and components and DNA damage, which leads to apoptosis (13). Therefore, alleviation of oxidative stress is crucial to inhibit inflammation and apoptosis in BMECs, and Nrf2 might be a potential therapeutic target for the reduction of oxidative stress.

Baicalin is one of the flavonoids extracted from the dried root of the herb *Scutellaria baicalensis*, exhibiting antibacterial, anti-inflammatory, antioxidant and anti-tumor properties. Numerous studies have demonstrated that baicalin could reduce malondialdehyde (MDA) content and increase the activity of superoxide dismutase (SOD) and total antioxidant capacity (T-AOC) through the Nrf2 pathway, thereby exerting antioxidant effects (14, 15). By suppressing NF-κB and its downstream inflammatory cytokines, as well as the caspase3 signaling pathways, baicalin could reduce oxidative stress by scavenging ROS and controlling inflammation and apoptosis (16, 17). However, there is still a lack of in-depth systematic studies on whether the effects of baicalin on inflammation and apoptosis are mediated by oxidative stress and whether the above changes also occur in BMECs. Therefore, the purpose of this study was to investigate the effect and mechanism of baicalin on H_2O_2-induced oxidative stress and apoptosis of BMECs.

Materials and methods

Cell culture

The BMECs were obtained from the Physiological and Biochemical Laboratory of Henan Agricultural University and retained the characteristics and functions of the primary cells. Add 10% fetal bovine serum (FBS; Gibco, New York, USA) at 37°C and culture using 5% CO_2 and 1% penicillin/streptomycin/amphotericin B sterile solution (Solarbio, Beijing, China). After 48 h of initial culture, the cells were cleaned with phosphate-buffered saline (PBS; BI, Israel) and the culture medium was removed. Then, the cells were digested with 0.25% trypsin-ethylenediamine tetraacetic acid (Gibco, New York, USA) for 3 min and stop digestion by adding DMEM/F12 containing 10% FBS. Centrifuge the cells for 4 min, then reculture in the medium and incubate at 37°C in a 5% CO_2 incubator at 37°C. The cells were used for follow-up experiments when the cell coverage reached 80%.

Sample treatment

Baicalin ($C_{21}H_{18}O_{11}$, purity > 98%, Macklin Biochemical Co., Ltd, Shanghai, China), hydrogen peroxide (H_2O_2, purity 3%, Merck & Co Inc, New Jersey, USA), and retinoic acid [RA, specific inhibitor of Nrf2 (Proteintech, Chicago, IL), Med Chem Express, New Jersey, USA] were stored at room temperature. Baicalin was fully dissolved in dimethyl sulfoxide (DMSO; Solarbio, Beijing, China) to the final concentration of 500 μM and stored at 4°C protected from light. Four experimental groups were set up in order to investigate whether reducing oxidative stress could decrease the inflammatory response, including control group (CON, untreated), H_2O_2 treatment group (H_2O_2), baicalin treatment group (baicalin) and baicalin with H_2O_2 group (baicalin +H_2O_2), baicalin and H_2O_2 group cells were finally treated with 10 and 200 μM for 18 and 6 h, and baicalin with H_2O_2 group cells were pretreated with 10 μM dose for 18 h. Then, the cells were treated with 200 μM H_2O_2 for 6 h. The baicalin solution was diluted with cell culture medium to 5, 10, 25, 50, and 100 μM for 12, 24, and 48 h to explore the alleviating effect of baicalin concentration on H_2O_2-induced oxidative stress, respectively. The same volume of DMSO was added to the CON group, ensuring that the concentration of DMSO in all treatment solutions prepared was <0.1% (v/v). The H_2O_2 was diluted with cell culture medium to concentrations of 100, 200, 400, 600, 800, and 1,000 μM, and treat the cells for 4, 6, and 8 h, respectively. According

TABLE 1 Primer sequences of the genes.

Gene	Primer sequences(5′-3′)	Length (bp)
NQO1	F GATCGTACTGGCCCACTCAG	169
	R GGGGTCCTTCAGTTTACCTGT	
HO-1	F CATCGACCCCACACCTACAC	192
	R AAGACGCCATCACCAGCTTA	
IL-6	F ATCTGGGTTCAATCAGGCGAT	200
	R CAGTGTTTGTGGCTGGAGTG	
IL-1β	F CCCCAGAGGGAAGAGCAGT	168
	R GAGGGCATTGGCATACGAGT	
TNF-α	F GAGCCTGTGAGCGTGCTTTT	163
	R TGGTGCTGAGGATGACATGG	
β-actin	F GATGATGATATTGCTGCGCTCG	159
	R TACGAGTCCTTCTGGCCCAT	

to the results, treating cells with 10 μM baicalin for 24 h and inducing cells with 200 μM H_2O_2 for 6 h were used as the optimal treatment conditions.

Cell viability assay

The appropriate H_2O_2 concentration and exposure time were screened for treating cells with Cell Counting kit-8 (CCK-8, Beijing Sun Biotechnology Co., LTD). The BMECs (2×10^5 cells/mL) were inoculated into 96-well plates, and the culture medium was discarded when the cells proliferated to the density of 70–80%. Then, 100 μL of different concentrations of baicalin and/or H_2O_2 solution was added to the wells and incubated at 37°C in a 5% CO_2 incubator for an appropriate time. A serum-free cell culture medium was used to wash the cells twice. Then, 100 μL 10% CCK-8 solution was added to each well. After incubation in the incubator for 2 h, the absorbance values at 450 nm were measured using an enzyme marker (BioTek Instruments, Inc, USA). Cell viability was calculated as follows: (OD of treatment group-OD of the blank group)/(OD of control group-OD of the blank group) × 100%.

ROS determination

The intracellular ROS were detected by fluorescent probe DCFH-DA staining using Reactive Oxygen Species Assay Ki (ROS Assay Kit, Shanghai Beyotime Biotechnology Co., Ltd. Shanghai, China). The cells were washed with PBS and then coincubated with 10 μM DCFH-DA in a cell incubator for 20 min. The cells were fully washed with PBS to remove DCFH-DA that had not entered the cells. Finally, the cells were resuspended in PBS and immediately visualized and imaged using fluorescence microscopy (AmScope, California, USA). After the same procedure, the fluorescence intensity at 488 nm excitation wavelength and 525 nm emission wavelength was measured using a Multifunctional Enzyme Labeler (SpectraMax i3x, Silicon Valley, USA).

Oxidative damage indicators measurement

Collecting the cells and lysing with RIPA lysis buffer (Beyotime, Shanghai, China) containing 1% phosphatase inhibitor mixture (CWBIO, Beijing, China) and protease inhibitor cocktail (Epizyme, Shanghai, China). The supernatant was removed after centrifugation, and the protein concentrations of samples were determined using a BCA kit (Beyotime, Shanghai, China). Then, the MDA content, SOD activity, and T-AOC level in the protein sample were measured according to the instructions of the Lipid MDA Assay Kit (S0131M), Total Superoxide Dismutase Assay Kit (S0101S), and Total Antioxidant Capacity Assay Kit (WST-8) (Beyotime Biotechnology Co. Shanghai, China).

Quantitative real-time PCR assay

Total RNA was extracted from BMECs by TRIzol reagent (Quan-Style Gold, Beijing, China), and the quality of RNA was detected. Then, 1 μg of total RNA samples was reverse-transcribed to cDNA according to the operation instructions (Vazyme, Nanjing, China), and the relative expression of mRNA was detected using a 7500 Real-Time PCR system (Applied Biosystems). The reactions were as follows: predenaturation (95°C for 30 s); cyclic reaction 40 times (95°C for 5 s, 60°C for 30 s); melting curve (95°C for 5 s, 65°C for 5 s). Three biological replicates and three technical replicates were set up for each treatment group to determine the mean crossover point value and standard deviation (SD). The fluorescence quantification results were calculated using the 2-ΔΔCT method, and the results were normalized using β-actin as an internal reference gene. The primers were designed by NCBI and synthesized by Shangya Biologicals. The sequences of the synthesized primers are shown in Table 1.

Western blot analysis

Proteins were extracted from BMECs using RIPA lysis buffers containing protease and phosphatase inhibitors, and their concentrations were determined. The protein samples were mixed with 5 × SDS–PAGE Protein Sample Loading Buffer (Beyotime, Shanghai, China) proportionally and then heated together in a water bath at 98°C for 5 min to denature the proteins. Separation and concentration gels were prepared according to the molecular weight requirement of the target protein, and the denatured protein samples and marker were added to the lane in turn. Subsequent to electrophoresis, the protein bands were transferred to polyvinylidene fluoride (PVDF) membranes according to the wet transfer method. The PVDF membranes were blocked in a shaker with 5% skimmed milk for 1 h before

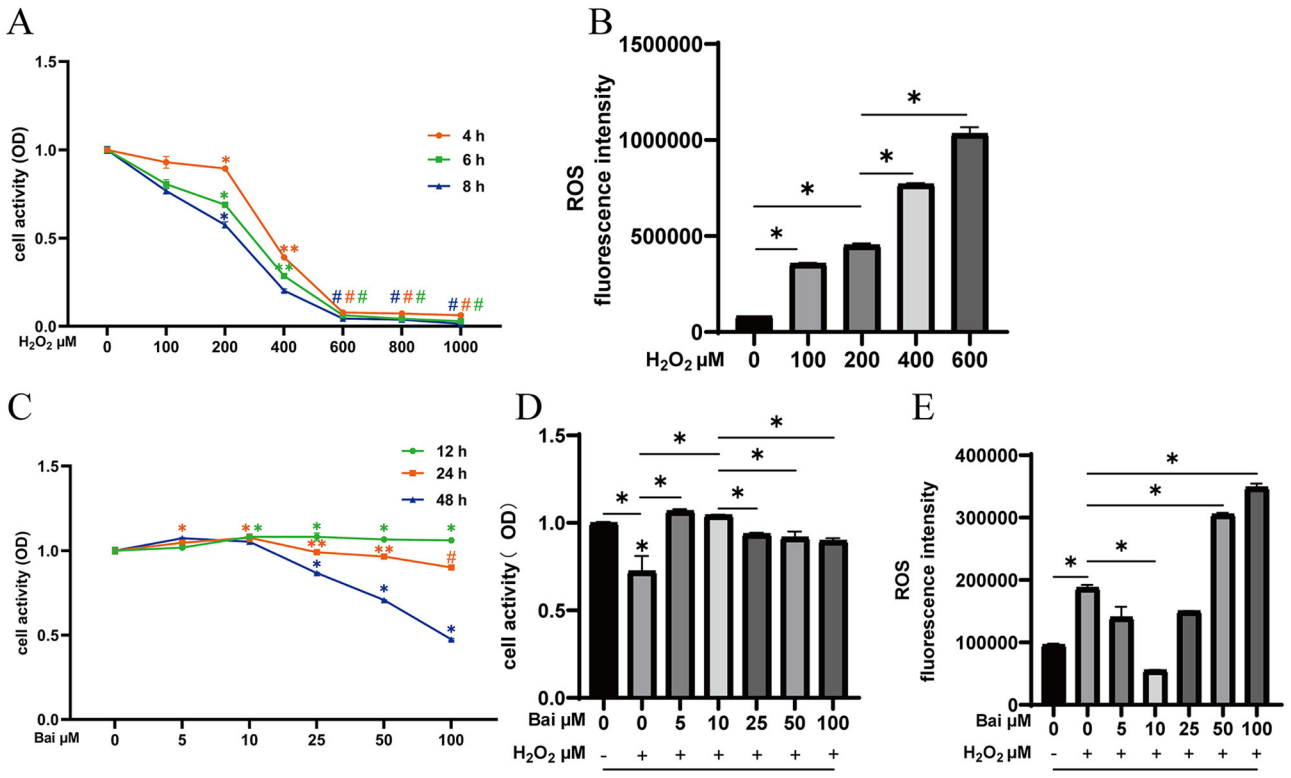

FIGURE 1
Exploration of the optimal concentration and time of H_2O_2 and baicalin. **(A)** Effect of different concentrations of H_2O_2 (0, 100, 200, 400, 600, 800, and 1,000 μM) on the cell viability of BMECs upon stimulation for 4, 6, and 8 h. $^*P < 0.05$ compared with the CON, $^{**}P < 0.05$ compared with the 200 μM H_2O_2 group, $^{\#}P < 0.05$ compared with the 400 μM H_2O_2 group. **(B)** Effect of different concentrations of H_2O_2 on ROS levels in BMECs upon stimulation for 6 h. $^*P < 0.05$. **(C)** Effect of different concentrations of baicalin (0, 5, 10, 25, 50, 100 μM) on the cell viability of BMECs upon stimulation for 12, 24 and 48 h. $^*P < 0.05$ compared with the CON, $^{**}P < 0.05$ compared with the 10 μM baicalin group, $^{\#}P < 0.05$ compared with the 50 μM baicalin group. **(D)** BMECs were treated with baicalin at different concentrations, and 200 μM H_2O_2 was added 6 h before the end of treatment to determine the cell viability at 24 h. $^*P < 0.05$. **(E)** Effect of different concentrations of baicalin on H_2O_2-induced ROS levels in BMECs. $^*P < 0.05$. The data from the CON were used to normalize the data of each treatment group. Each treatment was repeated three times, and the results are expressed as the means ± SEM.

incubation with the appropriate primary antibody overnight at 4°C. The next day, PVDF membranes were first washed 3 times with Tris Buffered Saline with Tween 20 (TBST) for 15 min each. Then, they were incubated for 1 h at room temperature using the corresponding matching fluorescent-coupled secondary antibody and washed again with TBST. The protein bands were visualized by Odyssey Infrared Imaging (LI-COR Biosciences, USA) using the Omni-ECL™ Femto Light Chemiluminescence Kit (Epizyme, Shanghai, China). The target proteins expression levels were normalized using β-actin bands as an internal control, and the protein bands were quantitatively analyzed by ImageJ software.

Statistical analysis

Data were collated and plotted by GraphPad Prism 8.0 software (San Diego, CA). One-way ANOVA and Duncan's multiple range test were used for multiple comparisons. The results are expressed as the means ± standard error (SEM) with three replicates for each treatment, and $P < 0.05$ indicated a significant difference.

Results

Protective effect of baicalin against H_2O_2-induced cell damage

Results of the protective effect of baicalin on H_2O_2-induced cell damage are shown in Figure 1. The cell viability of BMECs decreased to 68.9% after 6 h of stimulation with 200 μM H_2O_2, which the results satisfying the experimental requirements for establishing an oxidative damage model (Figure 1A). The treatment of 200 μM H_2O_2 significantly increased cells' ROS levels compared to the CON ($P < 0.05$) (Figure 1B). Different concentrations of baicalin were used to stimulate the cells for 12, 24 and 48 h, with varying effects ($P < 0.05$) (Figure 1C). Under the above conditions, the concentration of baicalin at 10 μM and the treatment time of 24 h could effectively inhibit H_2O_2-induced ROS level increase ($P < 0.05$) (Figures 1D, E).

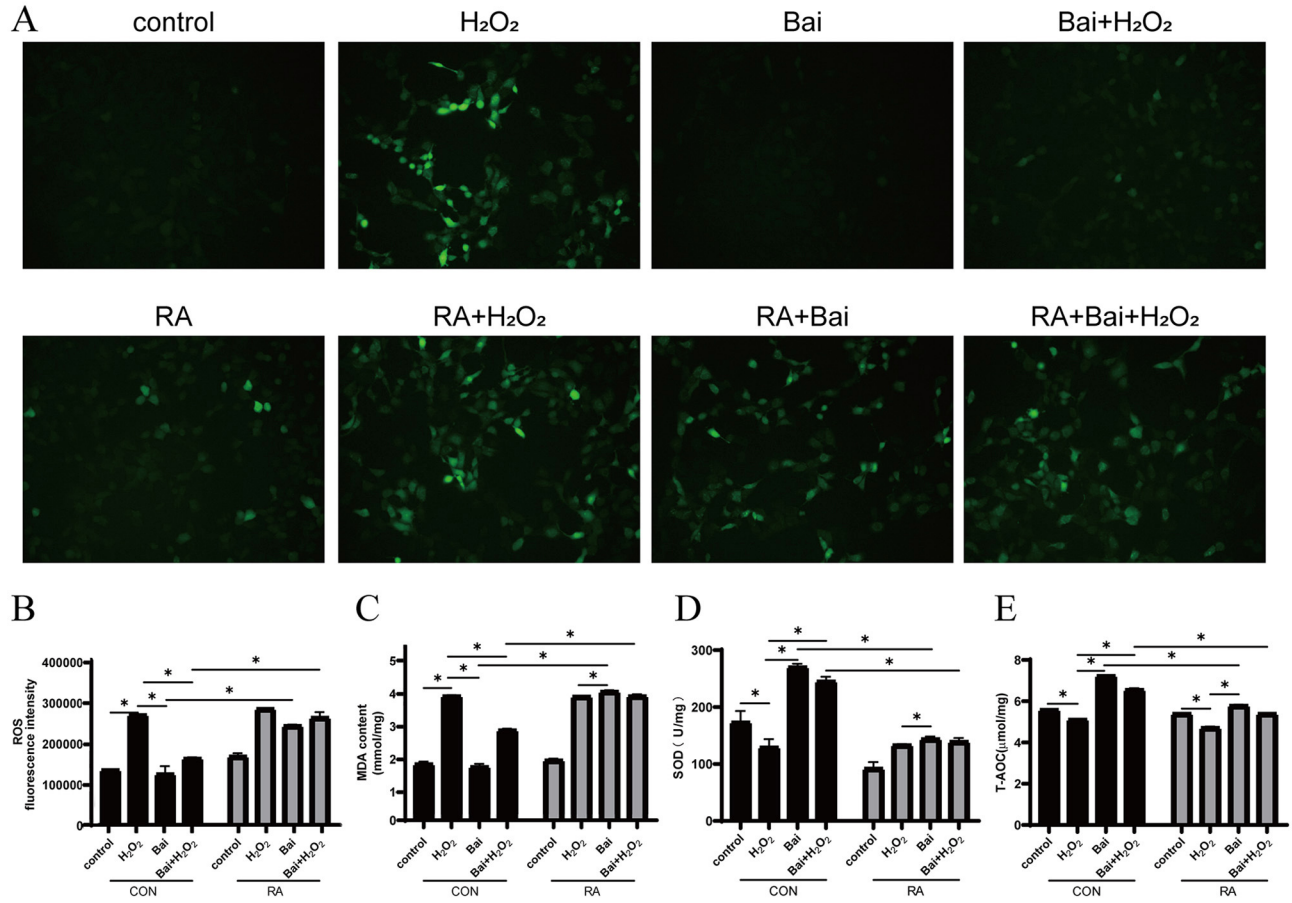

FIGURE 2
Effect of baicalin on H_2O_2-induced changes in ROS, MDA, SOD and T-AOC in BMECs. BMECs were treated with 5 μM RA for 1 h, and baicalin (10 μM) was then added for 18 h before cotreatment with 200 μM H_2O_2 for 6 h. **(A)** The ROS fluorescence intensity was imaged using a fluorescence microscope (scale bar is 200 μm). **(B)** Changes in ROS levels in BMECs. **(C)** Changes in MDA content in BMECs. **(D)** Changes in SOD activity in BMECs. **(E)** Changes in T-AOC levels in BMECs. *$P < 0.05$. RA, Nrf2 inhibitor.

Baicalin alleviates H_2O_2-induced oxidative stress

Next, it explored whether the antioxidant effect of baicalin was related to Nrf2 by adding the Nrf2 inhibitor RA. As shown in Figure 2, compared with the CON group, the H_2O_2 treatment increased ROS levels ($P < 0.05$) (Figures 2A, B) and MDA content in cells ($P < 0.05$) (Figure 2C), decreased SOD activity ($P < 0.05$) (Figure 2D) and T-AOC levels ($P < 0.05$) (Figure 2E). Furthermore, after the addition of Nrf2-specific inhibitor RA, baicalin was unable to effectively inhibit the increase of ROS and MDA levels ($P < 0.05$) (Figures 2A–C), and the levels of SOD and T-AOC were significantly decreased comparison to the CON group ($P < 0.05$) (Figures 2D, E).

Baicalin activates Keap1/Nrf2 signaling pathway

The H_2O_2 treatment significantly reduced Nrf2 and Keap1 protein expression levels ($P < 0.05$) compared to the CON group (Figure 3A). Then, the above results were further verified by qRT-PCR, and the expression of Nrf2 and Keap1 mRNA was consistent with the Western blotting results ($P < 0.05$) (Figures 3B, C). In addition, baicalin treatment also increased the mRNA abundance of the downstream antioxidant genes NQO1 and HO-1, while the addition of RA inhibited the activation of NQO1 and HO-1 by baicalin ($P < 0.05$) (Figures 3D, E).

Baicalin inhibits the H_2O_2-induced NF-κB signaling pathway

The effects of baicalin on BMECs inflammation and the regulatory effects of Nrf2 on inflammation are shown in Figure 4. Compared with the CON group, H_2O_2 treatment decreased the IκBα protein expression level and increased the p-p65/p65 ratio ($P < 0.05$) (Figure 4A), while baicalin had the opposite effect. This effect of baicalin was reversed after the addition of RA. Interleukin IL-6, IL-1β, and tumor necrosis factor TNF-α are the main cellular inflammatory factors and play an important role in BMECs inflammatory response. H_2O_2 treatment significantly increased the

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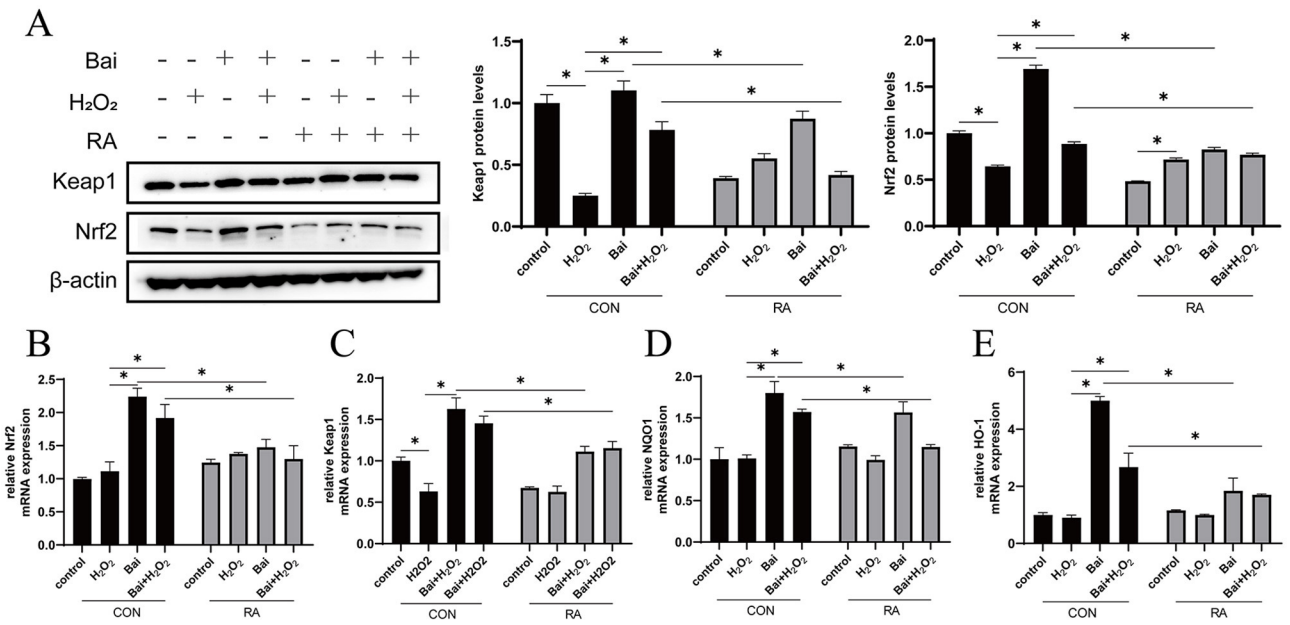

FIGURE 3
Activation of the Keap1/Nrf2 signaling pathway by baicalin. BMECs were treated with 5 μM RA for 1 h, and baicalin (10 μM) was then added for 18 h before cotreatment with 200 μM H_2O_2 for 6 h. **(A)** Western blot analysis of Keap1 and Nrf2 protein levels in BMECs. Immunoreactive bands are shown on the left, and quantitative results are shown on the right. **(B)** Nrf2 mRNA levels in BMECs. **(C)** Keap1 mRNA levels in BMECs. **(D)** HO-1 mRNA levels in BMECs. **(E)** NQO1 mRNA levels in BMECs. *$P < 0.05$.

mRNA content of the downstream pro-inflammatory cytokines IL-1β, IL-6, and TNF-α ($P < 0.05$) (Figures 4B–D). However, with or without cotreatment with H_2O_2, baicalin inhibited the mRNA expression of IL-1β, IL-6, and TNF-α, and the addition of RA effectively blocked the inhibitory effect of baicalin.

Baicalin inhibits the H_2O_2-induced caspase-3/Bcl-2 signaling pathway

The H_2O_2 treatment increased caspase-3 and Bax protein expression levels ($P < 0.05$) (Figure 5A), while baicalin treatment had an opposite effect on H_2O_2 with or without cotreatment with H_2O_2. This effect of baicalin was inhibited by the addition of RA. In addition, the above results were further validated using qRT–PCR, which showed that H_2O_2 treatment increased caspase-3 and Bax mRNA abundance and decreased the Bcl-2/Bax ratio ($P < 0.05$) (Figures 5B–E). However, baicalin treatment decreased caspase-3 and Bax mRNA abundance and increased Bcl-2 and Bcl-2/Bax ratios ($P < 0.05$), and the addition of RA effectively inhibited the effect of baicalin.

Discussion

The BMECs play a crucial role in the antioxidant process as the first line of defense against the invasion of pathogenic microorganisms and are an ideal model for establishing oxidative stress *in vitro*. In the current study, BMECs induced by H_2O_2 were used to construct an oxidative stress model. The results revealed that the induction of BMECs with 200 μM H_2O_2 for 6 h drastically decreased cell viability as well as increased ROS levels. In contrast, treating BMECs with 10 M baicalin for 24 h reversed the damage caused by H_2O_2 to BMECs. The biological effect of baicalin in enhancing cell growth might be the cause of these outcomes. Perruchot et al. demonstrated that baicalin treatment of BMECs could inhibit the synthesis of ROS and free radicals, providing a protective function in the body's antioxidant defense system (18). It illustrated that low-concentration baicalin (1–10 μg/mL) pre-treatment protected BMECs from H_2O_2-induced oxidative stress damage by regulating cell proliferation and the antioxidant response.

All organisms could produce ROS during cellular metabolism (19, 20). Numerous biological processes, such as oxidative stress, inflammation, and cell death, are linked to ROS. However, the breakdown of antioxidant defense systems leads to a decrease in antioxidant capacity and an overabundance of ROS, which triggers oxidative damage (20, 21). Relevant studies have shown that flavonoids, the primary active ingredient in baicalin, could effectively prevent peroxide and inhibit the occurrence of free radical chain reactions (14, 15). In this study, baicalin was found to reduce the accumulation of ROS in H_2O_2-induced BMECs. These results suggest that baicalin plays an antioxidant role in reducing H_2O_2-induced ROS accumulation. The MDA content and SOD activity in cells could reflect the degree of free radical damage (21, 22). The current research results have found that H_2O_2 increased the MDA content in BMECs, while baicalin treatment significantly reduced the MDA content in cells, increased SOD activity and

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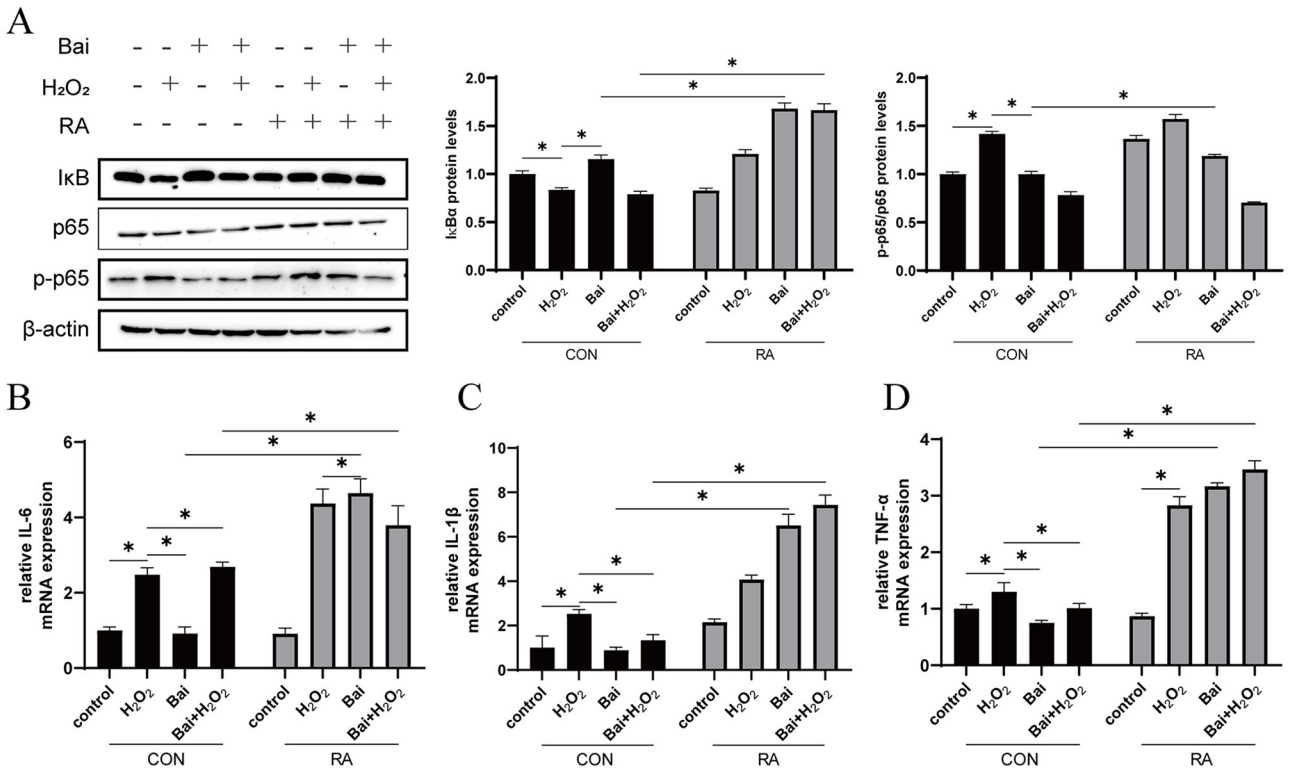

FIGURE 4
Baicalin inhibits the H_2O_2-induced NF-κB signaling pathway. BMECs were treated with 5 μM RA for 1 h, and baicalin (10 μM) was then added for 18 h before cotreatment with 200 μM H_2O_2 for 6 h. **(A)** Western blot analysis of IκBα, p65 and p-p65 protein levels in BMECs. Immunoreactive bands are shown on the left, and quantitative results are shown on the right. **(B)** IL-6 mRNA levels in BMECs. **(C)** IL-1β mRNA levels in BMECs. **(D)** TNF-α mRNA levels in BMECs. $^*P < 0.05$.

T-AOC levels. The results are consistent with a recent study by Wang et al. (22) and Zhou et al. (23), which showed that baicalin supplementation significantly reduced the levels of ROS and MDA. It is indicated that baicalin could enhance the antioxidant defense system and protect cells from oxidative stress and apoptosis. After Nrf2 is activated, it could regulate the gene expression of antioxidant-related enzymes and proteins (24). Therefore, the antioxidant effect of baicalin might be related to the Nrf2 signaling pathway. To further verify this, we added the Nrf2 inhibitor RA and found that RA effectively blocked the antioxidant effect of baicalin. Therefore, current research confirmed that baicalin has the potential to prevent oxidative stress and might mediate the action of Nrf2.

The Nrf2 pathway is a key regulator in the cellular anti-oxidative stress and defense system (25), and its expression could be activating and negatively regulated through the N-terminal structural domain by Kelch, like ECH-related protein 1 (26). Therefore, to verify the effect of baicalin on Nrf2, the expression of Nrf2 and its related proteins was examined in this study. The results showed that Nrf2 was activated by Keap1 degradation, which resulted in the subsequent translocation in the presence or absence of H_2O_2. Baicalin treatment significantly increased the expression levels of Nrf2, Keap1, NQO1 and HO-1 proteins, and the addition of RA inhibited the promotion of Nrf2 and Keap1 protein expression by baicalin. Ma et al. also discovered that baicalin could play an antioxidant role by activating the Nrf2-mediated antioxidant pathway and increasing the expression of HO-1, which is consistent with the conclusion of this study (27). Previous studies have shown that baicalin could prevent Keap1 from binding to Nrf2 through a modified Keap1 protein complex, resulting in reduced ubiquitination and thereby encouraging the expression of Nrf2 (28). These results demonstrated that baicalin could maintain the dynamic homeostasis of the body by activating the Keap1/Nrf2 signaling pathway, thus protecting BMECs from H_2O_2-induced oxidative stress.

It has been reported that baicalin could effectively inhibit allergic inflammation exudation, reduce capillary permeability, and inhibit intestinal inflammation (29). In this study, H_2O_2 treatment increased the mRNA levels of downstream pro-inflammatory cytokines IL-6, IL-1 β, and TNF-α, and this effect was reversed by baicalin supplementation. Shen reported that baicalin pretreatment could reduce the levels of IL-6, IL-1 β, and TNF-α in a mouse lung inflammation model, thus improving pathological changes in the lungs and reducing the body's inflammatory reaction (30). Consequently, the mechanism of anti-inflammatory baicalin might involve the inhibition of inflammation-related proteins and the blocking of NF-κB pathway activation (31). During the resting state, the NF-κB/Rel transcription factor binds to the upstream

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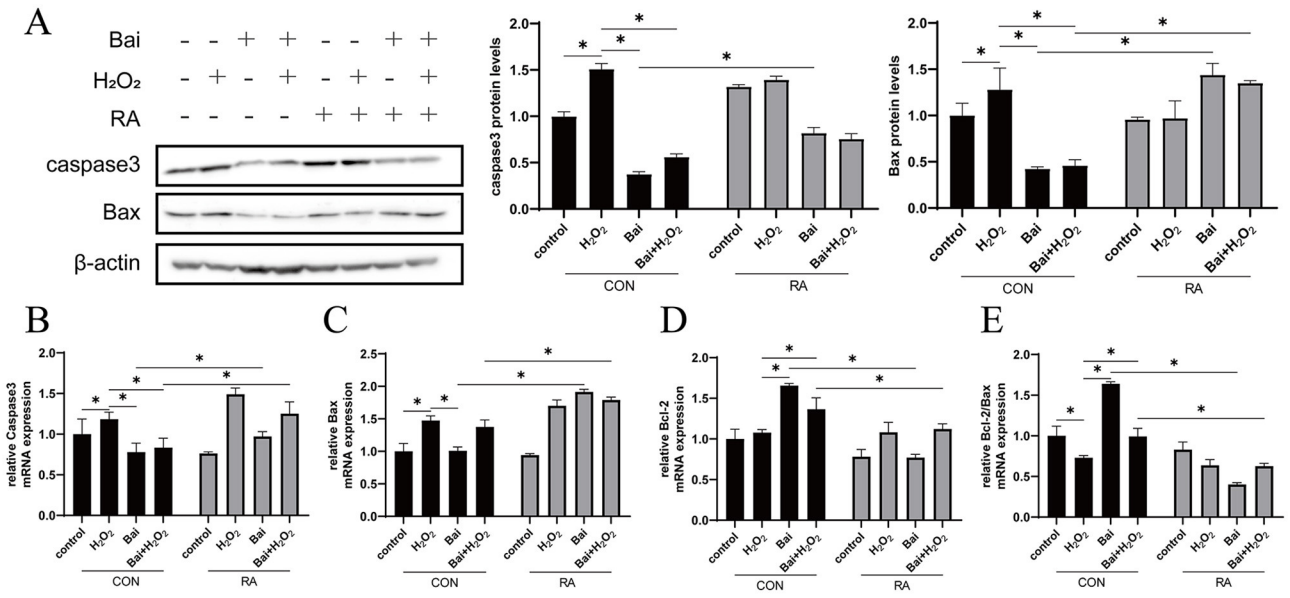

FIGURE 5
Baicalin inhibits the H_2O_2-induced caspase3/Bcl-2 signaling pathway. BMECs were treated with 5 μM RA for 1 h, and baicalin (10 μM) was then added for 18 h before cotreatment with 200 μM H_2O_2 for 6 h. **(A)** Western blot analysis of caspase3 and Bax protein levels in BMECs. Immunoreactive bands are shown on the left, and quantitative results are shown on the right. **(B)** Caspase3 mRNA levels in BMECs. **(C)** Bax mRNA levels in BMECs. **(D)** Bcl-2 mRNA levels in BMECs. **(E)** Ratio of Bcl-2 to Bax mRNA levels in BMECs. *$P < 0.05$.

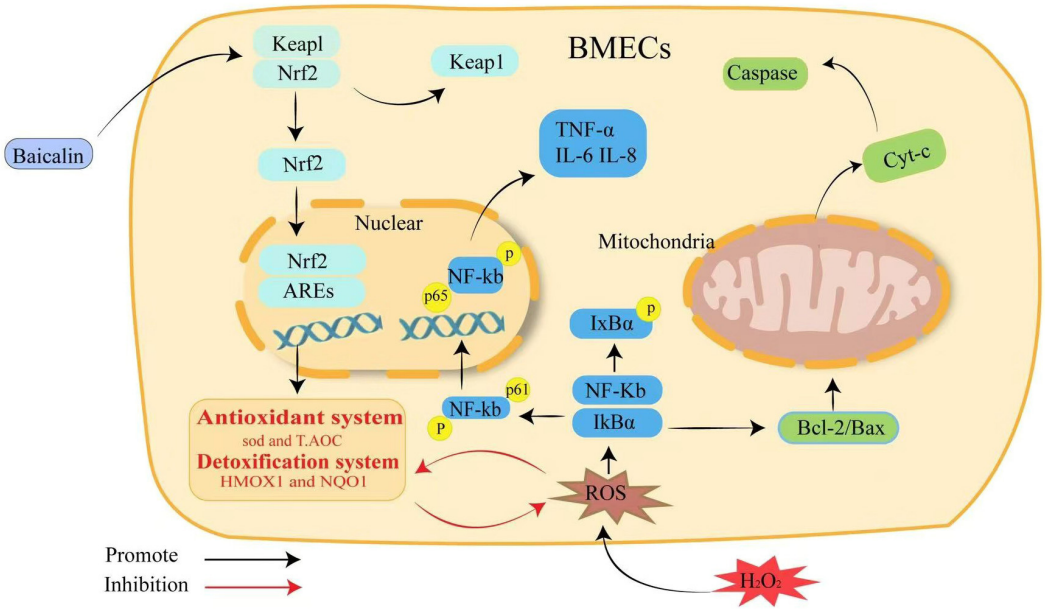

FIGURE 6
Schematic summary of the effect and mechanism of baicalin on H_2O_2-induced oxidative stress, inflammation and apoptosis in BMECs. Baicalin resists H_2O_2-induced oxidative damage in BMECs by activating the Keap1/Nrf2 signaling pathway and reducing ROS production, which in turn inhibits NF-κB inflammation and the caspase3/Bcl-2 apoptotic pathway to maintain intracellular redox homeostasis.

inhibitory κB kinase (IκB) to form a complex and is present in the cytoplasm in an inactive form. When cells are triggered, IκB kinase β (IKKβ) is phosphorylated, leading to the hydrolysis of IκBα by proteases and the release of NF-κB/Rel transcription factors into the nucleus as NF-κB p65, which in turn regulates the expression of downstream pro-inflammatory cytokines. ROS could directly activate IκB and induce nuclear translocation of the inflammatory mediator NF-κB, thereby regulating the expression of inflammation- and oxidative stress-related genes (32, 33). Interestingly, many of the downstream products encoded by NF-κB

are in turn activators of ROS, thus producing feedback effects exacerbating oxidative stress and ultimately creating a vicious cycle (34, 35). Therefore, we investigated the effect of baicalin on NF-κB related genes. The results indicated that baicalin could reduce the expression of NF-κB. Zhang et al. (36) and Li et al. (37) showed that baicalin protects cells from inflammatory damage by down-regulating the expression of the transcription factor NF-κB. Jiang reported that baicalin pretreatment could reduce the levels of IL-6, IL-1β and TNF-α in the cell supernatant, thereby significantly reducing the activity of NF-κB and reducing the inflammatory reaction of the body (38). It is consistent with the results of this study. Meanwhile, it has been discovered that activation of Nrf2 could inhibit the NF-κB inflammatory signaling pathway, reduce DNA oxidative damage, and thus alleviate inflammation and apoptosis (2). To further verify whether Nrf2 regulates the inflammatory response, we added the Nrf2 inhibitor RA and found that it blocked the inhibitory effect of baicalin on inflammatory cytokines, such as NF-κB. This result confirmed that Nrf2 mediates the regulatory effect of baicalin on inflammation.

Furthermore, apoptosis is also mediated by oxidative stress (39). We found that the modulation of oxidative stress by baicalin might inhibit apoptosis. Caspases are usually present as non-activated zymogens in most animal cells, and caspase zymogen activation is a central process in the onset of apoptosis, where caspase-3 is mainly involved in the execution of apoptosis. The apoptosis-related genes Bcl-2 and Bax belong to the Bcl-2 family. Bax could promote apoptosis, while Bcl-2 is an anti-apoptotic protein that could inhibit the activation of caspase to prevent apoptosis (40). It has been shown that activation of the Nrf2 signaling pathway could effectively alleviate mitochondrial damage and inhibit apoptosis (2, 41). Therefore, we explored the effect of Nrf2 signaling pathway inhibition on apoptosis. Baicalin significantly inhibited the H_2O_2-induced expression of caspase-3 and Bax and upregulated the expression of Bcl-2, while the inhibitor RA reversed these effects. Previous studies have shown that when apoptotic signals stimulate cells, the pro-apoptotic protein Bax oligomerizes and induces an increase in mitochondrial membrane permeability. It subsequently promotes the release of cytochrome c from the mitochondria, activates downstream caspase-3, and initiates the caspase cascade, resulting in the specific cleavage of downstream substrates, thereby triggering apoptosis. It is reported that baicalin supplementation could increase cell viability, elevate mitochondrial membrane potential, upgrade Bcl-2 levels and decrease caspase-3 and Bax levels, and our study also confirms this finding (42). This result suggests that baicalin might regulate the caspase/Bcl-2 signaling pathway through Nrf2 mediation, thereby inhibiting apoptosis.

Conclusions

In conclusion, BMECs are stimulated by H_2O_2, inducing oxidative damage and inflammation, disrupting cellular homeostasis, and leading to cell apoptosis. Baicalin could increase the expression of downstream antioxidant genes NQO1 and HO-1, as well as antioxidant system SOD and T-AOC, by activating Keap1/Nrf2 signaling pathway, thus alleviating H_2O_2-induced oxidative damage in BMECs. The NF-kB inflammatory pathway and the caspase/Bcl-2 apoptotic signaling pathway are further inhibited, thereby reducing ROS production via Nrf2-mediated mechanisms (Figure 6).

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

Ethical approval was not required for the studies on animals in accordance with the local legislation and institutional requirements because only commercially available established cell lines were used.

Author contributions

XK: Formal analysis, Investigation, Methodology, Writing – original draft. MW: Formal analysis, Investigation, Methodology, Writing – original draft. ZG: Investigation, Writing – original draft. XY: Investigation, Writing – original draft. HL: Resources, Writing – review & editing. TG: Funding acquisition, Supervision, Writing – review & editing. LZ: Conceptualization, Writing – review & editing. TF: Conceptualization, Funding acquisition, Resources, Supervision, Writing – review & editing.

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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.2024.1504887/full#supplementary-material

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Effects of *Gynura procumbens* extract supplementation on growth performance, carcass traits, antioxidant capacity, immunity and meat quality of meat ducks

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Introduction: *Gynura procumbens* (Lour.) Merr is a common traditional Chinese medicine with anti-tumor, anti-inflammatory and antioxidant activities. However, no related studies reported the potential application effect of *Gynura procumbens* on meat ducks. The study aims to investigate the potential effects of *Gynura procumbens* extract (GPE) supplementation on growth performance, carcass traits, antioxidant capacity, immunity and meat quality.

Methods: A total of 480 21-day-old female healthy ducks were randomly allocated to four treatments, each treatment containing six replicates with 20 ducks per replicate. The groups received a corn-soybean basal diet supplemented with 0 mg/kg GPE (CON), 200 mg/kg GPE (GPE200), 400 mg/kg GPE (GPE400), and 600 mg/kg GPE (GPE600), respectively. The entire experiment lasted for 7 weeks.

Results: The results showed that dietary supplementation with 600 mg/kg GPE significantly reduced the contents of serum urea nitrogen, triglyceride (TG) and total cholesterol (TC). GPE (200, 400, and 600 mg/kg) supplementation effectively reduced the contents of IL-2 and MDA. The levels of immunoglobulin M (IgM) as well as total antioxidative capacity (T-AOC) in GPE600 group dramatically elevated in comparison with the control group. Dietary GPE supplementation considerably increased the moisture content of the breast muscle. Furthermore, dietary supplementation with GPE markedly decreased the water loss rate and shear force.

Discussion: With the ban of antibiotics in poultry production, traditional Chinese medicines have been widely used in livestock and poultry production due to their high efficiency and low toxicity. *Gynura procumbens* extract GPE as a natural plant origin contains a series of biologically active components, including flavonoids, polyphenols, saponin, tannin and terpenoid. This study indicated that dietary supplementation with GPE can increase serum total antioxidant capacity, regulate immune function and improve meat quality to some extent in meat ducks. The recommended optimal GPE level in the diet of meat ducks is 600 mg/kg according to the results in this study.

KEYWORDS

***Gynura procumbens* extract, immunity, antioxidant capacity, meat quality, meat ducks**

1 Introduction

With the advent of biological and engineering technology, large numbers of new antibiotics were developed to treat diseases. In recent years, various antibiotics have been regularly used in medical treatment as well as livestock farming. The application of antibiotics in feed has greatly improved the growth performance of animals and promoted the rapid development of animal husbandry (1). Unfortunately, the extensive abuse of antibiotics resulted in significant antibiotic residues in meat and aquatic products, as well as increased the risks of drug-resistant strains mutations (2). Meanwhile, with the improvement in living standards, consumers have a stronger desire to pursue high quality natural foods. However, long-term misuse of antibiotics can undermine the original intention of ecological farming and simultaneously pose a substantial threat to public health (3–6). Therefore, there is an urgent need to search for alternative novel additives to antibiotics.

It is widely believed that nutritional regulation offers an effective strategy to replace antibiotics through the use of traditional Chinese medicine (TCM) additives. As the treasures of the Chinese nation, Chinese herbal medicines has a long history of being widely used in the treatment of various diseases among the people (7). Recently, traditional Chinese herbs have attracted increasing attention from agronomists due to their high efficacy and low toxicity. As we all know, with the gradual ban of antibiotics in livestock farming, more and more researchers have turned their attention toward the development of Chinese herbal additives. Indeed, many medicinal plants and bioactive compounds originated from natural plants have been widely used in livestock farming (8) For instance, Curcumin, a polyphenolic curcuminoid compound derived from the rhizomes of the plant *Curcuma longa*, has been used as feed additive for improving the intestinal health of broiler (9). Traditional Chinese medicinal herbs prescription composed of Cortex Fraxini, Pulsatilla chinensis and *Eucommia ulmoides* could improve ducks' antioxidant and immune capacity (10).

As a common folk TCM, GP (*Gynura procumbens (Lour.) Merr.*) is an annual evergreen shrub with a fleshly stem and purple tint and is considered to possess diverse pharmacological properties, such as antioxidation, antihypertension and cardioprotective, anti-inflammatory, antihyperglycemic and anticancer activities (11). It has been extensively used in clinic as remedy for eruptive fevers, rashes, kidney diseases, hypertension, diabetes mellitus and cancer (12, 13). These beneficial pharmacological activities of GP may be attributed to the presence of its active ingredients, including flavonoids, saponin, tannin, terpenoids and glycosides (14, 15). Currently, vast attention has been focused on the broad clinical application of GPE. However, no studies are exploring the potential effects of GPE supplementation on ducks.

Abbreviations: ADFI, Average daily feed intake; ADG, Average daily gain; ALB, Albumin; ALT, Alanine aminotransferase; AST, Aspartate aminotransferase; ALP, Alkaline phosphatase; BCA, Bicinchoninic acid; BUN, Blood urea nitrogen; BW, Body weight; ELISA, Enzyme-linked immunosorbent assay; F/G, Feed to weight gain ratio; GSH-Px, Glutathione peroxidase; GPE, *Gynura procumbens* extract; HFD, High fat diet; IMP, Inosine monophosphate; IgA, Immunoglobulin A; IgG, Immunoglobulin G; IgM, Immunoglobulin M; IL-6, Interleukin-6; IL-1β, Interleukin-1β; MDA, Malondialdehyde; SEM, Standard error of means; SOD, Superoxide dismutase; H&E, Hematoxylin and eosin; T-AOC, Total antioxidant capacity; TC, Total cholesterol; TCM, Traditional Chinese Medicine; TG, Triglyceride.

Currently, in the process of duck farming, Low immunity among duck flocks remains a pressing challenge for farmers and is widely recognized as a key factor contributing to high duck mortality rates. Additionally, ducks are highly susceptible to stress responses triggered by high-density farming, which results in oxidative damage and further causes the reduction of growth performance (16). Therefore, maintaining the balance of the oxidant/antioxidant and enhancing immunity is an effective approach to promote the health growth of ducks and plays a fundamental role in improving the quality of duck meat (17). Numerous clinical studies highlight that GPE is abundant in polyphenols and flavonoids, which plays an important role in boosting immunity and strengthening antioxidant defenses (18, 19). Despite GPE's widespread clinical applications, no related studies to date have explored its potential effects on ducks. Our preliminary study has indicated that dietary supplementation with GPE could boost duck's immunity. Therefore, the current study was designed to explore the potential effect of dietary supplementation with GP on growth performance, immunity and antioxidative capacity of ducks and further elucidate the important role of GPE in improving meat quality.

2 Materials and methods

2.1 Animals and experimental design

The experiment was carried out with permission from the Experimental Animal Ethics Committee in Jiangxi Academy of Agricultural Sciences. A total of 480 21-day-old female healthy ducks with similar body weight were bought from a local commercial hatchery (Jian, China). Subsequently, they were randomly assigned into four treatments with six replicates each comprising 20 ducks. The four dietary treatments consisted of the control and three levels of GPE (200 mg/kg, 400 mg/kg, and 600 mg/kg). The basal diet was formulated to meet the nutrient requirements of meat ducks (NY/T 2122–2012, China). The ingredient compositions and nutrient contents of the basal diets for ducks are listed in Table 1. These ducks were rearing on net bed with splash pool and received water *ad libitum* through the entire experiment period. The whole experiment was sustained for 7 weeks, the schematic diagram showing the study design is displayed in Figure 1.

2.2 Growth performance

After 12 h of fasting, the body weight (BW) from each replicate cage was measured on days 21, 42, and 70. Mortality was monitored daily, with the weight of dead ducks recorded and feed intake per cage was recorded every day through the experimental period. Subsequently, the average body weight, average daily feed intake, average daily gain, and the ratio of feed to weight gain (F/G) were calculated with correction for mortalities.

2.3 Blood sample collection

Two ducks from each replicate were randomly selected at 42 and 70 days of age, respectively. Blood was obtained from wing vein and centrifugated at 3,000 g, for 10 min at 4°C to prepare serum for subsequent measurement.

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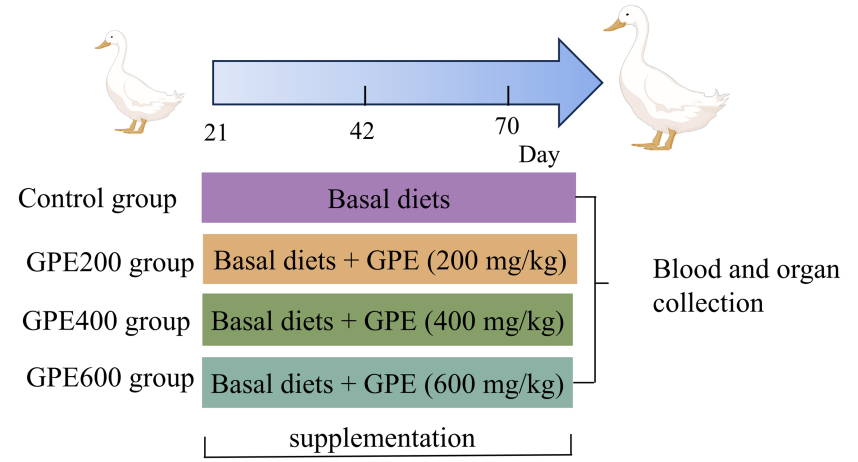

FIGURE 1
Schematic diagram of experiment protocol.

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

Items	Day 21–42	Day 43–70
Ingredients (%)		
Corn	63.82	71.01
Soybean meal	24.02	19.05
Wheat middling and reddog	8.00	6.00
Moringa oleifera leaf	0.00	0.00
Soybean oil	0.00	0.00
L-Lysine•HCl	0.02	0.00
DL-Methionine	0.17	0.12
Limestone	1.17	1.18
Dicalcium phosphate	1.28	1.28
Salt (NaCl)	0.35	0.36
Premix[a]	1.00	1.00
Total calculated nutrient levels	100	100
Metabolizable energy (MJ/kg)	2.87	2.92
Crude protein (%)	17.00	15.00
Lysine (%)	0.85	0.71
Methionine (%)	0.42	0.35
Calcium (%)	0.85	0.80
Available phosphorous (%)	0.60	0.35
Methionine + Cysteine (%)	0.70	0.60

[a]The premix provides the following per kg of diets: vitamin A, 11,500 IU; vitamin D3, 33,000 IU; vitamin E, 25 IU; vitamin K3, 3 mg; vitamin B1, 2 mg; vitamin B2, 8 mg; vitamin B6, 4.5 mg; vitamin B12, 0.03 mg; D-pantothenic acid, 12 mg; nicotinic acid, 50 mg; choline chloride, 1,000 mg; biotin, 0.2 mg; folic acid, 0.6 mg; antioxidant, 100 mg; Fe, 60 mg; Cu, 8 mg; Mn, 90 mg; Zn, 60 mg; I, 0.4 mg and Se, 0.2 mg.

2.4 Measurement of serum biochemical indices, antioxidant activity and immunity

For biochemical assays, the freshly collected serum samples were used to measure the activities of total antioxidant capacity (T-AOC, Cat no, A015-2-1), glutathione peroxidase (GSH-Px, Cat no, A005-1-2), superoxide dismutase (SOD, Cat no, A001-3-2), malondialdehyde (MDA, Cat no, A003-1-2), blood urea nitrogen (BUN, Cat no, C013-2-1), alkaline phosphatase (ALP, Cat no, A059-2-2), aspartate aminotransferase (AST, Cat no, C010-2-1), alanine aminotransferase (ALT, Cat no, C009-2-1), triglyceride (TG, Cat no, A110-2-1) and albumin (ALB, Cat no, A028-2-1) using corresponding commercial assay kits (Jiancheng Bioengineering, Nanjing, China). The contents of immunoglobulin A (IgA, Cat no, ml036901), immunoglobulin G (IgG, Cat no, ml036900) and immunoglobulin M (IgM, Cat no, ml061224) were quantified with ELISA assay kits (Shanghai Enzyme-linked Biotechnology, China) according to the manufacturer's instructions. Additionally, sample quality control and testing parameter setting were strictly adhered to the product specification.

2.5 Carcass traits and organ indices

At the end of the trial (70 days of age), two ducks from each replicate were randomly selected, weighed individually and sacrificed after 12 h of feed deprivation. Subsequently, ducks were sacrificed by exsanguination from the jugular vein. The weight of the defeather carcass was determined as the carcass weight. The carcass was weighed after removing the trachea, esophagus, spleen, pancreas, gallbladder, reproductive organ, intestinal tract, gizzard content and gizzard-membrane which was recorded as semi-eviscerated weight. The eviscerated weight was measured as eviscerated weight after removing the heart, liver, lung, gizzard, glandular stomach, and abdominal fat. Finally, the rate of dressing, semi-eviscerated, eviscerated, breast muscle, leg muscle, and abdominal fat were calculated. The weight of dissected heart, liver, spleen, lung, gizzard and glandular stomach was recorded, respectively, and organ index was calculated.

2.6 Evaluation of liver histopathological changes

For histopathological analysis, liver tissues of ducks were collected in ice-cold phosphate-buffered saline and subsequently

transferred into 4% fresh paraformaldehyde for fixation at room temperature for at least 24 h. Subsequently, the specimens were further processed for dehydration. After dehydration with ethanol and xylene, the tissues were embedded in paraffin, sectioned at a thickness of 5 μm and subjected to hematoxylin and eosin (H&E). Afterward, the above samples were photographed under a light microscope (BX53, Olympus).

2.7 Meat quality

After slaughter, the pH values of breast muscle and leg muscle were measured with a portable pH meter (pH-Star, Matthaus GmbH & Co. KG, Eckelsheim, Germany) in the pectoralis major muscle and leg muscle at about 1 cm depth as Huang previously described (20). Color changes were evaluated at each sampling by measuring L*, a*, and b* parameters using a reflectance spectrophotometer (X-Rite SP64, United States), L*, a*, and b* values represented the degree of lightness, greenness or redness, and blueness or yellowness, respectively. Subsequently, the shear force was measured. The procedure was as follows: the raw breast muscles were cut into three strips (1 × 1 × 3 cm) perpendicular to the muscle fiber direction to evaluate the tenderness using a digital-display muscle tenderness meter (C-LM3B, Tenova, Harbin, China). The water loss rate was measured according to the modified method of Li et al. (21). In brief, collected meat samples (0.125 cm^3) were weighed and carefully wrapped in absorbent and placed into the machine for testing. The program was set to 300 N for 5 min. After 5 min, the samples were weighed again. Finally, the water loss rate was calculated as a percentage:

$$\text{Water loss rate} = [(\text{initial muscle weight} - \text{final muscle weight}) / \text{initial muscle weight}] * 100\%.$$

2.8 Determination of IMP content in muscle

The inosine Monophosphate (IMP) concentration was determined with high-performance liquid chromatography (HPLC, Agilent 1290, Agilent Technologies, United States). The IMP content in the samples was measured using an acidic extraction method on approximately 1 g of dry weight samples. In brief, 25 mL of phosphoric acid was first added to the sample. Subsequently, it was gently mixed and incubated on ice for 30 min with intermittent gentle shaking. After extraction for 30 min in ice-water bath, the supernatant was collected after centrifugation (3,000 rpm/min, 5 min) and then transferred to a clean 50 mL centrifuge tube. Immediately afterward, the supernatant was adjusted to pH 6.5 with 5 M sodium hydroxide solution and topped to 50 mL with the additional supernatant. Afterward, the obtained solution was filtered through 0.45 μm organic filter membrane before being used for the HPLC analysis. Finally, the content of IMP in the filtrate was analyzed by HPLC. The HPLC conditions were as described above: column: Phenomenex Luna C18 100 Å column (250 mm × 4.6 mm, 5 μm); mobile phase: 0.05 M triethylammonium dihydrogen phosphate/acetonitrile (95/5); flow rate: 1 mL/min; detection wavelength: 254 nm; column temperature: 25°C.

2.9 Chemical composition of the duck breast muscle

Protein, fat and moisture contents in duck breast muscle (stored at 4°C) were determined following the guidelines from the Associate of Analytical Chemists.

2.10 Analysis of antioxidative enzyme activities in duck breast muscle

Duck breast muscle (100 mg, stored in air 4°C for 24 h) was accurately weighed and added with ice-cold phosphate buffer solution (4°C, 0.01 mol/L, pH = 7.2–7.4) to prepare 10% w/v homogenate. Firstly, the protein content of 10% homogenate was measured by bicinchoninic acid (BCA) protein assay reagent. Subsequently, the activities of SOD (Cat no, A001-3-2), GSH-Px (Cat no, A005-1-2), T-AOC (Cat no, A015-2-1), total cholesterol (TC, Cat no, A111-2-1) and the concentration of MDA (Cat no, A003-1-2) were determined using the commercial kits (Nanjing Jiancheng Biology Engineering Institute, Nanjing, China) following the instructions of the manufacturer, respectively.

2.11 Statistical analysis

All results are presented as the means ± (SEM) standard errors of the means. The experimental data were statistically analyzed using the SPSS version 19.0 software (SPSS Inc., Chicago, IL, United States). For the comparison between two experiment groups, statistical significance was assessed using Student's *t*-test. For multiple groups, the statistical significance of the differences was carried out by one-way analysis followed by Turkey test. Differences between groups were considered statistically significant for $p < 0.05$.

3 Results

3.1 Growth performance

As showcased in Table 2, in comparison to the control group, there was no significant difference in body weight, average daily feed intake (ADFI) and average daily gain (ADG) with increasing levels of GPE throughout the experimental period. Additionally, there was no statistically considerable variation in F/G between the control and the treatment groups.

3.2 Serum biochemical parameters

To probe whether GPE exerted potential protective effect on antioxidation, lipid metabolism and liver function, related biochemical indexes were measured by biochemical kits. The results are presented in Table 3. Relative to the control group, dietary GPE supplementation prominently (all for $p < 0.05$) decreased the activities of BUN and TG at 42 and 70 days of age. Moreover, dietary

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TABLE 2 Effect of GPE supplementation on growth performance of meat ducks.

Items[a]	Time	Diets				SEM	*p*
		CON	GPE200	GPE400	GPE600		
BW/g	Day 21	421.0	421.0	421.1	420.9	0.06	1.000
	Day 42	1282.34	1282.13	1282.10	1279.39	6.99	0.999
	Day 70	1943.20	1892.00	1937.00	1895.80	18.27	0.690
ADG/(g/d)	Day 21–42	40.29	41.04	41.00	40.78	0.31	0.843
	Day 43–70	23.03	21.52	22.92	20.96	0.51	0.414
	Day 21–70	30.43	29.89	30.67	29.45	0.36	0.670
ADFI/(g/d)	Day 21–42	123.38	123.98	124.49	123.89	1.13	0.991
	Day 43–70	151.09	148.88	151.58	148.05	2.01	0.925
	Day 21–70	139.22	138.21	139.97	137.69	1.44	0.955
F/G	Day 21–42	3.06	3.02	3.04	3.04	0.02	0.941
	Day 43–70	6.58	6.96	6.65	7.07	0.09	0.184
	Day 21–70	4.57	4.63	4.57	4.68	0.03	0.566

BW, ADG, ADFI, and F/G represent the means of 6 replicates. BW, body weight; ADG, average daily gain; ADFI, average daily feed intake; F/G, ratio of feed to weight gain; SEM, standard error (SE) of means.
[a]Data are the mean of 6 replicates with 20 ducks each replicate.

TABLE 3 Effect of GPE supplementation on serum biochemical parameters of meat ducks.

Items[1]	Diets				SEM	*p*
	CON	GPE200	GPE400	GPE600		
Day 42						
Total protein (g/L^{-1})	28.41^{c}	29.13^{bc}	30.87^{a}	30.26^{ab}	0.30	0.012
ALB (g/L^{-1})	12.78	12.99	13.31	12.95	0.18	0.779
BUN (μmol•L^{-1})	388.29^{a}	378.59^{ab}	365.39^{b}	346.78^{c}	3.71	<0.001
TG (mmol•L^{-1})	2.60^{a}	2.46^{ab}	2.38^{b}	2.32^{b}	0.03	0.019
TC (mmol•L^{-1})	2.71	2.56	2.49	2.51	0.03	0.084
ALP (U•L^{-1})	5504.30	5521.69	5543.72	5555.57	25.05	0.899
AST (U•L^{-1})	126.62	126.88	127.13	127.19	0.99	0.997
ALT (U•L^{-1})	2.34	2.33	2.38	2.37	0.03	0.923
Day 70						
Total protein (g/L^{-1})	28.82^{b}	30.67^{ab}	31.78^{a}	31.64^{a}	0.38	0.018
ALB (g/L^{-1})	12.62	13.04	13.48	13.36	0.13	0.084
BUN (μmol•L^{-1})	381.03^{a}	361.11^{b}	361.44^{b}	343.40^{c}	2.91	<0.001
TG (mmol•L^{-1})	2.69^{a}	2.33^{b}	2.29^{b}	2.25^{b}	0.04	<0.001
TC (mmol•L^{-1})	2.80^{a}	2.46^{b}	2.36^{b}	2.34^{b}	0.04	<0.001
ALP (U•L^{-1})	5507.99	5515.43	5520.27	5533.74	24.43	0.987

SEM, standard error (SE) of means.
[1]Different lowercase letters represent significant differences ($p < 0.05$) and data are the mean of 6 replicates with 2 ducks each replicate.

GPE inclusion significantly elevated the total protein level. Notably, at 42 and 70 days of age, as the concentration of GPE increased, BUN and TG activities progressively decreased, reaching their lowest at a dietary concentration of 600 mg/kg GPE. However, supplementation with GPE had no discernible effect on the contents of ALP, AST, ALT and ALB (all for $p > 0.05$).

3.3 Serum immunity and inflammatory parameters

To further interrogate the effect of GPE on cellular immunity, the ELISA analysis was used to detect the concentrations of serum IL-6, IL-1β, IgA, IgM, and IgG. As depicted in Table 4, with respect to the

TABLE 4 Effect of GPE supplementation on serum immunity related indicators of meat ducks.

Items[1]	Diets				SEM	*p*
	CON	GPE200	GPE400	GPE600		
Day 42						
IgA (g•L^{-1})	2.15	2.27	2.29	2.31	0.03	0.117
IgG (g•L^{-1})	3.83	3.94	4.04	3.91	0.03	0.051
IgM (g•L^{-1})	1.41^{b}	1.67^{a}	1.73^{a}	1.76^{a}	0.03	<0.001
IL-1β (mg•mL^{-1})	0.20	0.20	0.21	0.21	0.00	0.057
IL-6 (mg•mL^{-1})	0.21	0.19	0.18	0.18	0.01	0.457
IL-2 (mg•mL^{-1})	3.17^{a}	2.81^{b}	2.75^{b}	2.72^{b}	0.05	0.002
Day 70						
IgA (g•L^{-1})	2.16	2.25	2.27	2.31	0.02	0.156
IgG (g•L^{-1})	3.84^{b}	3.90^{ab}	4.05^{a}	4.05^{a}	0.03	0.029
IgM (g•L^{-1})	1.42^{b}	1.73^{a}	1.76^{a}	1.77^{a}	0.03	<0.001
IL-1β (mg•mL^{-1})	0.21	0.21	0.21	0.21	0.00	0.733
IL-6 (mg•mL^{-1})	0.20	0.19	0.19	0.18	0.00	0.439
IL-2 (mg•mL^{-1})	3.12^{a}	2.84^{b}	2.77^{b}	2.70^{b}	0.04	<0.001

SEM, standard error (SE) of means.
[1]Different lowercase letters represent significant differences ($p < 0.05$) and data are the mean of 6 replicates with 2 ducks each replicate.

control group, the IgM level was markedly ($p < 0.01$) enhanced at 42 and 70 days of age after dietary supplementation with GPE. Additionally, at 42 days of age, supplementation with GPE had no obvious effect on IgG activity. However, supplementation with GPE apparently elevated the IgG and IgM activities at 70 days of age. Furthermore, the concentration of IL-2 was notably lowered in GPE-treated groups at 42 and 70 days of age compared with the control group. Of note, there are no significant differences observed in IL-1β, IL-6, and IgA. These results demonstrated that dietary supplementation with GPE could improve immune function by enhancing the activities of immune factors and modulating the levels of inflammatory cytokines.

3.4 Serum antioxidant status

Antioxidant activity refers to the ability to eliminate free radicals in the body. To evaluate the antioxidant potential of GPE, the levels of oxidative stress-related indicators (T-AOC, SOD, MDA, and GSH-Px) were detected by corresponding assay kits. As displayed in Table 5, the level of T-AOC was distinctly elevated at 42 and 70 days of age following GPE supplementation, showing a dose-dependent manner with higher concentrations of GPE. Furthermore, in contrast to the control group, at 70 days of age, dietary supplementation with GPE conspicuously reduced MDA level, with 600 mg/kg GPE treatment showing the lowest levels. However, no prominent changes are observed in SOD and GSH-Px throughout the duration of the experiment after dietary supplementation with GPE.

3.5 Carcass traits

The effect of GPE supplementation on carcass traits of ducks were displayed in Table 6. Compared to the control group, all treatments did not show any significant effects on semi-eviscerated yield, dressed yield, eviscerated yield, breast muscle yield, leg muscle yield and abdominal fat yield. Although there were no differences in these indicators, it was worth noting that dietary supplementation with GPE decreased abdominal fat, especially 600 mg/kg.

3.6 Organ indexes

The effects of GPE on relative weights of liver, kidney, spleen, lung, gizzard and glandular stomach are illustrated in Table 7. Moreover, the kidney, spleen, lung, gizzard and glandular stomach weight were no obvious differences between the control group and the different dosages of GPE-treated groups. These results revealed that GPE had no obvious toxic and side effects to major organs *in vivo*.

3.7 Hepatic morphology

To further explore whether GPE exerted potential toxic effect on the liver, the histological architecture of the liver was observed by microscope following H&E staining. As showcased in Figure 2, in the control group, the structure of liver tissues was intact with regular arrangement of hepatocyte cells. However, in parallel to the control group, dietary inclusion of GPE did not result in any noticeable morphological changes in hepatocytes. This finding suggested that GPE supplementation had no potential cytotoxic effects on the liver, highlighting its suitability and safely as a feed additive.

3.8 Meat quality

To estimate the effect of GPE supplementation on muscle characteristics and meat quality traits, the related parameters of meat

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TABLE 5 Effect of GPE supplementation on serum anti-oxidant related indicators of meat ducks.

Items[1]	Diets				SEM	*p*
	CON	GPE200	GPE400	GPE600		
Day 42						
T-AOC (U•L^{-1})	3.33^{c}	3.56^{bc}	3.72^{a}	3.60^{ab}	0.03	0.000
SOD (U•L^{-1})	108.35	109.66	111.16	111.67	1.03	0.672
GSH-Px (U•L^{-1})	1.29	1.34	1.35	1.34	0.02	0.611
MDA (μmol•L^{-1})	8.28	7.96	8.07	7.98	0.08	0.556
Day 70						
T-AOC (U•L^{-1})	3.53^{b}	3.60^{b}	3.77^{a}	3.82^{a}	0.03	0.003
SOD (U•L^{-1})	114.61	116.05	117.68	117.01	1.52	0.908
GSH-Px (U•L^{-1})	1.15	1.19	1.24	1.21	0.02	0.162
MDA (μmol•L^{-1})	7.98^{a}	7.68^{b}	7.62^{b}	7.64^{b}	0.05	0.039

SEM, standard error (SE) of means.
[1]Different lowercase letters represent significant differences ($p < 0.05$) and data are the mean of 6 replicates with 2 ducks each replicate.

TABLE 6 Effect of GPE supplementation on carcass traits of meat ducks.

Items[1]	Diets				SEM	*p*
	CON	GPE200	GPE400	GPE600		
Dressed yield, %	90.11	89.88	89.68	89.36	0.17	0.451
Semi-eviscerated yield, %	83.27	82.89	83.07	82.51	0.19	0.544
Eviscerated yield, %	74.89	74.87	74.32	73.88	0.25	0.422
Breast muscle yield, %	13.38	13.51	12.54	13.25	0.21	0.413
Leg muscle yield, %	11.45	11.17	10.58	10.94	0.12	0.086
Abdominal fat yield, %	2.46	2.20	2.17	1.98	0.09	0.288

SEM, standard error (SE) of means.
[1]Data are the mean of 6 replicates with 2 ducks each replicate.

TABLE 7 Effect of GPE supplementation on organ index of meat ducks.

Items[1]	Diets				SEM	*p*
	CON	GPE200	GPE400	GPE600		
Heart, g/kg	7.57	7.65	7.71	7.94	0.14	0.819
Liver, g/kg	19.22	19.81	19.50	19.06	0.41	0.928
Spleen, g/kg	0.73	0.69	0.89	0.70	0.04	0.255
Lung, g/kg	9.45	9.89	9.02	10.43	0.28	0.338
Glandular stomach, g/kg	3.65	3.56	3.73	3.70	0.11	0.955
Gizzard, g/kg	24.92	26.68	26.19	27.72	0.60	0.421

SEM, standard error (SE) of means.
[1]Data are the mean of 6 replicates with 2 ducks each replicate.

quality (pH, color, water loss rate, and shear force) were measured. The effect of dietary GPE on the meat quality of ducks were presented in Table 8. Dietary supplementation with GPE showed no effect on the pH of breast or leg muscles. Additionally, no significant differences in meat color values were detected, including a*, b*, and L*. Notably, in parallel to the control group, dietary supplementation with GPE resulted in a substantial depression of water loss rate and shear force. These results indicated that diets with GPE supplementation could effectively improve shear force and reduce water loss rate, leading to an overall improvement in meat quality.

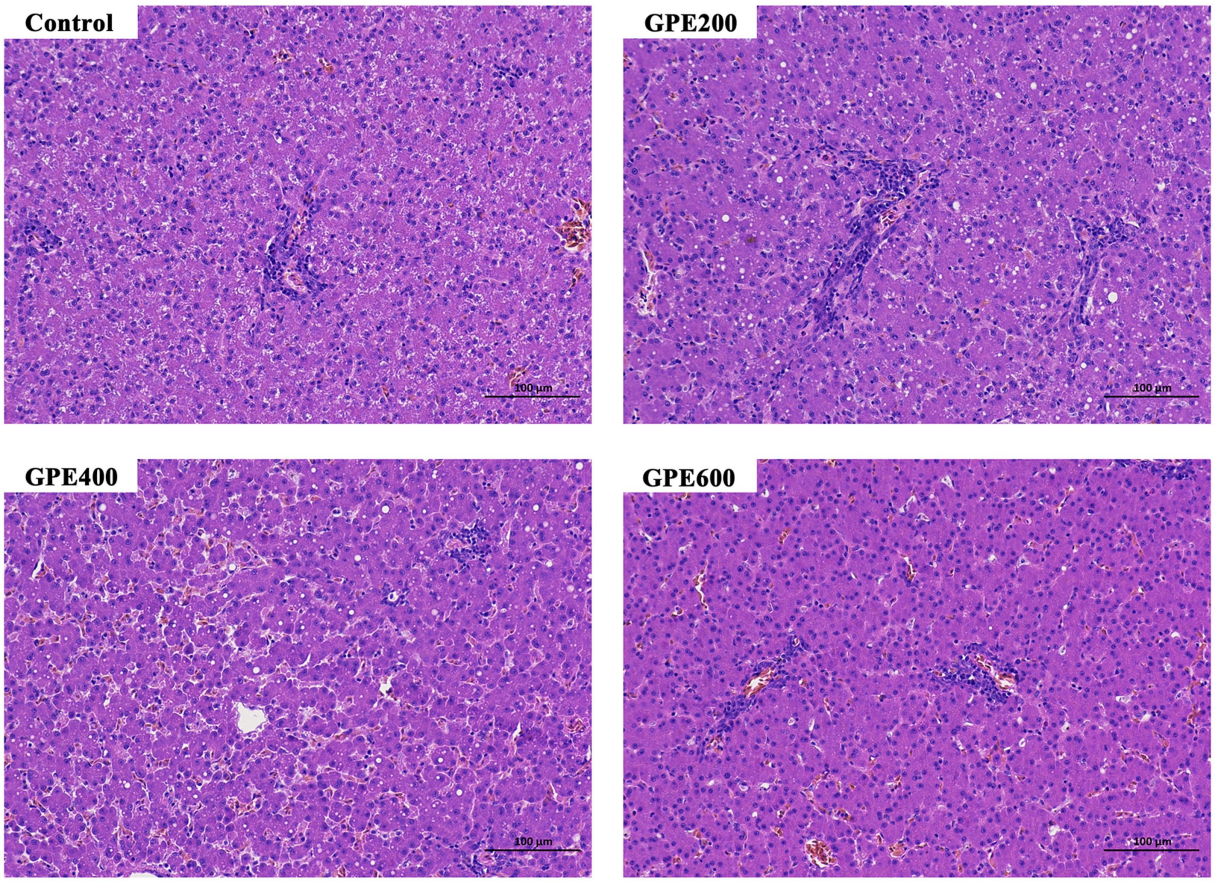

FIGURE 2
Effect of supplementation with GPE on histopathological alternations of liver tissue.

TABLE 8 Effect of GPE supplementation on meat quality of meat ducks.

Items[1]	Diets				SEM	*P*
	CON	GPE200	GPE400	GPE600		
Breast muscle pH	5.73	5.74	5.74	5.78	0.02	0.887
Leg muscle pH	6.72	6.70	6.77	6.82	0.04	0.685
L*	39.39	41.03	40.50	40.63	0.48	0.672
a*	16.24	16.26	16.91	16.94	0.39	0.868
b*	3.89	4.03	5.03	4.06	0.24	0.304
Water loss rate (%)	34.99^{a}	30.71^{b}	30.85^{b}	30.89^{b}	0.67	0.045
Shear force (kgf)	5.17^{a}	4.67^{b}	4.52^{b}	4.50^{b}	0.09	0.014
IMP (mg/g)	1.37	1.20	1.13	1.29	0.62	0.582

Values of "L*," "a*," "b*" represent brightness, redness and yellowness of meat surface. SEM, standard error (SE) of means.
[1]Different lowercase letters represent significant differences ($p < 0.05$) and data are the mean of 6 replicates with 2 ducks each replicate.

3.9 The IMP content of breast muscle

As shown in Table 8, the IMP content in the breast muscle meat of the GPE-treated groups was not significantly different from that of the control group at 70 days, indicating that GPE supplementation has no impact on modulating IMP level.

3.10 Chemical composition of the duck breast muscle

As displayed in Table 9, there was no noticeable effect of GPE on protein and fat ($p > 0.05$) content in the duck breast muscle. However, the inclusion of GPE (600 mg/kg) apparently improved the content

TABLE 9 Effect of GPE supplementation on protein, fat, moisture content and antioxidant capacity of duck breast muscle.

Items[1]	Diets				SEM	*p*
	CON	GPE200	GPE400	GPE600		
Moisture (%)	69.17[b]	68.74[b]	67.51[c]	70.47[a]	1.65	<0.001
Fat (%)	2.51	2.52	2.54	2.49	0.18	0.956
Protein (%)	20.07	20.21	18.78	19.21	0.49	0.715
TC (mmol/g)	0.25	0.24	0.25	0.26	0.01	0.495
MDA (nmol/mg prot)	0.47	0.51	0.57	0.53	0.15	0.102

SEM, standard error (SE) of means.
[1]Different lowercase letters represent significant differences ($p < 0.05$) and data are the mean of 6 replicates with 2 ducks each replicate.

of moisture in duck breast muscle. These findings suggested that GPE supplementation had no significant effect on the content of protein and fat but increased moisture, ultimately improving meat quality to some extent.

3.11 Antioxidant capacity of the duck breast muscle

As depicted in Table 9, in contrast to the control group, the levels of MDA and TC did not show any significant variations after GPE supplementation. These results demonstrated that GPE inclusion had no significant impact on improving antioxidant capacity of breast muscle or modulating its TC level.

4 Discussion

Over the last several decades, therapeutic practices in global animal production have heavily relied on synthetic antibiotics, with approximately 70% usage (22, 23). This widespread reliance is primarily due to their remarkable efficacy in boosting antioxidative defenses, mitigating inflammatory responses, and improving both overall health status and production performance (24). However, with increasing concern about the negative impact of antibiotic use aspects on public health, many countries have gradually been banning the use of antibiotics in animal husbandry production (25–27). Despite strict restrictions on the use of antibiotics, maintaining poultry health remains a formidable challenge in livestock and poultry production. In efforts to address this issue, researchers have gradually turned their attention to natural plants which could enhance immunity and antioxidant capacity of animals and birds (28). Therefore, it is imperative to search for efficient, eco-friendly, and safe feed additives that can enhance animals' production potential, improve body health, and serve as a viable alternative to antibiotics in feed.

Through a long history of clinical practice, TCM has developed a unique theoretical framework and abundant clinical experience aimed at enhancing the health of both humans and animals (29). In recent, with the ban on antibiotics, TCM has been widely used in livestock farming due to its high efficacy and low toxicity (30). Not only these, new natural compounds derived from TCM have been also increasingly used as feed additives (31, 32). Furthermore, many lines of evidence have indicated that dietary supplementation with TCM has a positive effect on improving animal health, providing a promising and effective alternative to antibiotics in livestock production (33). A large body of animal experiment results also confirmed that dietary supplementation with TCM has more advantages in promoting livestock growth and improving disease resistance in comparison to conventional feed additives (34). Therefore, more and more researchers are also aware of the vital role of TCM in livestock farming (35). It can thus be stated that TCM has the potential to be a new feed additive to improve livestock productivity and health status.

GP is a traditional folk herbal medicine widely used in China, which is clinically used for the treatment of eruptive fevers and inflammatory-related diseases. It contained a series of biologically active components, including flavonoids, polyphenols, saponin, tannin and terpenoid (18, 36). Research has indicated that GP leaf extract could enhance hepatic antioxidation capacity and decrease pro-inflammatory factor levels in mice, effectively mitigating cadmium-induced liver toxicity (37). Furthermore, other investigators have found that dietary supplementation of GP or GP-containing herbal prescriptions could reduce the egg yolk cholesterol, suppress harmful excreta microflora and improve layers performance, which was ascribed to the bioactive compounds in GP (38). From this, GPE has the potential to be a new feed additive to improve livestock farming applications. Therefore, to delve deeper into whether the potential effects of GPE on ducks are associated with immune enhancement and improved antioxidant functions, we measured immune and anti-oxidation-related parameters.

Serum biochemical parameters provide valuable information for evaluating the health status of ducks and reflect many metabolic alternations of organs and tissues. ALT, AST and ALP are the commonly used indicators for assessing liver function. Once liver was injured, hepatocytes were destroyed contributing to the sharply increased serum aminotransferases (39). Urea nitrogen is the primary metabolite of protein and amino acid, which reflects the utilization efficiency of nitrogen (40). Serum total protein and ALB could reflect the metabolic status of protein in the body to a certain extent (41). TC is an important index that could indirectly reflect the ability of hepatic lipid synthesis (42). Additionally, TG is the recognized indicator, which is used to further evaluate the function of hepatic lipid metabolism (43).

In this work, there is no statistically significant difference in ALT, AST, and ALP levels between the control group and treatment groups, indicating dietary supplementation of GPE did not exert any toxic effect on the liver. Notably, dietary supplementation of GPE dramatically reduced the levels of total protein and BUN, suggesting the utilization efficiency of nitrogen was dramatically increased after GPE supplementation. Furthermore, dietary

supplementation of GPE improved hepatic lipid metabolism, as evidenced by the reduced levels of TC and TG. Concordant with our results, Murugaiyah et al. reported that serum TC level was decreased in chemical and high fat diet (HFD)-induced hyperlipidemic rats treated with GPE (44). Hence, these results illustrated that dietary supplementation with GPE could modulate hepatic lipid metabolism and improve nitrogen utilization efficiency in ducks.

Accumulated lines of evidence have indicated that immunity is intimately correlated with livestock health (45). The immune system guards the body against foreign substances and protects it from invasion by pathogenic organisms. As the important regulators of immune function, Ig is a class of specific active proteins that can be converted into antibodies by antigen induction, such as IgM, IgG, IgA and so on (46). The immunoglobulin activities (IgA, IgM, and IgG) in the serum are important indicators to estimate the non-specific immunity status of the animal. Immunoglobulin A (IgA), an important serum immunoglobulin, plays a crucial role in the immune defense of mucosal surfaces (47). Additionally, when the body is exposed to an external stimulus, the immune system is primed, triggering the release of several inflammatory factors, such as IL-1β and IL-6 (48). In the present experiment, dietary inclusion of GPE could dramatically elevate the contents of IgM and IgG at day 70 of age in compared to the control group. The IL-2 level was considerably inhibited by dietary GP supplementation. Curiously, treatment with GPE has no evident difference in IL-6 and IL-1β activities. Consistent with our observations, Wu et al. found that dietary supplementation with flavonoids from bamboo leaf could elevate the serum IgM activity in comparison to the control group, which might be attributed to the potent immunomodulatory functions of flavonoids (49). Additionally, Huang et al. also noted that supplementation with flavonoid-rich Fenugreek extract had a positive effect on modulating immunity in broiler (50). The enhanced immunity may result from GPE'S ability to stimulate immune cells to release immune factors, thereby attenuating inflammation (51).

In addition to modulating immunity function, numerous studies have revealed that oxidative stress was also a crucial contributor to inferior growth performance of ducks (52). To further elucidate whether the potential effect of GPE was intimately associated with its antioxidant function, the oxidative stress-related indicators were determined. MDA, as the final product of lipid peroxidation, is widely considered a reliable marker reflecting the degree of oxidative damage (53). GSH-Px are the key antioxidant enzymes that can scavenge ROS generated from oxidant stress (54). SOD is a pivotal endogenous antioxidant enzyme that acts as a component of first-line defense against oxidative damage. It catalyzes the dismutation of superoxide anion, forming hydrogen peroxide and molecular oxygen (55). T-AOC was the main parameter to measure the total antioxidant level of the enzymatic and nonenzymatic systems (56). In the present experiment, our results indicated that dietary supplementation with GPE could dramatically elevate the activity of T-AOC and reduce MDA content in serum, which might largely be attributed to its primary bioactive ingredient, polyphenolic. Similar to our finding, Ao et al. demonstrated that grape seeds were also richer in polyphenolic compounds and confirmed that supplementation with grape seeds dramatically enhanced antioxidative activity by improving T-AOC in broilers (57). The excellent antioxidant capacity might be closely associated with polyphenol, flavonoids in GPE, all of which exhibit potent antioxidant and free radical scavenging activities (36).

Carcass characteristics have been recognized as crucial response parameters to assess dietary energy and amino acid status in livestock dietary, such as yields of breast muscle, thigh muscle, and abdominal fat (58). Our findings suggested that no significant variance in dressing yield was observed between the control group and the three treatment groups. Additionally, there was no statistically significant difference in dressing percentage, semi-eviscerated yield, eviscerated yield, breast muscle yield and abdominal fat yield between the various treated groups. In agreement with our results, Ma et al. reported that there were no pronounced effects on dressing percentage, eviscerated yield, breast and thigh percentage after supplementation with flavonoids derived from sea buckthorn fruits (59). Altogether, these findings indicated that dietary supplementation with GPE did not show any substantial effect on the carcass characteristics.

The organ indexes are important indicators, which could partially reflect the health status of the body. When the body is challenged by an external stimulus, the internal organs of the body will undergo corresponding changes. Among them, the spleen is an important immune organ, which protects the host from microbial infections and mechanical injuries. The liver is a central organ for lipid metabolism and plays a vital role in detoxification. The lung is a particularly sensitive organ, which defenses the body from external stimulus. Our present results showed that dietary supplementation of GPE had no implications on the various organs, such as heart, liver, kidney, spleen, gizzard and glandular stomach. These data demonstrated that dietary supplementation with GPE did not exert toxic side effects on these organs, confirming that dietary supplementation with GPE is a safe strategy for improving animal health.

As we all know, in addition to their impact on the growth and health of livestock, abnormal oxidative stress and immunity influence have also a negative effect on the quality of livestock meat (60). Meat quality is an essential element that influences consumer's purchasing decisions. As the consumer's demand for meat quality is increasing, improving the quality of meat becomes a highly effective approach to boost the consumer's purchase desire (61). Recently, more and more traditional Chinese medicine, such as *Astragalus mongholicus Bunge* and *Largehead Atractylodes* Rh, have been confirmed to exhibit a favorable effect on the improvement of meat quality (33, 62). Therefore, to investigate whether the potential effect of GPE was closely linked to improving meat quality, the meat quality relevant parameters were measured. In this work, dietary supplementation with GPE apparently improved the moisture content of breast muscle, while it had no notable variation in protein, fat and IMP contents of the breast muscle. In keeping with our observation, Ahmad et al. found that *β*-GOS and methionine co-supplementation did not show any promising effects on meat quality in broilers (63). Furthermore, dietary supplementation of GPE could apparently reduce the shear force and the water loss rate. In addition, no marked difference in breast muscle pH, leg muscle pH and breast muscle color were observed in any of the experimental groups. As we all know, pH value is a vital reference for judging the change of acidity in the process of muscle tissue

fermentation and speed of fermentation after slaughtering, which has a substantial impact on meat quality. Therefore, maintaining a stable pH is essential for the proper maturation of muscles. Recent studies have demonstrated that a rapid pH reduction causes denaturation of myofibrillar proteins, leading to poor water-holding capacity and deteriorated drip loss (64). In this investigation, the reduced water loss in the breast muscle may be attributed to a stable pH value. The above results suggested that dietary supplementation with GPE prominently enhanced the tenderness and water-holding capacity of duck meat, leading to a notable improvement in its overall quality. Consistent with our results, Jin et al. reported that dietary supplementation with curcumin influenced the color of leg muscle while enhanced a prominent trend toward improving the water-holding capacity in breast muscle of meat ducks (65). The integrity of the cell membrane is a crucial factor that influences the water-holding capacity and tenderness of meat. Enhancement in meat quality may be caused by the fact the stability and integrity of the cell membrane were changed due to the improved antioxidant capacity of meat (66).

Taken together, our study first demonstrated that there was no significant effect on growth performance after dietary supplementation with GPE. Noteworthy, dietary supplementation with GPE could enhance immunity and regulate oxidative stress. Moreover, supplementation of GPE could improve meat quality, which was primarily manifested by improvements in the shear force and water loss rate. It might offer a strong rationale for developing and utilizing GPE and GP-containing traditional Chinese herbal prescriptions as a novel feed additive. Furthermore, our discovery would have far-reaching implications for expanding the potential utility of GPE.

Apart from these strengths, there are several additional limitations to our study. Firstly, despite GPE exhibiting favorable effect on ducks, it is not clearly established which bioactive ingredients are associated with its excellent modulation effect. Secondly, the study is currently restricted to ducks only and has not been extended to other conventional animals. Thirdly, our study only focused on the modulation effect of GPE in ducks, which neglected the dynamic process of GPE *in vivo*. To address these limitations, the detailed bioactive ingredients still need a deeper investigation. In addition, the specific regulatory mechanism of GPE as immunomodulators and antioxidants deserves to be further investigated. Furthermore, more different kinds of animals were required to investigate its potential effect before widespread application. By explicitly noting these constricts, we aim to elucidate which active components exhibited the best modulation effect on ducks. Besides, we also encourage ongoing research to investigate the dynamic process of GPE *in vivo* for a clearer understanding of the underlying molecular mechanisms.

5 Conclusion

In conclusion, this study showed that dietary supplementation with GPE could increase serum total antioxidant capacity, regulate immunity function and improve meat quality to some extent in meat ducks. These results can provide further evidence using GPE as a feed additive in meat duck production. The recommended optimal GPE level in the diet of meat ducks is 600 mg/kg according to the results in this study.

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 approved by the Experimental Animal Ethics Committee in Jiangxi Academy of Agricultural Sciences. The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

GA: Conceptualization, Writing – original draft. PX: Formal analysis, Writing – original draft. JC: Data curation, Methodology, Writing – review & editing. WSo: Data curation, Writing – review & editing. QS: Validation, Writing – review & editing. CX: Data curation, Writing – review & editing, Investigation. WSu: Data curation, Writing – review & editing, Visualization. ZZ: Formal analysis, Visualization, Writing – review & editing. QW: Funding acquisition, Project administration, Writing – review & editing. XC: Funding acquisition, Resources, Writing – review & editing.

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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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Organic trace elements enhance growth performance, antioxidant capacity, and gut microbiota in finishing pigs

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Excessive inorganic trace elements are added to livestock and poultry feed to meet the needs of animals, accompanied by frequent occurrence of excretion and gastrointestinal stress. Replacing inorganic trace elements with organic trace elements provides a promising solution to alleviate these problems. Therefore, this study aimed to assess the impact of replacing all inorganic trace elements (ITMs) in feed on the growth performance, meat quality, serum parameters, trace element metabolism, and gut microbiota of finishing pigs. A total of 128 Duroc × Landrace × Yorkshire growing-finishing pigs (33.88 ± 0.62 kg) were assigned to four treatments in a randomized complete block design. Each treatment was divided into four replicates, each containing eight pigs. The control group received a basal diet containing 100% inorganic trace elements, while the experimental groups were provided with diets where all inorganic trace elements were substituted with 30, 50, and 70% organic trace elements. The experiment spanned 56 days. Results indicated that replacing all ITMs with 30, 50, and 70% OTMs demonstrated no adverse effects on average daily feed intake, average daily gain, feed conversion ratio, eye muscle area, backfat thickness, and relative organ weight of finishing pigs compared to the control group. Furthermore, the replacement led to increased serum immunoglobulin A concentration and Cu-SOD enzyme activity, and decreased serum MDA level, and GSH-Px activity in the liver. Notably, 50 and 70% OTMs increased serum Mn-SOD activity, and 70% OTMs increased serum T-AOC content. Moreover, it significantly decreased the excretion of trace elements in feces without compromising their deposition in the muscle. Additionally, replacing 100% ITMs with 30% OTMs resulted in an improved Shannon index of colonic microbiota in finishing pigs. In conclusion, replacing 100% inorganic trace elements with 30, 50, and 70% organic trace elements exhibited no detrimental effects on the performance of finishing pigs. In conclusion, replacing 100% inorganic trace elements with 70% organic trace elements had certain potential to improve the production performance of finishing pigs. This replacement strategy can enhance meat quality, boost antioxidant capacity, reduce trace element excretion, facilitate trace element absorption and deposition, and enhance gut microbiota homeostasis.

KEYWORDS

trace minerals, antioxidant capacity, mineral metabolism, mineral element pollution, gut microbiota

1 Introduction

Trace mineral elements are crucial for ensuring the healthy growth, productivity, and reproductive capacity of animals. Serving as cofactors, they play a vital role in synthesizing essential enzymes and proteins within the animal organism (1). These elements are currently categorized into two groups based on their sources: organic and inorganic (2). Inorganic trace elements are predominantly found in the form of inorganic salts and are extensively utilized in modern pig farming due to their cost-effectiveness. Nevertheless, their limitations have become increasingly apparent. Inorganic trace elements are susceptible to dissociation and can form complexes with substances like tannins, phytates, oxalates, and silicates, thereby hindering optimal absorption rates and bioavailability (3). Consequently, the excessive amounts added during production surpass the recommended levels outlined in feeding guidelines, resulting in a significant excretion of metal elements in fecal matter, leading to severe environmental and soil contamination (4). Research indicates that pig manure in China contributes to around 30% of the total waste emissions in the livestock sector, with heavy metal discharge from pig farming making up 71.52% of the overall heavy metal emissions from livestock and poultry manure, the highest share among all types of livestock waste (5, 6). Hence, there is an urgent need to develop strategies to minimize the release of trace minerals from pigs.

Enhancing the absorption efficiency of trace elements, while meeting the nutritional demands of animals, is a crucial strategy to reduce trace mineral emissions. Initially denoting organic acids like zinc gluconate and ferrous fumarate, the definition of organic trace elements has broadened to encompass metal amino acid chelates, metal proteinates, and polysaccharide complexes (7, 8). Extensive research underscores the superior stability of organic trace elements in the animal gut, their reduced susceptibility to degradation, and fewer antagonistic interactions with other compounds. This translates to heightened bioavailability within the animal, ensuring that even modest amounts of trace minerals fulfill the animal's nutritional requirements. Studies indicate that substituting inorganic trace minerals with lower doses of organic trace minerals has no adverse effects on key economic parameters, including production and egg-laying performance, in sows, piglets, broilers, and laying hens (2, 3, 9, 10). Simultaneously, this substitution increased the absorption rate of trace elements and significantly reduced the excretion of mineral micronutrients in manure, thereby contributing to environmental protection (11). In a prior investigation, we observed that replacing all inorganic trace elements in the diet of weaned piglets with 30 to 50% organic trace elements did not impede growth performance and reduced trace element excretion (12). However, another study demonstrated that replacing inorganic trace elements with glycine- or methionine-chelated copper and zinc significantly improved the growth performance of piglets, while also enhancing the utilization of copper and zinc in the feed (13). Nevertheless, there is a paucity of discourse on the impact of substituting inorganic trace minerals with organic trace minerals on muscle trace mineral deposition and gut microbiota in fattening pigs. Additionally, existing literature predominantly focuses on single organic trace elements like zinc methionine, zinc glycine, and copper methionine, with limited evaluation of multiple complex organic compounds (14).

Hence, this study aimed to examine the impact of substituting 100% inorganic trace elements in pig feed with compound organic trace elements at levels of 30, 50, and 70% on growth performance, carcass traits, meat quality, serum parameters, trace element metabolism, and gut microbiota in growing and finishing pigs. This investigation sought to assess the feasibility of replacing inorganic trace elements in feed with reduced organic trace elements.

2 Materials and methods

2.1 Animal management, diets, and experimental design

A total of 128 growing-finishing pigs (Duroc × Landrace × Yorkshire) with an initial average body weight of 33.88 ± 0.62 kg were assigned to four treatments in a randomized complete block design based on body weight. Each treatment was divided into four replicates, each containing eight pigs. The basal diet (Table 1) was formulated to meet NRC (2012) standards and supplemented with trace minerals excluding Fe, Cu, Zn, Se, I, and Mn. The control group diet contained 100% inorganic trace minerals (sulfates) commonly used in the Chinese pig industry with additional Fe, Cu, Zn, Se, I, and Mn at 80, 6, 100, 0.3, 0.3, and 40 mg/kg, respectively. Organically complexed Fe, Cu, Zn, and Mn were added to the basal diet at levels of 30, 45, and 60% (30% OTMs, 50% OTMs, and 70% OTMs) compared to the control group using MINEXO™ (Table 2). The molecular weight of copper, iron, manganese, and zinc is <500 Da. Furthermore, all amino acids are from high quality plant proteolysis and contains 18 amino acids. The measured values of copper, iron, manganese, and zinc included in the test diet are shown in Table 3. The study lasted 56 days during which feed consumption was monitored weekly, and body weight was measured at the end to calculate average daily gain (ADG), average daily feed intake (ADFI), and feed to gain ratio (F/G). Pigs had *ad libitum* access to feed and water.

2.2 Sample collection

Fresh fecal samples were collected from each replicate consecutively for 3 days before the end of the trail and stored at −20°C. The collected feces were mixed, dried, and ground. Using the quartering method, 50 g of dry fecal samples were obtained to assess the trace element content. Post-experiment, all pigs were weighed following a 12-h fast. One pig closest to the average body weight in each replicate was selected for slaughter and sampling. Blood samples were collected from anterior vena cava puncture without anticoagulants, centrifuged at 3000 × g for 10 min at room temperature, and then immediately stored at −80°C for serum biochemical parameters analysis. Following stunning, bleeding, dehairing, evisceration, and midline splitting, the hot carcass weight and intestinal weight were measured. Backfat thickness on the left side of the carcass was recorded at multiple regions, and the mean value was noted. Approximately a 15 cm segment of the intestine was ligated near the proximal colon, followed by a precise incision made at the

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

Ingredients	Content, %	Nutrient levels[b]	Content
Corn	56.00	Digestive energy, kcal/kg	3190.98
Soybean meal (46%)	18.14	Crude protein, %	15.47
Rice bran meal	8.40	Crude fiber, %	3.99
Brown	6.00	Crude fat, %	2.73
Paddy	8.00	Dry matter, %	85.15
Limestone	1.00	Ca, %	0.54
$CaHPO_4$	0.30	TP, %	0.54
NaCl	0.40	Available phosphorus, %	0.17
Choline chloride	0.10	SID Lys, %	1.23
L-Lysine hydrochloride	0.50	SIDMet + Cys, %	0.55
DL-Methionine	0.01	SID Thr, %	0.55
Threonine	0.05	SID Met, %	0.27
Premix[a]	1.10	SID Lys, %	1.23
Total	100		

[a]The premix provided the following per kg of diets: VA 12400 IU, VD3 2,800 IU, VE 30 IU, VK 5 mg, VB12 40 μg, VB1 3 mg, VB2 10 mg, nicotinic acid 40 mg, D-pantothenic acid 15 mg, folic acid 1 mg, VB6 8 mg, biotin 0.08 mg, CaI_2O_6 0.92 mg, Na_2SeO_3 0.77 mg. [b]Nutrient levels were determined, except that digestible energy, SID Lys, SID Met, SID Thr, and SID Trp were calculated. The crude protein, crude fiber, crude fat, neutral detergent fiber, acid detergent fiber, moisture, ash, Ca, and TP were all determined according to the China National Standard, and the document numbers are GB/T 6432–2018, GB/T 6434–2006, GB/T 6433–2006, GB/T 20806–2006, NY/T 1459–2007, GB/T 6435–2014, GB/T 6438–2007, GB/T6436-2002, and GB/T 6437–2018, respectively.

TABLE 2 Experiment design.

Items	Forms	100% ITMs	OTMs		
			30%	50%	70%
Cu, mg/kg	Amino acid-chelated		1.8	3.0	4.2
	Sulfates	6			
Fe, mg/kg	Amino acid-chelated		2.4	40	56
	Sulfates	80			
Mn, mg/kg	Amino acid-chelated		12	20	28
	Sulfates	40			
Zn, mg/kg	Amino acid-chelated		30	50	70
	Sulfates	100			

ITMs, inorganic trace minerals; OTMs, organic trace minerals.

TABLE 3 Measured values of trace minerals in experimental diets (air-dry basis).

Items	100% ITMs	OTMs		
		30%	50%	70%
Cu, mg/kg	6.11	2.04	3.21	4.43
Fe, mg/kg	81.76	25.91	42.71	59.83
Mn, mg/kg	40.91	13.83	22.93	28.40
Zn, mg/kg	106.51	32.56	53.89	75.49

ITMs, inorganic trace minerals; OTMs, organic trace minerals.

center of the colon using a sterile scalpel. Subsequently, the colonic contents were collected in a 2 mL sterile centrifuge tube, rapidly frozen in liquid nitrogen, and stored at −80°C. Moreover, a muscle sample approximately 10 cm thick was taken from the longissimus muscle between the fifth and sixth ribs on the left side for meat quality evaluation.

2.3 Growth performance

Pigs were weighed on day 1 and day 56 of the fattening period, with daily feed intake being recorded. The gathered data were utilized to determine the average daily feed intake (ADFI), average daily gain (ADG), and feed-to-gain ratio (F/G) according to the following formula:

ADFI = total feed intake/ (test days × test number);
ADG = total weight gain/ (number of test days × number of tests);
F/G = ADFI/ ADG.

2.4 Organ index

After slaughtering the growing-finishing pigs, the heart, liver, spleen, intestines, and kidneys were excised. Surface tissue fluid was then dried using absorbent paper and weighed. The organ weight percentage was calculated using the formula: organ index (g/kg) = organ weight/body weight.

2.5 Meat quality analysis

Meat color was assessed at 45 min and 24 h postmortem using a portable colorimeter (CR-410, Minolta, Chiyoda, Japan) under a D-65 light source. pH values were also measured at these time points postmortem with a portable pH meter (testo-205, Testo, Lenzkirch, Germany). A muscle sample weighing approximately 120 g was steamed over boiling water for 30 min, then cooled on a hook for another 30 min, dried with filter paper, and reweighed (15). Cooking loss was determined by calculating the weight change percentage. Shear force values were assessed using a Warner-Bratzler shear force device (TA.XT Plus, Stable Micro Systems, Godalming, UK) based on previous studies (16).

2.6 Serum biochemical parameter analysis

Serum levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), lactate dehydrogenase (LDH), total bile acid (TBA), total protein (TP), albumin (ALB), globulin (GLB), glucose (GLU), total cholesterol (TC), triglycerides (TG), high-density lipoprotein cholesterol (HDL-C), and low-density lipoprotein cholesterol (LDL-C) were quantified using an automated biochemical analyzer and the respective reagents (KHB 450, Shanghai Kehua Bioengineering Co., Ltd., Shanghai, China).

2.7 Immunoglobulin analysis

Serum levels of IgA, IgG, and IgM were determined using ELISA kits following the manufacturer's instructions (Jiangsu Meimian Industrial Co., Ltd., Yancheng, China). All detection procedures were carried out in accordance with the manufacturer's instructions for each respective kit.

2.8 Antioxidant capacity analysis

Malondialdehyde (MDA, cat: A003-2), total antioxidant capacity (T-AOC, cat: A015-2-1), total superoxide dismutase (T-SOD, cat: A001-2), copper-zinc superoxide dismutase (Cu/Zn-SOD, cat: A001-2), manganese superoxide dismutase (Mn-SOD, cat: A001-2), and glutathione peroxidase (GSH-px, cat: A005) levels were analyzed using commercial kits obtained from Nanjing Jiancheng Bioengineering Institute (NJJCBIO, Nanjing, China). Liver tissue samples (0.1–0.2 g) were excised and rinsed in cold physiological saline to remove residual blood. The tissues were blotted dry using filter paper and accurately weighed. Each sample was placed into a 5 mL homogenizer tube, and 0.86% physiological saline was added at a weight-to-volume ratio of 1:9. Using ophthalmic scissors, the tissue was finely minced, followed by homogenization to prepare a 10% tissue homogenate. Homogenization was performed in an ice-water bath using a tissue grinder at 15,000 rpm for 5 cycles, with each cycle consisting of 10 s of homogenization followed by a 30-s interval. All procedures were conducted under low-temperature conditions to preserve sample integrity. Liver tissue samples were homogenized in physiological saline, and the resulting supernatant was collected post-centrifugation for subsequent analysis. The final results were adjusted based on the total tissue protein concentration.

2.9 Trace mineral element analysis

Approximately 15.0 g of *longissimus dorsi* muscle was freeze-dried and ground, and fecal samples were dried in an oven and ground. A 0.20 g portion of the ground sample was placed in a microwave digestion vessel, to which 10 mL of concentrated nitric acid and 1 mL of hydrogen peroxide were added. The sample underwent digestion in a microwave digestion system. Subsequently, 0.5 mL of perchloric acid was introduced to the vessel, and the solution was heated at 180°C for 2 h to eliminate residual acids. The solution was then diluted to 10 mL with 1% nitric acid, and the concentrations of Cu, Fe, Mn, and Zn in the sample were determined using inductively coupled plasma optical emission spectrometry (5,110 ICP-OES, Agilent, US).

2.10 Microbial analysis

Microbial diversity in colonic contents was assessed through high-throughput sequencing of bacterial 16S rDNA. The total DNA of intestinal flora in colon contents was extracted using the Cetyltrimethylammonium bromide (CTAB) method. Subsequently, PCR amplification was carried out following electrophoresis detection and quantification. For this purpose, the Primers 341F (5'-CCTACGGGNGGCWGCAG-3') and 805R (5'-GACTACHVG GGTATCTAATCC-3') were utilized. The resulting amplified products were purified using AMPure Xbeads and measured with a Qubit. Evaluation of the purified amplified products was conducted using the Agilent 2100 Bioanalyzer and the lumina library quantification kit from Kapa Biosciences. The lumina MiSeq sequencing platform from Shanghai Meiji Biomedical Technology Co., Ltd. facilitated two-end sequencing. Subsequently, the double-end data obtained from sequencing were processed by splitting, concatenating, filtering, and denoising using the DADA2 algorithm to obtain the final Amplicon Sequence Variant (ASV) signature sequence and ASV abundance table. Further analysis, including alpha diversity, species differentiation, and functional prediction, was performed based on the characteristic sequences and abundance tables of ASVs. The significance of alpha diversity was assessed using the Kruskal-Wallis test. Differential intestinal flora were identified through Linear Discriminant Analysis Effect Size (LEfSe) analysis, whereby a significance level of $p < 0.05$ and

a Linear Discriminant Analysis (LDA) value >4 were considered as the criteria for differentiation. Additionally, the PICRUSt2 software was utilized for predicting the function of gut microbiota.

2.11 Statistical analysis

Data were managed and analyzed using the IBM SPSS Statistics V26.0 software package (IBM Corp., Armonk, NY, USA), with results reported as the mean and pooled standard error. Prior to conducting group comparison analyses, the normality of the data was confirmed through the Shapiro–Wilk test. In cases where the data exhibited a non-normal distribution, statistical analysis involved a one-way ANOVA followed by the Kruskal–Wallis test with multiple FDR corrections. For normally distributed data, a one-way ANOVA analysis followed by LSD test was applied. Significant differences were defined at $p < 0.05$, with a trend toward significance noted at $0.05 \leq p < 0.10$.

3 Results

3.1 Performance, carcass traits, and organ index

The average daily gain, average daily feed intake, feed-to-gain ratio, carcass weight, loin eye area, and backfat thickness of fattening pigs were not influenced by either the level or source of trace element supplementation, as depicted in Table 4 ($p > 0.10$). Moreover, the substitution of 100% ITMs with 30–70% OTMs in Table 5 showed no significant impact on the heart, liver, spleen, kidney, or intestinal indices of fattening pigs ($p > 0.10$).

3.2 Meat quality

As shown in Table 6, the *longissimus dorsi* muscle color, pH at 45 min, shear force, and cooking loss in fattening pigs were not influenced by the source or level of trace element supplementation in the diet ($p > 0.10$). Notwithstanding, the pH at 24 h in the 50% OTMs and 70% OTMs groups showed a significantly higher level compared to the 100% ITMs group ($p < 0.05$).

3.3 Serum biochemical parameters and immunoglobulin contents

Tables 7, 8 illustrates that substituting 100% ITMs with reduced OTMs in dietary supplementation had no significant impact on the serum levels of ALT, AST, LDH, TBA, TP, ALB, GLB, A/G, BUN, TC, HDL-C, LDL-C, IgG, and IgM ($p > 0.10$). Furthermore, IgA and TG levels were significantly higher in the 30, 50, and 70% OTM groups compared to the 100% ITM group ($p < 0.05$). Notably, 70% OTMs exhibited a marked reduction in serum ALP activity ($p < 0.05$).

TABLE 4 Effects of the dietary replacement of inorganic trace minerals with lower levels of organic trace minerals on the growth performance and carcass traits of growing-finishing pigs.

Items	100% ITMs	OTMs			SEM	*p*-value
		30%	50%	70%		
Initial BW, kg	33.94	33.78	33.81	33.81	0.49	0.992
Final BW, kg	79.53	80.73	77.34	80.00	3.96	0.843
ADG, kg/d	0.76	0.75	0.78	0.82	0.05	0.614
ADFI, kg/d	2.01	1.92	1.93	2.04	0.13	0.832
F/G	2.64	2.56	2.49	2.48	0.07	0.168
Carcass weight, kg	27.82	30.26	34.24	35.22	1.25	0.098
Loin eye muscle area, cm^2	27.82	30.26	35.22	37.00	1.53	0.097
Average back-fat thickness, mm	14.12	13.77	9.44	8.08	1.10	0.070

Values are presented as mean and pooled SEM, $n = 6$. Data in the same row with no or the same letter indicate no significant difference ($P > 0.05$), while with different letters mean significant difference ($P < 0.05$). ITMs, inorganic trace minerals; OTMs, organic trace minerals; BW, body weight; ADG, average daily gain; ADFI, average daily feed intake; F/G, feed-to-gain ratio.

TABLE 5 Effects of dietary replacing inorganic with lower levels of organic trace minerals on the organ index of finishing pigs.

Items	100% ITMs	OTMs			SEM	*P*-value
		30%	50%	70%		
Heart index, g/kg	3.60	3.90	4.00	4.11	0.16	0.748
Liver index, g/kg	17.11	17.61	17.28	16.44	0.64	0.924
Spleen index, g/kg	2.01	2.22	1.87	1.89	0.13	0.781
Kidney index, g/kg	1.47	1.67	1.49	1.69	0.06	0.435
Intestinal index, g/kg	4.42	4.25	4.28	4.80	0.16	0.673

Values are presented as mean and pooled SEM, $n = 6$. Data in the same row with no or the same letter indicate no significant difference ($P > 0.05$), while with different letters mean significant difference ($p < 0.05$). ITMs, inorganic trace minerals; OTMs, organic trace minerals.

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TABLE 6 Effects of the dietary replacement of inorganic trace minerals with lower levels of organic trace minerals on the meat quality traits of growing-finishing pigs.

Items	100% ITMs	OTMs			SEM	*P*-value
		30%	50%	70%		
Meat color						
L^*_{45min}	49.67	48.08	47.91	50.95	0.49	0.061
a^*_{45min}	13.54	14.22	13.78	13.47	0.20	0.606
b^*_{45min}	6.34	6.12	6.18	6.94	0.19	0.462
L^*_{24h}	52.38	51.44	55.17	53.07	0.70	0.309
a^*_{24h}	13.68	13.49	13.49	13.34	0.25	0.980
b^*_{24h}	4.96	5.84	6.05	5.90	0.29	0.608
pH value						
pH_{45min}	6.65	6.66	6.76	7.07	0.10	0.402
pH_{24h}	5.58^b	5.63^b	6.31^a	6.59^a	0.14	0.002
Drip loss						
Shear force, N	78.36	73.97	67.59	51.47	5.09	0.278
Moisture, %	0.71	0.72	0.72	0.80	0.02	0.275

Values are presented as mean and pooled SEM, $n = 6$. Data in the same row with no or the same letter indicate no significant difference ($P > 0.05$), while with different letters mean a significant difference ($P < 0.05$). ITMs, inorganic trace minerals; OTMs, organic trace minerals; L∗, lightness; a∗, redness; b∗, yellowness.

TABLE 7 Effects of the dietary replacement of inorganic trace minerals with lower levels of organic trace minerals on the serum biochemical parameters of growing-finishing pigs.

Items	100% ITMs	OTMs			SEM	*P*-value
		30%	50%	70%		
ALT (999U/mg prot)	58.29	55.20	46.33	62.82	2.91	0.242
AST (999U/mg prot)	60.74	56.40	53.00	46.27	2.38	0.168
ALP (999U/mg prot)	151.70^a	154.99^a	105.30^b	156.80^a	6.85	0.000
LDH (999U/mg prot)	1009.00	1117.00	1127.33	1227.33	71.12	0.810
TBA (999U/mg prot)	36.08	33.85	45.34	43.15	7.97	0.964
TP (999U/mg prot)	79.32	74.90	76.32	84.35	2.27	0.533
ALB (999U/mg prot)	44.41	44.41	44.08	49.21	1.17	0.387
GLB (999U/mg prot)	35.00	30.67	32.33	35.00	1.75	0.824
GLU (999U/mg prot)	5.38^{ab}	3.46^c	4.43^b	6.13^a	0.36	0.017
TC (999U/mg prot)	2.74	2.91	3.02	3.00	0.08	0.611
TG (999U/mg prot)	0.73^b	4.38^a	3.43^a	5.23^a	0.59	0.010
HDL-C (999U/mg prot)	0.83	0.93	0.76	0.85	0.02	0.078
LDL-C (999U/mg prot)	1.35	1.48	1.92	1.61	0.09	0.157

Values are presented as mean and pooled SEM, $n = 6$. Data in the same row with no or the same letter indicate no significant difference ($P > 0.05$), while data with different letters indicate a significant difference ($P < 0.05$). ITMs, inorganic trace minerals; OTMs, organic trace minerals; ALT, alanine aminotransferase; AST, aspartate transaminase; ALP, alkaline phosphatase; LDH, Lactate dehydrogenase; TBA, Total serum bile acids; TP, total protein; ALB, albumin; GLB, globin; GLU, glucose; TC, total cholesterol; TG, triglyceride; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol.

3.4 Antioxidant capacity

As shown in Table 9, the serum MDA levels and CuZn-SOD activity were significantly lower in the 30, 50, and 70% OTMs groups compared to the control group ($p < 0.05$). Conversely, the Mn-SOD activity and T-AOC levels in the 70% OTMs group were significantly higher than those in the control group ($p < 0.05$). No significant differences were found in serum GSH-px and T-SOD activities among the groups ($p > 0.10$). Furthermore, the liver GSH-px activity was significantly higher in all experimental groups than in the control group ($p < 0.05$). Additionally, the liver CuZn-SOD and T-SOD activities were significantly higher in the 50 and 70% OTMs groups compared to the control group ($p < 0.05$). There were no significant differences in liver T-AOC content, MDA content, or Mn-SOD activity among the groups ($p > 0.10$).

TABLE 8 Effect of low levels of organic trace elements on serum immune indexes of growing fertile pigs.

Items	100% ITMs	OTMs			SEM	*P*-value
		30%	50%	70%		
IgA (μg/mL)	12.73^{b}	15.79^{a}	14.69^{a}	15.29^{a}	0.42	0.020
IgG (μg/mL)	20.24	19.97	17.91	20.16	0.46	0.235
IgM (μg/mL)	1.18	1.12	1.08	1.18	0.02	0.263

Values are presented as mean and pooled SEM, $n = 6$. Data in the same row with no or the same letter indicate no significant difference ($P > 0.05$), while with different letters mean a significant difference ($P < 0.05$). ITMs, inorganic trace minerals; OTMs, organic trace minerals.

TABLE 9 Effects of the dietary replacement of inorganic trace minerals with lower levels of organic trace minerals on the antioxidant capacity of growing-finishing pigs.

Items	100% ITMs	OTMs			SEM	*P*-value
		30%	50%	70%		
Serum						
T-AOC (mM/L)	0.58^{b}	0.68^{ab}	0.67^{ab}	0.73^{a}	0.02	0.040
MDA (nM/mL)	1.65^{a}	1.46^{b}	1.44^{b}	1.36^{b}	0.04	0.029
GSH-px (IU/L)	509.53	532.40	628.97	684.88	32.31	0.177
CuZn-SOD (IU/mL)	89.96^{b}	109.47^{a}	116.64^{a}	108.47^{a}	3.71	0.035
Mn-SOD (IU/mL)	48.67^{a}	35.46^{ab}	22.43^{bc}	16.21^{c}	4.23	0.004
T-SOD (IU/mL)	124.67	138.63	139.08	144.93	3.42	0.191
Liver						
T-AOC (nM/g prot)	0.06	0.06	0.06	0.07	0.003	0.664
MDA (nM/mg prot)	0.13	0.14	0.11	0.09	0.002	0.318
GSH-Px (IU/mg prot)	31.10^{c}	35.06^{b}	37.94^{b}	41.56^{a}	0.59	0.001
CuZn-SOD (IU/mg prot)	368.73^{c}	375.66^{bc}	390.57^{ab}	398.21^{a}	4.43	0.037
Mn-SOD (IU/mg prot)	78.19	82.33	81.65	83.29	0.84	0.136
T-SOD (IU/mg prot)	446.92^{c}	457.99^{bc}	472.22^{ab}	481.50^{a}	4.76	0.017

Values are presented as mean and pooled SEM, $n = 6$. Data in the same row with no or the same letter indicate no significant difference ($P > 0.05$), while data with different letters indicate a significant difference ($P < 0.05$). ITMs, inorganic trace minerals; OTMs, organic trace minerals; T-AOC, total antioxidant capacity; MDA, malondialdehyde; GSH-px, glutathione peroxidase; prot, protein; CuZn-SOD, copper-zinc superoxide dismutase; Mn-SOD, manganese superoxide dismutase; T-SOD, total superoxide dismutase.

3.5 Contents of trace elements in feces and muscles

Table 10 illustrates that fecal trace element concentrations were notably lower in the 30, 50, and 70% OTMs groups compared to the 100% ITMs group ($p < 0.05$). Conversely, no significant variations were observed in the trace element content of the *longissimus dorsi* muscle across the groups. Noteworthy, the Mn and Zn content in the muscle exhibited an upward trend with increased OTMs supplementation ($0.05 < p < 0.10$).

3.6 Microbial analysis

3.6.1 Dilution curve

Figure 1A illustrates that the dilution curve starts to level off once the sample size reaches 5,000, signaling that the microbial communities' detection in the samples is adequate for microbial flora analysis. Figure 1F reveals a total of 1,464 operational taxonomic units (OTUs) identified across the four groups, with 740 OTUs being common to all groups. The counts of OTUs in the 100% ITMs, 30% OTMs, 50% OTMs, and 70% OTMs groups were 1,144, 1,198, 1,122, and 995, respectively.

3.6.2 Gut microbiota diversity

As shown in Figures 1B–E, there were no significant differences in the Ace index, Simpson index, and Chao index of fecal microbiota between the 30% OTMs, 50% OTMs, and 70% OTMs groups compared to the 100% ITMs group ($p > 0.05$). However, the Shannon index of the fecal microbiota in the 30% OTMs group was significantly higher than that in the 100% ITMs group ($p < 0.05$). When the contribution rate of principal component 1 was 15.82% and that of principal component 2 was 8.09%, the 30% OTMs group was distributed further from the 100% ITMs, 50% OTMs, and 70% OTMs groups, indicating a significant difference in microbial diversity between the 30% OTMs group and the other three groups (Figure 1G).

3.6.3 Gut microbial composition

As shown in Figure 2A, the top five phylum-level microbial abundances in the 100% ITMs, 30% OTMs, 50% OTMs, and 70% OTMs groups were *Firmicutes*, *Bacteroidota*, *Proteobacteria*, *Spirochaetota*, and

TABLE 10 Effect of lower levels of inorganic trace minerals on fecal trace element excretion and muscle trace element deposition in growing and fattening pigs.

Items	100% ITMs	OTMs			SEM	*P*-value
		30%	50%	70%		
Fecal excretion						
Cu, mg/kg	54.85[a]	35.22[c]	37.63[c]	40.30[b]	1.64	<0.001
Fe, mg/kg	877.67[a]	430.48[d]	464.59[c]	561.95[b]	37.18	<0.001
Mn, mg/kg	328.95[a]	167.78[d]	189.32[c]	226.78[b]	13.12	<0.001
Zn, mg/kg	981.68[a]	461.72[d]	566.28[c]	622.76[b]	40.89	<0.001
Muscle						
Cu, mg/kg	0.51	0.46	0.49	0.55	0.02	0.278
Fe, mg/kg	5.10	5.14	5.33	5.73	0.18	0.558
Mn, mg/kg	0.52	0.37	0.42	0.52	0.02	0.087
Zn, mg/kg	8.04	7.71	8.53	8.92	0.18	0.049

Values are presented as mean and pooled SEM, $n = 6$. Data in the same row with no or the same letter indicate no significant difference ($P > 0.05$), while data with different letters indicate a significant difference ($P < 0.05$). ITMs, inorganic trace minerals; OTMs, organic trace minerals.

Actinobacteriota, respectively. According to Figures 2B,C, the abundance of *Firmicutes* was significantly higher in the 70% OTMs group compared to the 30% OTMs group ($p < 0.05$), with no significant differences observed among the other groups ($p > 0.05$). Additionally, the abundance of *Actinobacteriota* was significantly higher in the 50% OTMs group compared to the 30% OTMs and 70% OTMs groups ($p < 0.05$).

As shown in Figure 3A, at the genus level, the top five microbial compositions in the 100% ITMs group were *Lactobacillus* (13.40%), *Streptococcus* (13.39%), *Clostridium_sensu_stricto_1* (11.94%), *UCG-005* (11.62%), and *Terrisporobacter* (10.70%). In the 30% OTMs group, the top five were *Lactobacillus* (11.63%), *Clostridium_sensu_stricto_1* (10.95%), *Streptococcus* (9.65%), *Terrisporobacter* (9.14%), and *UCG-005* (8.01%). For the 50% OTMs group, the top five were *Terrisporobacter* (15.89%), *Clostridium_sensu_stricto_1* (14.35%), *Streptococcus* (11.42%), *UCG-005* (7.61%), and *Lactobacillus* (6.80%). In the 70% OTMs group, the top five were *Lactobacillus* (22.86%), *Clostridium_sensu_stricto_1* (15.99%), *Terrisporobacter* (12.19%), *Streptococcus* (10.30%), and *UCG-005* (6.98%).

According to Figures 3B–H, compared to the 100% ITMs group, the relative abundance of *Turicibacter* in the 30% OTMs group was significantly lower ($p < 0.05$), while the relative abundances of *Eubacterium_siraeum_group*, *norank_f__Eubacterium_coprostanoligenes_group* and *norank_f__Oscillospiraceae* were significantly higher ($p < 0.05$). The relative abundance of *NK4A214_group* in the 100% ITMs group was significantly higher than in the 50% OTMs and 70% OTMs groups ($p < 0.05$). The relative abundance of *Solobacterium* in the 50% OTMs group was significantly higher than in the 100% ITMs and 30% OTMs groups ($p < 0.05$). Additionally, the relative abundance of *Holdemanella* in the 50% OTMs group and *Eubacterium_siraeum_group* in the 30% OTMs group were significantly higher than in the other groups ($p < 0.05$).

3.6.4 Correlation analysis between gut microbiota and serum antioxidant indicators, serum immune indicators, and fecal trace element content in fattening pigs

Spearman correlation analysis was also conducted to investigate the relationships among serum antioxidant index, immunoglobulin levels, fecal trace element content, and the gut microbiota of the piglets (Figure 4). In the gut microbiota, *Escherichia-Shigella*, *Blautia*, and *norank_f__Eubacterium_coprostanoligenes_group* were significantly positively correlated with GSH-px activity ($p < 0.05$).

There were significant positive correlations between *norank_f__Eubacterium_coprostanoligenes_group* and T-SOD activity ($p < 0.05$). ALP activity was significantly positively correlated with both *Escherichia-Shigella* and *norank_f__Eubacterium_coprostanoligenes_group* ($p < 0.05$). Mn-SOD activity showed significant positive correlations with *UCG-005*, *NK4A214_group*, and *Lachnospiraceae_XPB1014_group* ($p < 0.05$), and significant negative correlations with *Subdoligranulum* and *Clostridium_sensu_stricto_1* ($p < 0.05$). Serum IgM levels were significantly positively correlated with *NK4A214_group* ($p < 0.05$) and significantly negatively correlated with *Terrisporobacter* ($p < 0.05$). CuZn-SOD activity had significant positive correlations with *Subdoligranulum*, *Blautia*, and *Terrisporobacter* ($p < 0.05$), and significant negative correlations with *UCG-005* and *NK4A214_group* ($p < 0.05$). T-AOC content was significantly positively correlated with *Subdoligranulum* and *norank_f__T34* ($p < 0.05$), and significantly negatively correlated with *NK4A214_group* ($p < 0.05$). IgA levels showed significant positive correlations with *Subdoligranulum*, *norank_o__Clostridia_UCG-014*, *norank_f__T34*, and *Blautia* ($p < 0.05$), and significant negative correlations with *Corynebacterium* and *NK4A214_group* ($p < 0.05$). TG content had a significant positive correlation with *Subdoligranulum* ($p < 0.05$), and significant negative correlations with *UCG-005* and *NK4A214_group* ($p < 0.05$). GSH content was significantly negatively correlated with *Corynebacterium* ($p < 0.05$). MDA content showed a significant negative correlation with *norank_f__Eubacterium_coprostanoligenes_group* ($p < 0.05$). GLU content was significantly negatively correlated with *Escherichia-Shigella*, *norank_f__Eubacterium_coprostanoligenes_group*, and *Rikenellaceae_RC9_gut_group* ($p < 0.05$). IgG levels were significantly negatively correlated with *norank_f__Eubacterium_coprostanoligenes_group* ($p < 0.05$). Fecal Mn excretion was significantly negatively correlated with *norank_f__T34* and *Rikenellaceae_RC9_gut_group* ($p < 0.05$).

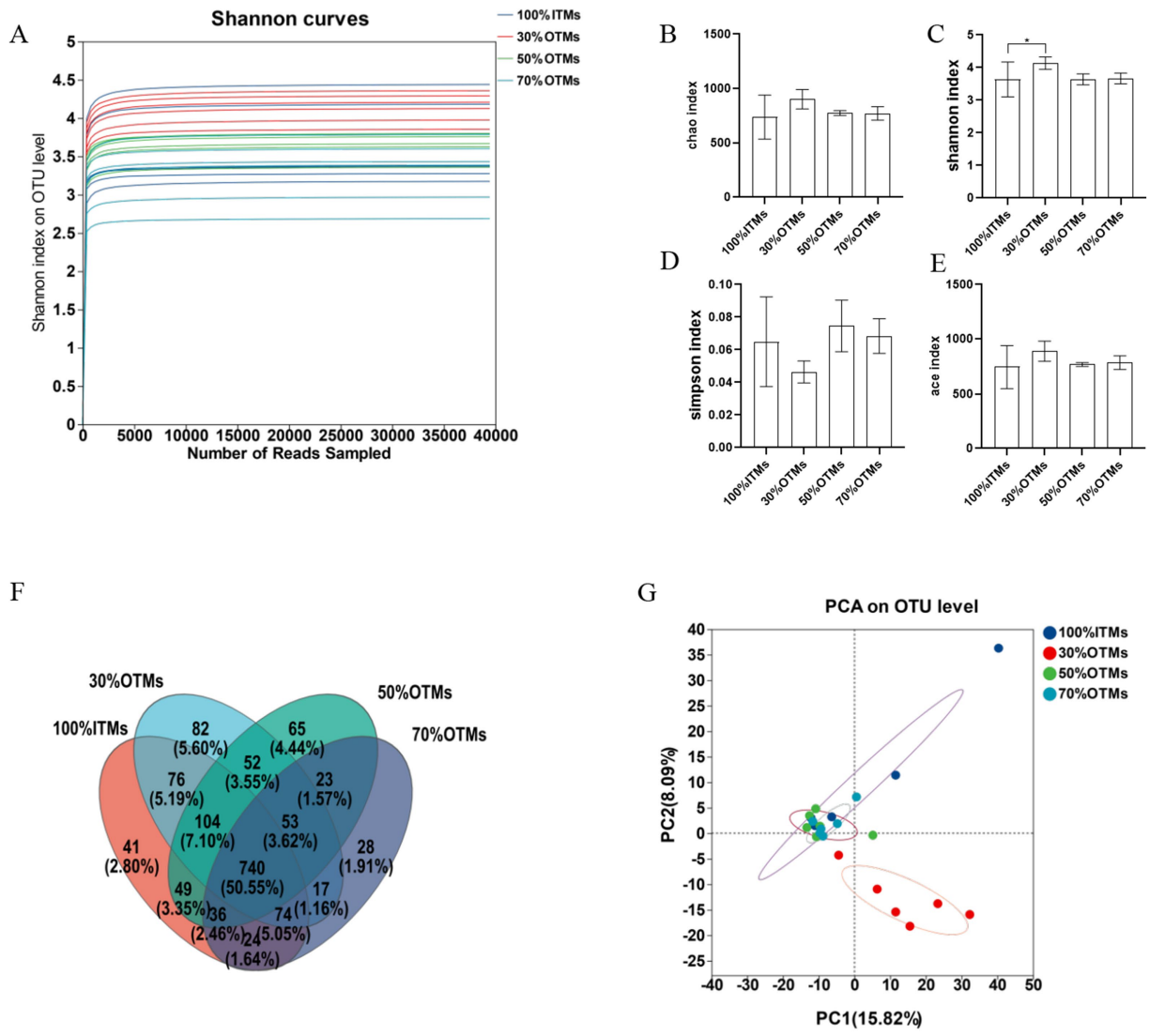

FIGURE 1
The effects of reducing inorganic trace elements with organic trace elements on the microbial diversity in the colon of finishing pigs. **(A)** Shannon curves, **(B)** Chao index, **(C)** Shannon index, **(D)** Simpson index, **(E)** ace index, **(F)** Wayne figure, **(G)** Principal component analysis (PCA) plot of bacterial communities.

4 Discussion

In contemporary traditional intensive pig farming, trace elements are often added in excess to pig diets to prevent deficiencies, while the naturally occurring trace elements in the feed are frequently disregarded. The NRC (2012) stipulates that for fattening pigs (25–80 kg), the required trace element levels are Cu 3.50 mg/kg, Fe 50.00 mg/kg, Mn 2.00 mg/kg, and Zn 50 mg/kg. In this study, the basal diet in this experiment adhered to the NRC standards for the control group. Moreover, all trace elements in the 70% organic trace element substitution group met the NRC standards, whereas in the 50% substitution group, Mn and Zn levels exceeded the standards, and in the 30% substitution group, Mn levels surpassed the recommended levels. The levels of the other trace elements fell below the NRC standards. Recent research suggests that replacing 50% of the NRC standard inorganic trace elements (Cu, Fe, Mn, Zn) with organic trace elements has no significant impact on the growth performance, slaughter performance, or backfat thickness of fattening pigs (17). Consequently, we conclude that the feed used in this experiment adequately meets the nutritional needs of the test animals.

A significant body of research suggests that organic trace elements can increase the bioavailability of trace elements in livestock and poultry, enhance production performance, meat quality, and immunity, and decrease the excretion of trace elements in feces, thereby mitigating environmental pollution (11). Due to their superior bioavailability and performance in production, organic trace elements are extensively utilized in pigs (18). Prior studies have demonstrated that substituting 30–60% of inorganic trace elements with organic trace elements in the diet of fattening pigs does not adversely affect their production performance (2). Similarly, using a low dose (one-third) of organic trace elements to entirely replace a high dose (100%) of inorganic trace elements does not negatively impact the production performance of weaned piglets (19).

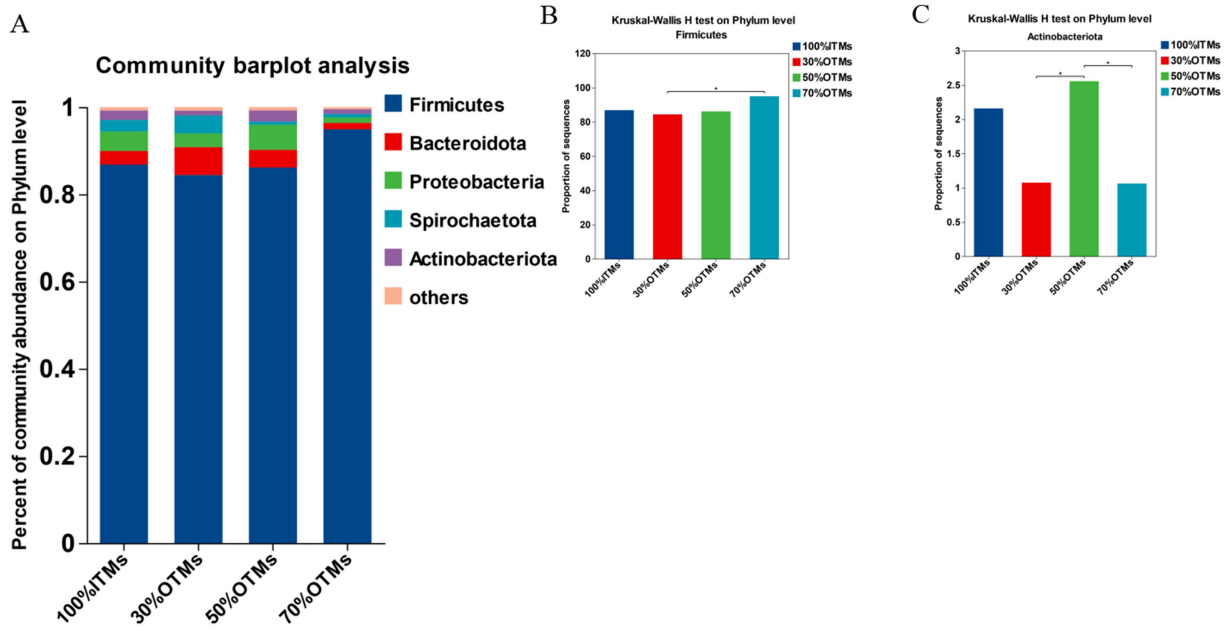

FIGURE 2
Analysis of phylum-level differences in colonic microbiota of finishing pigs following the replacement of inorganic trace elements with reduced levels of organic trace elements.

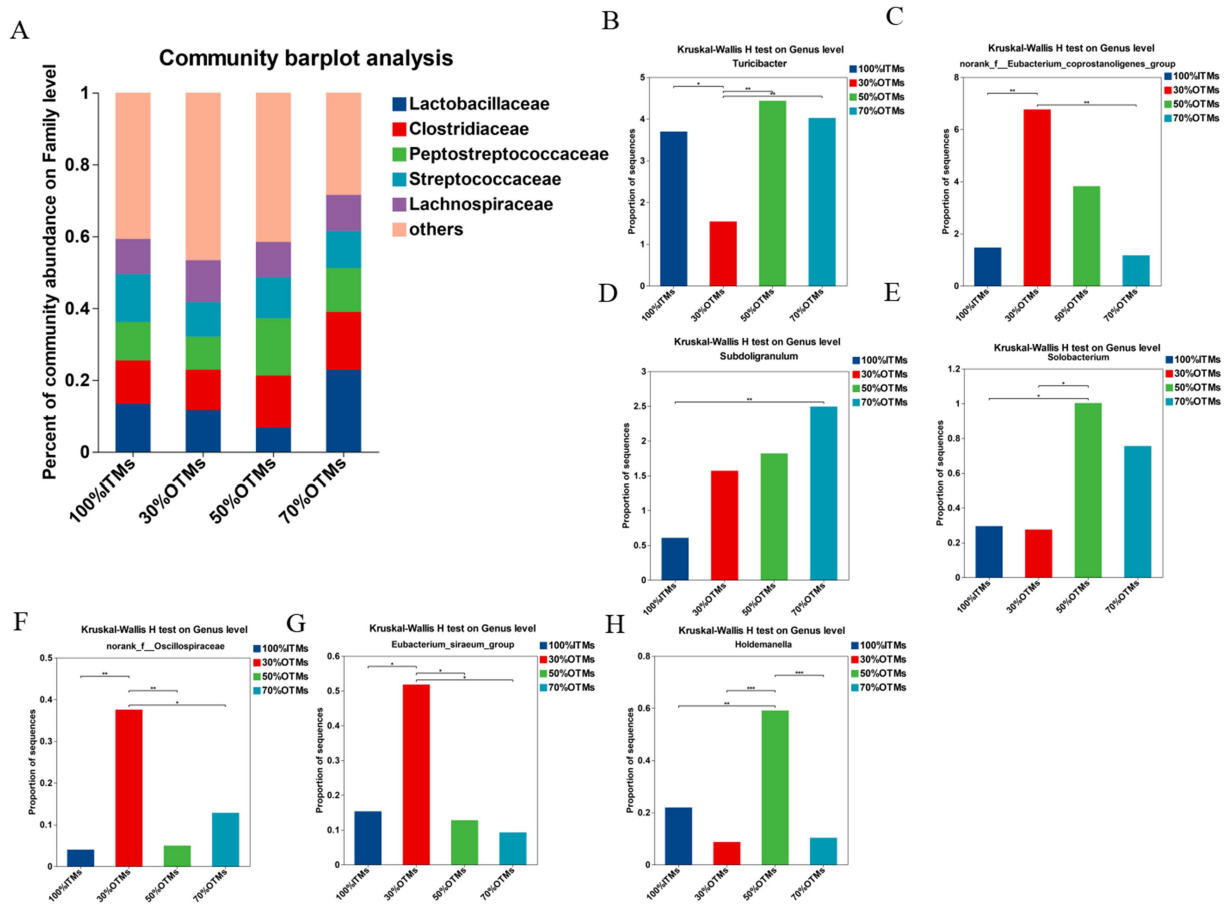

FIGURE 3
Analysis of family-level differences in colonic microbiota of finishing pigs following the replacement of inorganic trace elements with reduced levels of organic trace elements.

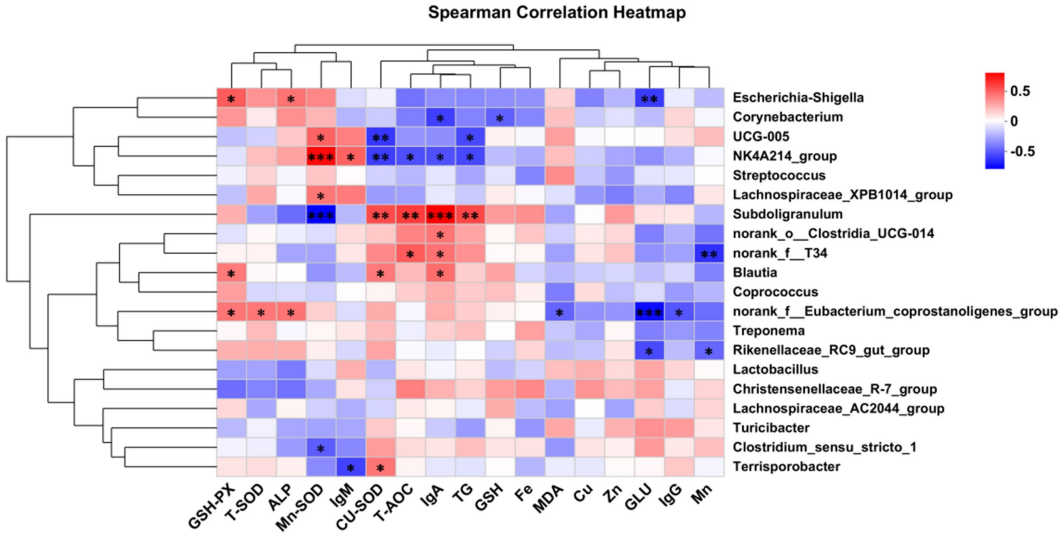

FIGURE 4
Correlation analysis of microbial species with muscle trace element deposition, immune indicators, serum antioxidant capacity, and serum biochemical parameters. GSH-PX, Glutathione peroxidase; T-SOD, Superoxide dismutase; ALP, Alkaline phosphatase; Mn-SOD, Mn Superoxide dismutase; IgM, immunoglobulin M; Cu-SOD, Cu Superoxide dismutase; T-AOC, Total antioxidant capacity; IgA, immunoglobulin A; TG, Triglyceride; GSH, Glutathione; Fe, Iron content in longissimus dorsi muscle; MDA, Malondialdehyde; Cu, Copper content in *longissimus dorsi* muscle; Zn, Zinc content in *longissimus dorsi* muscle; GLU, Glucose; IgG, immunoglobulin G; Mn, Manganese content in *longissimus dorsi* muscle. Differences were set to $*p < 0.05$ and $**p < 0.01$.

Consistent with earlier research, this study observed that replacing 100% of inorganic trace elements with 30% organic trace elements had no detrimental effect on the growth and slaughter performance of fattening pigs. Conversely, reducing the proportion of inorganic trace elements (100%) by substituting them with organic trace elements (30, 50, and 70%) could potentially enhance slaughter performance, increase loin eye area, and reduce backfat thickness in fattening pigs.

The organic trace elements utilized in this study were amino acid chelated trace elements, which are abundant in various amino acids and are more readily absorbed by animals. Furthermore, this experiment indicated that once organic trace elements fulfill the growth requirements of the animals, excessive supplementation does not enhance the production performance of fattening pigs. Replacing inorganic trace elements with organic counterparts in the diet shows promise in enhancing meat quality in livestock and poultry. Previous research implies that organic trace elements might enhance meat quality in fattening pigs through the activation of antioxidant enzymes (20). The inclusion of yeast chromium in the diet can notably decrease lipid peroxidation in fattening pigs, hence improving meat tenderness and antioxidant capacity (21). Moreover, studies have revealed that substituting 12.5% of inorganic trace elements with organic variants can considerably reduce the a* value of breast muscle in white-feather broilers and lower abdominal fat percentage. In alignment with these findings, this study determined that employing 50 and 70% organic trace elements instead of inorganic ones resulted in a significant increase in 24-h muscle pH compared to the 100% organic trace element group. This indicates the potential of substituting inorganic trace elements with organic ones for enhancing meat quality.

Previous research has shown that trace elements from diverse sources do not notably impact serum biochemistry in livestock and poultry; however, they can enhance immune function. For instance, substituting one-third of inorganic trace elements with commercial-level organic alternatives can markedly elevate serum IgG levels in fattening pigs while not significantly altering other serum biochemical parameters (12). Similarly, the replacement of inorganic selenium with organic selenium has been reported to have no noteworthy effect on serum biochemical parameters in laying hens (22). In deviation from earlier studies, this investigation reveals that substituting inorganic trace elements with 30% organic counterparts leads to a significant reduction in serum glucose levels in fattening pigs, whereas replacements of 50 and 70% show no substantial impact. Additionally, all levels of organic trace element substitution (30, 50, and 70%) result in a remarkable increase in serum TG and IgA levels in fattening pigs. TG, linked to lipid metabolism and fat deposition, acts as a vital energy source for animals. The findings observed may be attributed to the utilization of amino acid chelated organic trace elements in this study, which encompass various amino acids absorbed in the small intestine and utilized by the body for lipid metabolism. Moreover, the surge in serum IgA levels with organic trace element supplementation could be associated with the activation of the immune system by these trace elements.

Antioxidant function is a vital system for maintaining the health of livestock and poultry, with Cu, Fe, Mn, and Zn serving as essential components of antioxidant enzymes in animals. Research has demonstrated that substituting 100% of inorganic trace elements with 30% organic counterparts can notably boost serum T-SOD and muscle Mn-SOD enzyme activity in fattening pigs, thereby enhancing their overall antioxidant capacity (2). Prior studies have shown that supplementing the diet with 70% organic trace elements significantly elevates serum glutathione peroxidase activity in laying hens. Moreover, incorporating 40–70% organic

trace elements can significantly increase the total antioxidant capacity in the serum of laying hens (23). Similarly, the findings of this study suggest that replacing 100% of inorganic trace elements with 70% organic elements significantly raises serum T-AOC levels and Cu-SOD enzyme activity in fattening pigs while decreasing serum MDA levels. Furthermore, substituting inorganic trace elements with 50 and 70% of organic versions notably enhances liver GSH-Px, Cu-SOD, and T-SOD enzyme activities. Even with a reduction in organic trace element supplementation to 30%, there is still an enhancement in antioxidant capacity in fattening pigs, as indicated by increased serum Cu-SOD and liver GSH-Px enzyme activities.

Amino acid-chelated trace elements exhibit higher absorption rates than inorganic trace elements due to sharing the same absorption pathways as amino acids. This distinct absorption profile reduces competition and antagonistic effects (12). Numerous studies have indicated that substituting inorganic trace elements with low-dose organic variants can diminish mineral excretion in feces and enhance the efficiency of trace element absorption. For instance, replacing 100% of commercial inorganic trace minerals with 1/3 organic trace elements showed no adverse effects on the production performance, yolk mineral retention, or egg quality of laying hens, while notably decreasing trace element excretion in feces (9). Similarly, a study supplementing the diet of yellow-feathered broilers with organic trace elements revealed higher levels of serum Mn and Se, breast muscle Fe, Zn, and Se, as well as heart Se, compared to the inorganic trace element group. Moreover, fecal mineral excretion was reduced in the organic trace element-fed group (24). In line with prior research, the outcomes of this study illustrate that replacing inorganic trace elements with 30–70% organic counterparts considerably decreases fecal trace element content in fattening pigs without compromising trace element deposition in muscle tissue, offering significant environmental benefit. Forthermore, at a 70% replacement rate, organic trace elements tend to enhance trace element deposition in muscle.

Gut microbiota play a critical role in maintaining animal health by supporting gut health through the diversity of metabolic functions, preservation of the intestinal mucosa integrity, and reinforcement of innate immunity as the primary defense against foreign and toxic substances (25, 26). Studies have shown that trace minerals like Cu, Fe, Mn, and Zn from different sources can enhance gut health by adjusting the composition and function of the intestinal microbiota (27). For instance, substituting copper sulfate with 20 mg/kg of copper glycinate notably increased the colonic microbiota abundance in fattening pigs and decreased trace element emissions (28). In our investigation, we utilized 16S rDNA sequencing to assess the α-diversity of colonic microbiota in fattening pigs and observed a significant increase in the Shannon index when replacing 100% ITMs with 30% OTMs. Moreover, PCA analysis based on bray_curti illustrated a substantial difference in microbial composition between the 30% OTMs group and the control group. A higher abundance of beneficial gut bacteria is generally beneficial for host health (29). At the phylum level, the 70% OTMs group exhibited the highest *Firmicutes* abundance. As the organic trace minerals' proportion rose, Firmicutes' abundance also increased, while *Campylobacterota*'s relative abundance decreased with the rising replacement ratio. *Campylobacterota* are pathogenic bacteria associated with various diseases in humans and animals. At the genus level, the 30% OTMs group demonstrated significantly higher abundances of *norank_f__Oscillospiraceae*, *norank_f__Eubacterium_coprostanoligenes_group*, and *Eubacterium_siraeum_group* compared to the 100% ITMs group, with the pathogenic genus *Terrisporobacter* significantly less prevalent than in the control group. *Norank_f__Oscillospiraceae* and *Eubacterium_siraeum_group* are commonly found in animal intestines and can produce beneficial short-chain fatty acids like butyrate, essential for gut health. Furthermore, the 50% OTMs group displayed notably higher abundances of *Streptococcus* and *Holdemanella* compared to the 100% ITMs group. *Streptococcus* aids in gut lactose digestion and is crucial for maintaining intestinal wall health, while *Holdemanella* is identified as a potential contributor to gut health (30). Previous studies have found a positive correlation between *Holdemanella* and the digestibility of carbohydrates and fibers (31). The 70% OTMs group showed significantly higher relative abundance of *Subdoligranulum* compared to the 100% ITMs group. *Subdoligranulum* plays a role in lactate conversion to butyrate (32). Overall, our results suggest that replacing inorganic trace elements with organic ones at varying ratios can notably increase the abundance of beneficial gut bacteria, suppress harmful bacteria proliferation, and promote gut health in fattening pigs.

5 Conclusion

In this trial, we observed that replacing inorganic trace elements with organic alternatives, under the given feeding conditions, had no adverse effects on growth performance or the relative organ weight in fattening pigs and showed potential for enhancing meat quality. Notably, the substitution improved lipid metabolism, immune function, antioxidant levels, trace element emissions reduction, and gut microbiota structure. In conclusion, replacing 100% inorganic trace elements with 70% organic trace elements had certain potential to improve the production performance of finishing pigs. This study highlights the viability of reducing inorganic trace elements with organic counterparts, which bears substantial implications for trace element reduction and environmental conservation. Further extensive research is essential to elucidate the precise mechanisms of efficient organic trace element absorption.

Ethics approval and consent to participate.

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: National Center for Biotechnology Information (NCBI) Sequence Read Archive (SRA), https://www.ncbi.nlm.nih.gov/bioproject/1170518, project number is: PRJNA1170518.

Ethics statement

The animal studies were approved by Institutional Animal Care and Use Committee at Hunan Agricultural University, Changsha,

China (approval no. 202205). The studies were conducted in accordance with the local legislation and institutional requirements. Written informed consent was obtained from the owners for the participation of their animals in this study.

Author contributions

WX: Data curation, Validation, Visualization, Writing – original draft. MiZ: Investigation, Software, Validation, Writing – original draft. ZY: Formal analysis, Writing – original draft. MeZ: Resources, Writing – original draft. QC: Conceptualization, Funding acquisition, Methodology, Project administration, Supervision, Writing – review & editing.

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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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Physicochemical profiles of mixed ruminal microbes in response to surface tension and specific surface area

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Introduction: In ruminants, a symbiotic rumen microbiota is responsible for supporting the digestion of dietary fiber and contributes to health traits closely associated with meat and milk quality. A holistic view of the physicochemical profiles of mixed rumen microbiota (MRM) is not well-illustrated.

Methods: The experiment was performed with a 3 × 4 factorial arrangement of the specific surface area (SSA: 3.37, 3.73, and 4.44 m^2/g) of NDF extracted from rice straw and the surface tension (ST: 54, 46, 43, and 36 dyn/cm) of a fermented medium in a fermentation time series of 6, 12, 24, 48 h with three experimental units. Here, we used three rumen-fistulated adult Liuyang black goats as the rumen liquid donors for this experiment.

Results: It was found that increasing SSA decreased the average acetate/propionate ratio (A/P, $p < 0.05$) and increased the molarity of propionate ($p < 0.05$). Increasing ST decreased total volatile fatty acid (tVFA) concentration ($p < 0.01$). Greater SSA increased ($p < 0.01$) MRM hydrophobicity, whereas increasing ST increased MRM cell membrane permeability ($p < 0.01$). The neutral detergent fiber digestibility (NDFD, $r = 0.937$) and tVFA ($r = 0.809$) were positively correlated with the membrane permeability of MRM.

Discussion: The surface tension of the artificial medium and substrate-specific surface area had a significant influence on MRM's fermentation profiles, hydrophobicity, and permeability. The results suggest that physical environmental properties are key in regulating rumen fermentation function and homeostasis in the gastrointestinal tract ecosystem.

KEYWORDS

physicochemical properties, rumen microbes, specific surface area, surface tension, physical properties

Implications

Enzyme activity and microbes' adhesion to the substrate are directly related to the physical characteristics of the substrate and rumen fluid. We measured the *in vitro* fermentation of neutral detergent fiber and the surface physical properties of microbes under the different specific surface areas of the neutral detergent fiber and surface tension of the culture medium. This findings indicate that rumen fermentation and animal production can be improved by modifying the physical characteristics of the substrate and rumen fluid.

Introduction

For ruminants, the rumen microbiota is responsible for degrading indigestible fibers into energy and nutrients (1). The ability of the microbiota to digest fiber is enhanced when they transform from planktonic to irreversible adhesion model (2). Microbial biofilms are the prerequisite for fiber digestion and microbial adhesion (3). Microbial biofilms are multicellular microbial communities formed after planktonic cells adhere to solid surfaces (4). The transition from a free-living planktonic lifestyle to a sessile, attached state, forming biofilms, is a multifactorial process governed by biological, chemical, and physical properties of the environment, surface, and bacterial cell (5). Bacterial biofilm is a matrix of extracellular polymeric substances (EPS) (6) that provide mechanical stability and protection against environmental adversities (7). The biofilm is influenced by the availability of environmental nutrients and some nucleotide second messengers, such as cyclic adenosine monophosphate (cAMP) and cyclic di-guanosine monophosphate (c-di-GMP) through cAMP receptor protein (CRP) signaling (8).

The initial bacterial adhesion process is influenced by the properties of the material surface, including surface roughness, topography, wettability, and stiffness (7, 9, 10). The structured channels within the biofilm (3) facilitate the exchange of nutrients between the embedded microbes and the external environments, which contribute to microbial colonization (11) and quorum sensing (12). Specific surface area (SSA) is a crucial parameter in quantifying interactive functions at liquid-solid/gas interfaces, especially in the case of adsorption, heterogeneous catalysis (13), and reaction at the surface of materials (14, 15). Feed particles with greater SSA provide more adsorption sites, resulting in higher fiber digestion efficiency (7). Bacterial attachment and biofilm formation on substrate surfaces highly depend on the available surface area (16, 17). However, comprehensive data linking SSA with microbial adhesion and biofilm formation are still lacking, representing a significant knowledge gap.

Surface tension (ST) of the media also plays a critical role in microbial attachment abilities and the adhesion process, such as its superhydrophobic or super-hydrophilic properties (7, 18). This study is novel in its approach to systematically evaluating the combined effects of SSA and ST on the physical properties of the rumen microbiota and fermentation profiles, an area that has not been extensively explored. The interaction between these factors and their influence on microbial cell membrane permeability, hydrophobicity, and fermentation efficiency remain poorly understood, despite their potential to optimize microbial activity and feed digestibility.

From an economic perspective, improving the efficiency of rumen fermentation is crucial for sustainable livestock production (19). Feed costs account for up to 70% of total expenses in ruminant farming (20). Enhancing fiber digestibility through physicochemical modifications to feed properties offers a cost-effective strategy to maximize feed utilization and minimize waste (21). Additionally, optimizing fermentation conditions, such as reducing ST, can further improve microbial efficiency, leading to higher productivity and lower feed costs (22, 23). These strategies are particularly valuable in the context of increasing global demand for livestock products and the need for sustainable agricultural practices.

This study aims to address these gaps by employing a factorial experimental design to evaluate how varying SSA and ST levels influence the physical and chemical properties of rumen microbes, as well as their fermentation capabilities. By integrating these physicochemical parameters, our findings offer actionable strategies for improving feed efficiency, livestock productivity, and environmental sustainability (24, 25). Additionally, the strong correlations observed between microbial cell membrane permeability and key fermentation metrics, such as fiber digestibility and volatile fatty acid (VFA) production, provide a mechanistic understanding that could guide future advancements in ruminant nutrition and biotechnology.

An in-depth understanding of the physical properties of the environmental surface on fermentation kinetics and physicochemical properties of the rumen microbiota could be conducive to regulating the fiber degradation process. Therefore, this study was conducted to investigate the effects of ST of the fermentation inoculum and SSA of the substrate on the physical properties of the rumen microbes and the consequences of fiber degradation.

Materials and methods

Experimental design

The experiment consisted of a series of *in vitro* batch cultures performed as a completely randomized experimental design with 12 treatments in a 3 × 4 factorial arrangement, each comprising three individual runs. The present work used three SSAs of neutral detergent fiber (NDF) (3.37, 3.73, and 4.44 m^2/g) and four STs (36, 43, 46, and 54 dyn/cm) of the incubation medium. Another run was performed on separate days using the same SSA preparations. The new ST was similar to the previous run and was prepared with fresh rumen inoculum collected from three experimental goats at a similar ratio. The experiment was approved by the Animal Care Committee of the Institute of Subtropical Agriculture, Chinese Academy of Sciences, Changsha, Hunan (No. ISA-2012-018).

Inoculum donator and *in vitro* fermentation

In the present work, animal welfare and management strategies of rumen inoculum donors were described in our previous work

Abbreviations: APG, alkyl polyglucoside; A/P, acetate/propionate ratio; cAMP, cyclic adenosine monophosphate; c-di-GMP, cyclic di-guanosine monophosphate; CRP, cAMP receptor protein (CRP); EPS, extracellular polymeric substances; FITC-dextran, fluorescein isothiocyanate-dextran; GPL, glycerolphospholipids; LOS, lipooligosaccharide; LPS, lipopolysaccharide; MRM, mixed rumen microbiota; NDF, neutral detergent fiber; NDFD, neutral detergent fiber digestibility; OD, optical density; PBP1A, penicillin-binding protein; PBS, phosphate-buffered saline; SSA, specific surface area; ST, surface tension; tVFA, total volatile fatty acid; VFA, volatile fatty acid; ξ, Zeta potential.

(23, 26). Briefly, three rumen-fistulated adult black Liuyang goats of similar age, weight, and good health status were used to provide equal rumen fluids of about 150 ml rumen liquid of each donor for *in vitro* fermentation to reveal the physical characteristics of ST and SSA. The *in vitro* fermentation procedure and chemical compositions of the buffer medium were explained in our previous publications (15, 27). About 50 mL of fermentation medium at a ratio of 1:2 (v/v, rumen fluid: buffer medium) with 4 ST properties, combined with 500 ± 50 mg neutral detergent fiber (NDF) of rice straw (3 SSA levels) at 39°C under continuous flushing with CO_2, was performed in a time series of 6, 12, 24, and 48 h to collect fermented microbial samples. The fermented samples were used to determine volatile fatty acid (VFA) concentrations and physical properties (zeta potential, also called electrokinetic potential, hydrophobicity, and cell membrane permeability) of the microbiota in the rumen. The diet for goats consisted of a concentrate (corn 47%, soybean meal 24%, wheat bran 22%, salt 0.77%, limestone powder 2.23%, and premix 4%) and forage (corn stover) in a ratio of 40:60. The goats were provided free access to water and were fed twice daily at 08:00 and 18:00, receiving 200 g of concentrate and 300 g of forage per day.

Specific surface area and surface tension

The NDF resource was derived from rice straw, while the surface tension system was constructed as described in our previous work (23). Briefly, 3 SSAs (3.37, 3.73, and 4.44 m^2/g) of NDF were ground, sieved through a mill with different sieve pore sizes, and measured using a surface area analyzer (Quadrasorb-*SI*, Quantachrome Inc. Florida, CA, USA). Four STs (36, 43, 46, and 54 dynes/cm) of inoculum medium were prepared by adding alkyl polyglucoside (APG, 28.7 dynes/cm) and immediately measured using a model K100 tensiometer (KRÜSS GmbH, Hamburg, Germany).

Analysis of physical properties of microbes

The mixed fermentation samples, consisting of a mixture of fermentation substrate and microbes, were collected at a fermentation time series of 6, 12, 24, and 48 h. The mixed fermentation samples of 2, 3, and 2 mL of supernatant were collected by centrifugation (5,000 × g at 4°C for 10 min to remove the substrate) to analyze the zeta potential (ξ), cell membrane permeability, and hydrophobicity of the mixed rumen microbes, respectively. To measure the mixed rumen microbiota's physical properties, we followed our previous work's detailed descriptions (15, 23, 28).

Zeta potential (ξ)

The microbial pellet was prepared by rinsing and resuspending the collected residue in 5 mL of 1 mM KNO_3 (pH 6.6) as described by Pelletier et al. (29) and determined using a zeta potential analyzer (Brookhaven Instruments Corp., Holtsville, NY, USA) equipped with a He-Ne laser (658.0 nm) as the light source in a high-precision system (30 cycles) at 39°C.

Permeability of cell membrane

Three milliliter of supernatant was mixed with 1 mL of 100 mg/L fluorescein isothiocyanate-dextran (FITC-dextran, Sigma, molecular weight ≈ 38 kDa) solution and incubated for 1 h at 37°C and then centrifuged to collect 2 mL of sub-supernatant for fluorescence intensity determination (15, 30). The blank negative control was prepared using 3 mL supernatant and 1 mL distilled water.

Hydrophobicity of the cell surface

The microbiota pellet from each fermented flask was centrifuged, collected, rinsed, homogenized, and resuspended in 6 mL of 0.1 M KNO_3 phosphate-buffered saline (PBS) solution (pH 6.6) as described in previous reports (23, 31). The hydrophobicity and permeability of the microbial cell membrane were calculated according to Equations 1, 2 (23), respectively.

$$Hydrophobicity(\%) = \frac{H_{a0} - H_{a1}}{H_{a0}} \times 100\% \quad (1)$$

$$Permeability(\%) = \frac{P_{a0} - P_{a1}}{P_{a0}} \times 100\% \quad (2)$$

Hydrophobicity was defined as the difference between the percentage of cells retained by the hydrocarbon and aqueous layers. H_{a0} represents the absorbance value of optical density (OD) value at 400 nm, OD400, before the addition of hexadecane, and H_{a1} represents the absorbance value of OD400 after hexadecane addition. P_{a0} represents the absorbance value of 25 mg/L FITC-dextran rumen microbe-free liquid, and P_{a1} represents the incubated microbe-free liquid absorbance value.

Chemical analysis

The concentration of VFA in the fermentation liquid was determined according to our previous report (28). Two milliliter of fermented liquid was centrifuged, collected, immediately mixed, and homogenized with 0.15 mL metaphosphoric acid (conc. 25%). The sub-supernatant was then centrifuged and collected for analysis of VFA concentrations at incubation time series of 6, 12, 24, and 48 h by gas chromatography (HP5890, Agilent 5890; Agilent Technologies Co. Ltd, USA).

Statistical analysis

Statistical analysis of a completely randomized experimental design was performed using the MIXED procedure of SAS software (32). For VFA and surface physical variables, the SSA, ST, time series, and interactions were included in the model as fixed effects, while the run was used as a random effect and incubation time as a repeated effect. The SLICE statement

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TABLE 1 Combinatorial interactions of SSA of NDF, ST, and incubation time on VFA profiles.

Item[1]	NDFD (%)[2]	tVFA (mM)[3]	Profiles of volatile fatty acids (mol/100 mol)						A:P[4]
			Acetate	Propionate	Butyrate	Isobutyrate	Isovalerate	Valerate	
SSA (m^2/g)									
3.37	22.6 [a]	28.3	41.0	24.1 [b]	21.4	4.63	5.50	3.35	1.72[a]
3.73	21.9[b]	28.4	38.4	25.3 [ab]	22.3	4.89	5.74	3.50	1.53[ab]
4.44	22.3[ab]	28.7	39.3	25.9 [a]	22.2	4.79	5.56	3.30	1.51[b]
SEM[a]	0.16	0.78	0.61	0.27	0.23	0.079	0.085	0.053	0.035
ST (dynes/cm)									
36	17.6[c]	29.6[ab]	39.3	24.3[b]	22.1	4.82	6.05 [a]	3.43	1.64
43	24.1[a]	30.7[a]	39.3	24.5[b]	22.3	4.83	5.64 [ab]	3.37	1.62
46	24.0[a]	27.3[b]	39.3	25.1[b]	21.9	4.75	5.46 [b]	3.44	1.59
54	23.2[b]	26.4[b]	38.7	26.4[a]	21.6	4.68	5.25 [b]	3.30	1.49
SEM[b]	0.19	0.86	0.74	0.31	0.29	0.091	0.102	0.065	0.042
Significance of effects									
SSA	*	NS	NS	*	NS	NS	NS	NS	*
ST	***	***	NS	**	NS	NS	**	NS	NS
SSA * ST	NS	NS	NS	NS	NS	NS	NS	NS	NS
Time	***	***	***	**	NS	***	***	***	**
SSA * time	***	*	NS	NS	*	NS	*	*	NS
ST * time	***	NS	NS	NS	NS	*	NS	**	NS
SSA * ST * time	***	NS	NS	NS	NS	NS	NS	**	NS

[1]SSA, specific surface area; ST, surface tension; SEM[a], standard error of least squares means for SSA; SEM, standard error of least squares means for ST; SSA * ST, the interaction between SSA and ST; Time, incubation time; SSA * time, the interaction between specific surface area and incubation time; ST * time, the interaction between surface tension and time; SSA * ST * time, interaction among SSA, ST, and time.

[2]NDFD, neutral detergent fiber disappearance.

[3]tVFA, total volatile fatty acid; NS means no significance; *, ** and *** mean $P < 0.05$, $P < 0.01$, and $P < 0.001$, respectively.

[4]A:P, the ratio of acetate to propionate.

[a,b,c]Means with different superscripts differed ($P < 0.05$).

of SAS software was used to detect differences between means at specific SSA levels, and the pairwise difference (PDIFF) statement of SAS software was used to compare ST effects within each SSA level. Pearson correlation analysis was performed using the *Proc Corr* procedure of SAS (32), and $r > 0.5$ was considered biologically significant. Least-squares means are given throughout the text, and significance was reported as $P \leq 0.05$.

Results

Volatile fatty acids

The propionate was affected by SSA, which increased with increasing SSA ($P < 0.05$) (Table 1). In addition, the ratio of acetate to propionate decreased with increasing SSA ($P < 0.05$). The tVFA and isovalerate decreased ($P < 0.01$), but propionate increased ($P < 0.01$) with increasing ST. Interactive effects for NDFD, tVFA, and individual VFA were not observed between SSA and ST. A similar interaction for tVFA (Figure 1A), butyrate (Figure 1B), and isobutyrate (Figure 1C) was observed between time and SSA. Valerate and isovalerate increased with increasing time (SSA = 4.44 m^2/g), whereas they decreased after a fermentation time longer than 24 h (SSA = 3.37 and 3.73 m^2/g, respectively) (SSA × time interaction, $P < 0.05$; Figures 1D, E). In addition, isobutyrate and valerate increased with fermentation time as a function of ST of the fermentation liquid (ST × time interaction, $P < 0.05$ for isobutyrate, Figure 1D, and ST × time interaction, $P < 0.01$ for isobutyrate, Figure 1F).

Physical properties of the rumen microbiota

Zeta potential (ξ) changed with increasing incubation time ($P < 0.01$), and the effects of incubation time on ξ depended on the SSA of the substrate (SSA × time interaction, $P < 0.05$) (Table 2). At SSA = 3.37 m^2/g, the increasing and decreasing magnitude of ξ from 12 to 24 h and 24 to 48 h, respectively, was greater than that of SSA = 3.73 m^2/g (Figure 2A).

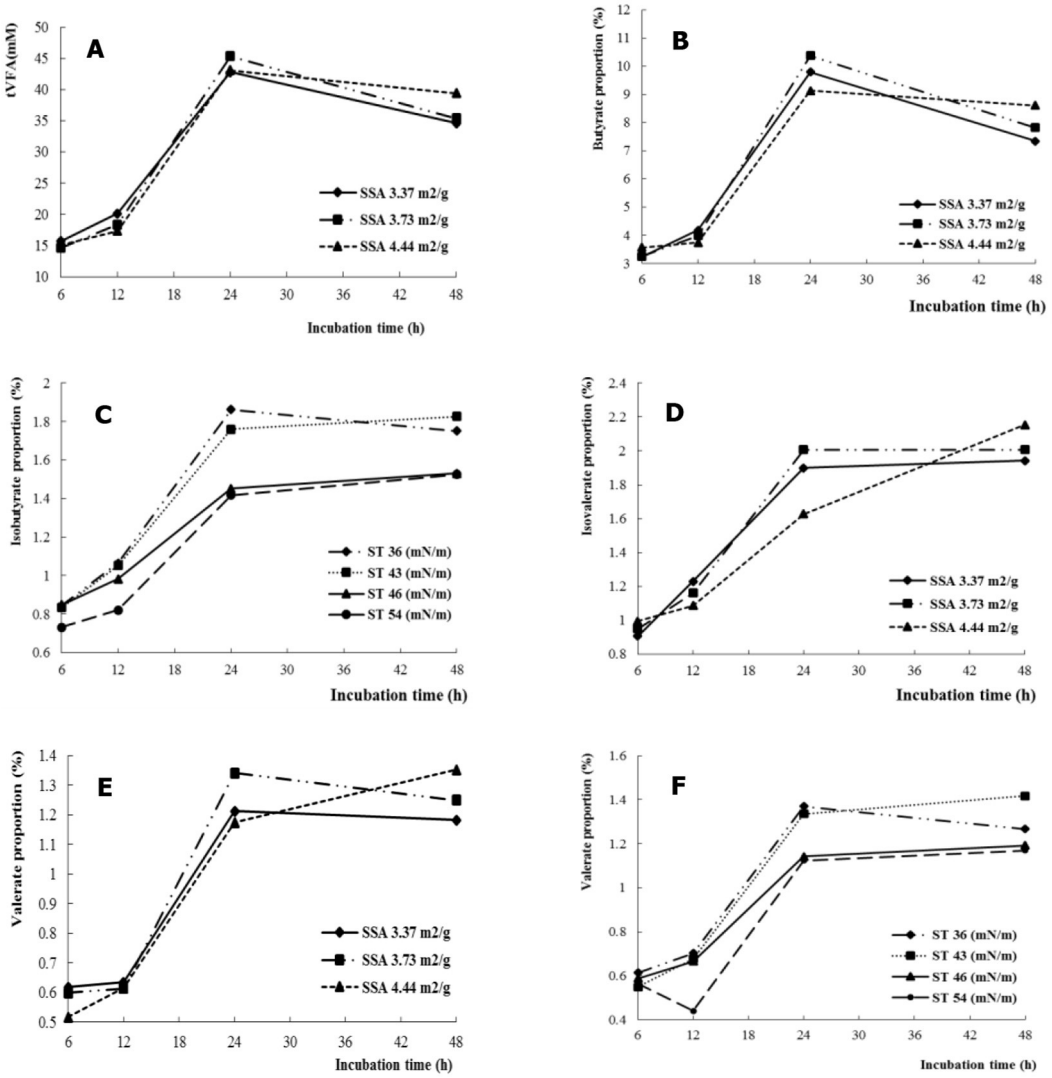

FIGURE 1
The SSA or ST interacted with fermentation time on VFA profiles, tVFA **(A)**, butyrate **(B)**, isobutyrate **(C)**, isovalerate **(D)**, and valerate **(E, F)**.

The hydrophobicity of the cell membrane of mixed rumen bacteria increased ($P < 0.01$) with increasing SSA of NDF. The effect of incubation time on hydrophobicity depended on the SSA of the substrate (SSA × time interaction, $P < 0.05$). The hydrophobicity of the cell surface of rumen bacteria decreased with increasing fermentation time (SSA = 3.37 and 3.73 m^2/g), whereas it increased at 24 h (SSA = 4.44 m^2/g) (Figure 2B).

Cell membrane permeability was higher ($P < 0.01$) for ST = 43 and 46 dynes/cm compared with ST = 36 dynes/cm. The effects of incubation time on cell membrane permeability were dependent on the SSA (SSA × time interactions, $P < 0.05$) and ST (ST × time interactions, $P < 0.001$). The increase in cell membrane permeability was greater for SSA = 4.44 m^2/g ($P < 0.05$) than for SSA = 3.37 m^2/g when the incubation time increased from 12 to 24 hours (Figure 2C). For ST = 36 dynes/cm, cell membrane permeability decreased with incubation time from 6 to 12 h, whereas it increased numerically for ST = 43 dynes/cm, and increased ($P < 0.01$) sharply with increasing fermentation time from 12 to 24 h than for ST = 36 dynes/cm (Figure 2D).

Linear correlation analysis

The correlation between cell membrane permeability of the microbiota was calculated, and the important fermentation value indices, NDFD, and tVFA (Figure 3). A high correlation ($r = 0.937$, $P < 0.001$) was found between permeability and NDFD. In addition, a high correlation ($r = 0.809$, $P < 0.001$) was established between microbiota permeability and tVFA concentration between all treatments and incubation times.

Discussion

Volatile fatty acids (VFAs) are not only the primary end-products of carbohydrate metabolism in the rumen but also play

TABLE 2 Combinatorial interactions of SSA, ST, and incubation time on the physicochemical properties of rumen microbes *in vitro*.

Item[1]	Physicochemical properties of ruminal microbiota[2]		
	Zeta potential (ξ, mV)	Hydrophobicity (%)	Permeability (%)
SSA (m^2/g)			
3.37	−32.8	16.2[b]	35.8
3.73	−32.5	18.2[a]	35.4
4.44	−31.5	19.8[a]	36.5
SEM[a]	0.73	0.74	0.46
ST (dynes/cm)			
36	−33.3	18.6	34.4[b]
43	−33.3	19.1	36.7[a]
46	−30.3	18.1	37.0[a]
54	−32.2	16.3	35.5[ab]
SEM[b]	0.89	0.85	0.55
Significance of effects			
SSA	NS	**	NS
ST	NS	NS	**
SSA * ST	NS	NS	NS
Time	***	***	***
SSA * time	*	*	*
ST * time	NS	NS	***
SSA * ST * time	NS	NS	NS

[1]SSA, specific surface area; ST, surface tension; SEM[a], standard error of least squares means for SSA; SEM[b], standard error of least squares means for ST; SSA * ST, interaction between specific surface area and surface tension; Time, incubation time; SSA * time, interaction between specific surface area and incubation time; ST * time, interaction between surface tension and time; SSA * ST * time, interaction among SSA, ST and time.

[2] [a,b,c]Means with different superscripts differed ($P < 0.05$); *, **, and *** mean $P < 0.05$, $P < 0.01$, and $P < 0.001$, respectively.

critical roles in host energy metabolism and microbial homeostasis (33). The observed increase in tVFA with reduced surface tension suggests that the physicochemical properties of the fermentation medium can significantly influence microbial metabolic efficiency (17, 19). Reduced ST with nonionic surfactants or biosurfactants may enhance microbial interaction with substrates, promoting enzymatic action (34, 35). This finding aligns with studies demonstrating that microbial surfactants lower interfacial tension to improve fermentation efficiency (22, 36).

Mechanistic insights into membrane permeability

The increased cell membrane permeability observed with lower ST values is consistent with findings that nonionic surfactants enhance microbial membrane fluidity, facilitating metabolite exchange and enzymatic leakage (15, 37). This mechanism allows extracellular enzymes to act more effectively on substrates, supporting the breakdown of complex carbohydrates (38, 39). The extracellular enzymatic activity of laccase, lignin peroxidase, and manganese peroxidase of *Phanerochaete chrysosporium* was increased with increasing permeability under the electronic field (30). Gram-negative bacteria are surrounded by an outer membrane consisting of a complex arrangement of glycerolphospholipids (GPL) (40). Their main function is to serve as a permeability barrier to allow the influx of essential nutrients while excluding harmful compounds, such as antibiotics and antimicrobial peptides (41, 42). There are strong positive correlations between cell membrane permeability and NDFD or tVFA. The high linear co-relationship between cell membrane permeability and fermentation parameters, such as NDFD ($r = 0.937$) and tVFA production ($r = 0.809$), underscore the critical role of permeability in microbial fermentation dynamics (15). Theoretical models suggest that optimal membrane permeability supports both nutrient uptake and the secretion of secondary metabolites, such as VFAs. This dual role highlights the centrality of permeability in microbial ecosystem stability and efficiency (43, 44). In general, the functions of signal transduction, secretion of active substances, nutrient absorption, metabolite excretion, and biological membrane function of the microbiota are influenced by cell membrane permeability (45), which in turn is related to the zeta potential and hydrophobicity properties of the membrane (46).

Role of specific surface area and surface tension in hydrophobicity and adhesion

SSA significantly influenced microbial hydrophobicity, which is essential for microbial adhesion to feed particles and biofilm formation. Increased SSA provides more adsorption sites, enhancing microbial colonization and fiber degradation efficiency. These results are consistent with earlier findings showing that hydrophobicity promotes microbial adhesion, an essential precursor to effective fermentation.

Cell adhesion of microbes to the substrate, which is a prerequisite for bacterial colonization and proliferation, is influenced by cell surface hydrophobicity (47, 48). Higher hydrophobic strains have stronger adhesion ability (49, 50). We reported that hydrophobicity increased with increasing SSA, and higher hydrophobicity occurred in the first fermentation phage. We hypothesize that higher SSA increases the activity and adhesiveness of the microbiota in the rumen by altering its surface hydrophobicity. Interestingly, while SSA had a pronounced effect on microbial hydrophobicity, ST did not significantly alter this property. This observation underscores the dominant role of substrate characteristics in influencing microbial adhesion, emphasizing the importance of feed preparation in optimizing ruminal fermentation. Surface hydrophobicity was not affected by the ST of the medium. The lower hydrophobicity of the cell surface was evident during the incubation period. In previous studies, rumen microbes'

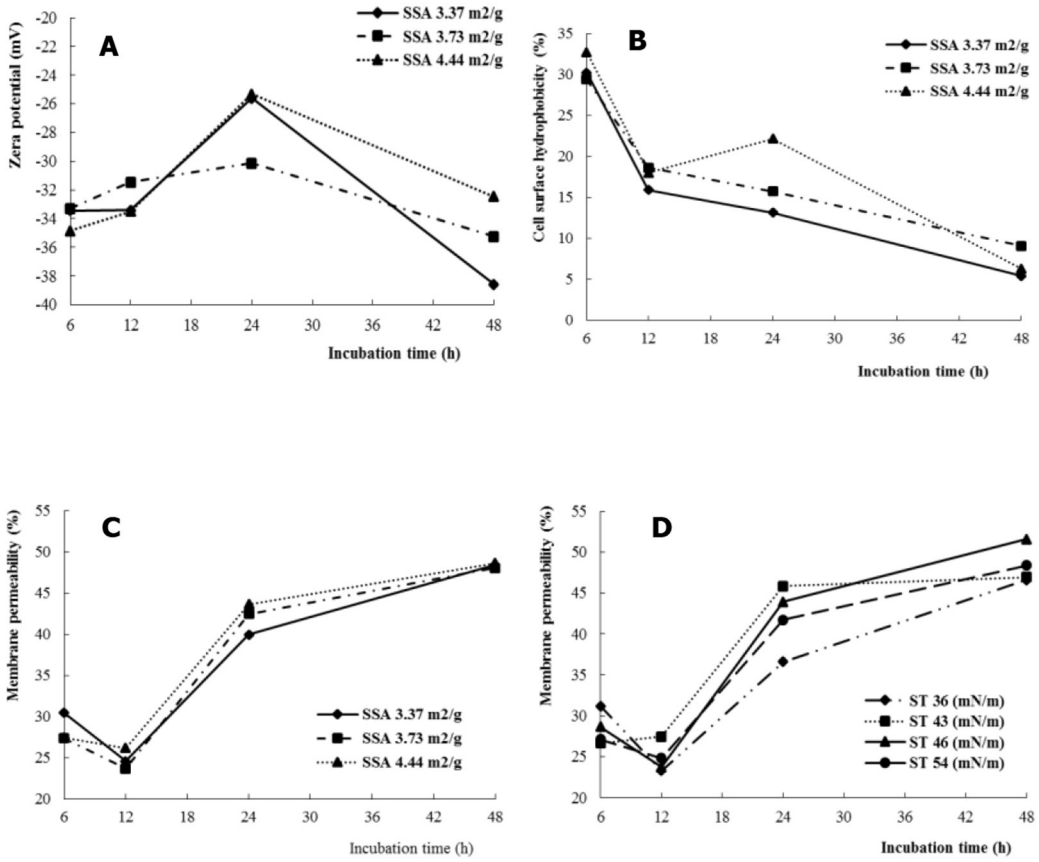

FIGURE 2
The SSA or ST interacted with fermentation time on cell membrane permeability of rumen microbes, Zeta potential **(A)**, cell surface hydrophobicity **(B)**, and membrane permeability **(C, D)**.

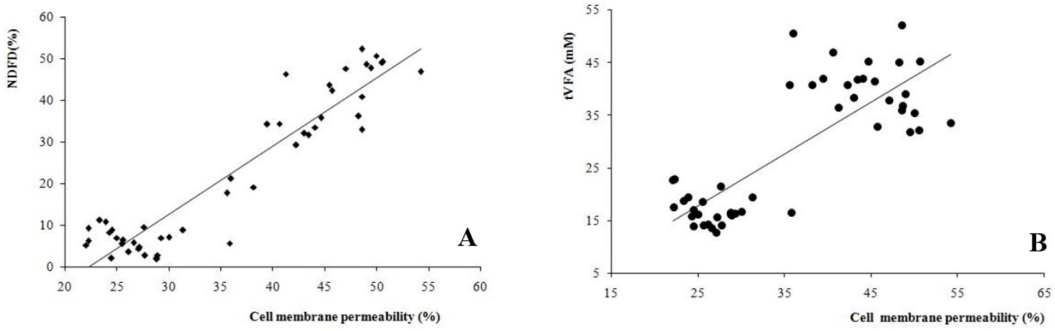

FIGURE 3
Correlations between bacteria cell membrane permeability and **(A)** neutral detergent fiber digestibility (NDFD, $r = 0.937$, $P < 0.001$), and **(B)** total volatile fatty acids (tVFA, $r = 0.809$, $P < 0.001$) among all the treatments and incubation times.

hydrophobicity was related to bacterial aging, and a high value was found in freshly isolated strains of *Staphylococcus aureus* or *Serratia* spp. (51, 52). Given the complexity of the rumen microecosystem, further studies are needed to explain the relationships between the surface properties of the rumen microbiota and the adhesion process, microbial proliferation, and microbial enzyme secretion.

Zeta potential and microbial stability

Zeta potential (ξ), a measure of surface charge, reflects the electrostatic interactions governing microbial and dispersion. In this study, ξ remained unaffected by ST or SSA, except for temporal changes. The reduction in ξ after 24 h may result from microbial aggregation and the accumulation of dead cells, which alter surface

charge distribution (53). Although ξ showed a minimal direct correlation with membrane permeability, its role in maintaining microbial dispersion and biofilm integrity is essential (54, 55). In addition, the surface charge of bacteria is also influenced by the growth medium, bacterial phages, and bacterial surface structure (56). Membrane permeability can be directly affected by membrane zeta potential through a class of special channels known as voltage-gated ion channels or voltage-dependent ion channels, such as sodium/calcium/channel proteins (α-helical transmembrane segments, S1-S6) with a particular ion selectivity and voltage dependence (57, 58). The cell surface of the rumen microbiota was negatively charged, referred to as a net negative charge (56). The negative charges of the membranes increased with increasing fermentation time up to 24 h and decreased the resistance of the microbe attached to the substrate (59, 60). This indicates that the main adhesion of rumen microbes to the substrate occurred before 24 h.

Interdependence of physicochemical properties

The interplay between hydrophobicity, membrane permeability, and zeta potential provides a comprehensive understanding of microbial responses to environmental changes (48). Manipulating SSA and ST can selectively enhance desirable microbial properties, such as adhesion (61, 62) and enzyme secretion while mitigating inhibitory factors like excessive aggregation (63, 64). These findings align with current research strategies to tailor fermentation environments for optimized microbial activity (10, 65).

Practical implications for livestock nutrition and future directions

This study highlights actionable strategies for improving feed formulation in livestock nutrition. Increasing SSA through particle processing or additive application enhances microbial adhesion and fiber degradation, while moderate reductions in ST using bio-/nonionic- surfactants or other agents optimize enzymatic activity. These approaches can significantly enhance feed efficiency, reduce waste, and promote sustainable livestock production. Future studies should explore the molecular mechanisms underlying the observed effects using advanced omics technologies. Metagenomics and metabolomics can provide deeper insights into how SSA and ST influence microbial community composition and metabolic pathways, paving the way for precision management of ruminal fermentation.

Conclusions

In the rumen, both the large specific surface area of the fiber and the low surface tension of the inoculum result in increased propionate production and decreased acetate-to-propionate ratios by disrupting the microbiome ecosystem. Cell surface properties of rumen microbes, including hydrophobicity and permeability, change with substrate properties and interfacial properties of the medium. In addition, neutral detergent fiber digestibility and total volatile fatty acid correlate strongly with the cell membrane permeability of the rumen microbiota. These results suggest that physical environmental properties may be critical in regulating the physical properties of the rumen microbiota and the balanced ecosystem.

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 studies were approved by the trial was conducted at the experimental farm of the Institute of Subtropical Agriculture (ISA), the Chinese Academy of Sciences. The protocol and experiment procedures were approved by the Animal Care Committee of ISA (Approval ISA-2012-018). The studies were conducted in accordance with the local legislation and institutional requirements. Written informed consent was obtained from the owners for the participation of their animals in this study.

Author contributions

YL: Conceptualization, Formal analysis, Methodology, Writing – original draft. JL: Conceptualization, Software, Validation, Writing – original draft. ST: Resources, Visualization, Writing – original draft. CZ: Project administration, Validation, Writing – original draft. ZT: Supervision, Validation, Writing – original draft. AS: Writing – original draft.

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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 *in ovo* feeding of vitamin C on embryonic development, hatching process, and chick rectal temperature of broiler embryos

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Maternal nutritional status plays a crucial role in embryonic development and has persistent effects on postnatal chicks. Vitamin C (VC) plays an important role in embryonic and postnatal development involved in nutri-epigenetics. The present study was conducted to investigate the effects of *in ovo* feeding (IOF) of VC on embryonic development, egg hatching time, and chick rectal temperature. Trial 1 was conducted under normal incubation conditions without the IOF procedure and was designed to analyze the characteristics of embryonic development and establish the scoring standards for yolk absorption and the rupture of the shell membrane. The results showed that the relative weight of the embryo and residual yolk and the organ indexes were reliable indicators of embryonic development. Yolk absorption was scored 0, 1, 2, 3, and 4, with a higher score indicating more complete absorption. In addition, the rupture of the shell membrane was divided into two cases: YES and NO. Trial 2 included three groups, control (CON), normal saline (NS), and vitamin C (VC), and was designed to detect the effects of IOF of VC on the indicators in trial 1, as well as the plasma biochemical indicators. At embryonic age 11 (E11), each egg in the CON group was non-injected, each egg in the NS group was injected with 0.1 mL of sterile normal saline, and each egg in the VC group was injected with 0.1 mL of sterile normal saline containing 3 mg vitamin C. The whole day of E21 was evenly divided into three time periods: early (incubation hours 480–488), middle (incubation hours 488–496), and late (incubation hours 496–504). Among the CON, NS, and VC groups, the percentages of the early-hatched chicks (egg hatching time) were 29.31, 12.00, and 33.90%, respectively. The proportions of early and middle hatched chicks in these groups were 51.72, 42.00, and 38.27%, respectively. The rectal temperature of chicks was lower ($p < 0.05$) in the VC group than in the CON and NS groups. Compared to the NS group, the plasma biochemical indicators in the VC group showed significantly lower levels of alkaline phosphatase (ALP), total protein (TP), albumin (ALB), GLB, total bilirubin (TBIL), TBA, uric acid (UA), high-density lipoprotein cholesterol (HDL-C), and corticosterone (CORT) ($p < 0.05$). Additionally, alanine aminotransferase (ALT) had an increasing trend ($p = 0.059$) in the VC group. In conclusion, our data demonstrated that VC accelerated the hatching process and reduced chicks' rectal temperature, which may be related to the improvement of liver function and changes in metabolism, as indicated by blood biochemical indicators.

KEYWORDS

vitamin C, *in ovo* feeding, embryonic development, hatching time, rectal temperature

1 Introduction

The developmental origins of health and disease (DOHaD) theory suggests that factors present during the gestational and early-postnatal period (nutrition, climate, stress, toxins, and exercise) can significantly influence adult health and likelihood of developing chronic diseases later in life (1). Growing evidence has shown that an impoverished *in utero* environment may cause many diseases after birth, such as cardiovascular disease, metabolic syndrome, obesity, and diabetes (2). As with livestock animals, the abovementioned effects directly reduce production performance and lead to economic losses. Malnutrition during incubation (achieved by removing quantities of albumen from the egg) can lead to low hatch rates in chicks and decreased post-hatch body weight up to 7 days of age (3). Using Arbor Acres broilers as an example, the incubation period accounts for up to one-third of the entire life cycle, and this proportion is expected to increase with the development of breeding, which will lead to the embryonic environment playing a more prominent role in the production performance of broilers.

Nutrition is an important factor affecting embryonic development and also serves as an intervention strategy that neutralizes the negative effects of other factors on embryonic development (4). Prenatal epigenetic diets, including vitamin C (VC) from fruits, resveratrol from grapes, and isothiocyanates from broccoli, help protect against environmental pollution and improve fetal and offspring health outcomes (5). Embryos derive nutrients from the mother through the placenta in mammals, while avian embryos only rely on the nutrients deposited in the eggs. If there is insufficient nutrient deposition in breeder eggs, it may directly affect embryonic development and the offspring's production performance. Our previous studies reported that the endogenous synthetic ability of VC was weak during embryonic development, and VC supply was mainly from endogenous absorption from the yolk and albumen (6). However, dietary VC supplementation failed to increase VC deposition in the yolk and albumen (7). In addition, stress during the breeding process reduced the synthesis capacity of VC, and VC was preferentially used to eliminate stress at the same time (8), leading to less deposition in breeder eggs.

VC plays an important role in embryonic or postnatal development, and vitamin C supplementation during pregnancy improves placental function (9). Some studies have shown that *in ovo* feeding (IOF) of VC has positive effects on growth performance in postnatal broilers (10), suggesting that VC might improve embryonic development in broilers based on the DOHaD theory. In addition, the elimination and reconstruction of DNA methylation occur during embryonic development in broilers (11), and VC can act as a cofactor for related enzymes involved in DNA and histone demethylation (12).

The hatching process is closely related to embryonic development and is also reflected in the rupture of the shell membrane (or shell), yolk absorption, and hatching time (13). In addition, rectal temperature is a reliable indicator for evaluating a chick's stress resistance both inside and outside of the egg (14). In this study, we investigated the effects of IOF of VC on embryonic development, the hatching process, and the rectal temperature of newly hatched chicks. We also explored the potential underlying mechanisms.

2 Materials and methods

The animal experimental procedures were approved by the Institutional Animal Care and Use Committee of Southwest University of Science and Technology (Permit Number: L2023013).

2.1 Broiler breeder flocks, egg collection, and incubation

At embryonic age 11 (E11), the eggs were candled, and the unfertilized eggs and dead embryos were removed. At E19, all the eggs were transferred to hatching baskets. All the eggs, sourced from Arbor Acres broiler breeder flocks, were purchased from the Xianyang Dacheng Poultry Industry Co. Ltd. The fertilized eggs were collected from the same flock at 27 weeks of age for trial 1 and 31 weeks of age for trial 2. All the eggs were incubated in an automatic incubator, and the hatching program was set to chicken mode.

2.1.1 Trial 1

A total of 300 disinfected eggs were randomly divided into 10 replicates, with 30 eggs per replicate. Each egg was weighed, and its weight was recorded on the eggshell. At E19, each egg was put into a mesh bag. At E11, E13, E15, E17, E19, E20, E21, and on the postnatal 1st day (D1), one egg or chick per replicate was selected for the measurement of embryonic or chick weight and organ indexes. Every 2 h, starting from E19.5 to E20.5, one egg per replicate was selected for the standard establishment of yolk absorption and shell membrane rupture scores. The eggs were incubated using an automatic incubator (9TV-2A, Beijing Blue Sky Electronic Technology Co., Ltd.). The incubation procedures were as follows: (1) E1–E6: temperature 38°C, humidity 60%; (2) E7–E12: temperature 37.8°C, humidity 55%; (3) E13–E18: temperature 37.6°C, humidity 60%; and (4) E19–E21: temperature 37.2°C, humidity 70%. The eggs were turned every 2 h before E19 and not turned after E19.

2.1.2 Trial 2

Two incubators (A and B) were used in trial 2. Three groups were set up in both incubators A and B: control (CON), normal saline (NS), and vitamin C (VC) groups. At E11, each egg in the CON group was non-injected, each egg in the NS group was injected with 0.1 mL of sterile normal saline, and each egg in the VC group was with injected 0.1 mL of sterile normal saline containing 3 mg of vitamin C. The injection site was the yolk sac. The egg injection method is based on a previous publication (15). A total of 450 disinfected eggs were randomly divided into three groups with 10 replicates in incubator A, while a total of 210 disinfected eggs were randomly divided into three groups without replicates in incubator B. At E13, E15, E17, E19, E20, E21, and on D1, one egg or chick per replicate was selected for the measurement of embryonic or chick weight and organ indexes in incubator A. At the end of E20, two eggs per replicate were selected for yolk absorption and shell membrane rupture scoring in incubator A. After hatching, two chicks per replicate were selected for the measurement of rectal temperature and then one chick per replicate was selected for plasma collection in incubator A. All the eggs were used for analyzing the hatching time in incubator B.

2.2 Embryonic weight and organ indexes

After the embryo or chick was slaughtered, the weight of the embryo or chick, residual yolk, liver, heart, lung, and gallbladder was measured. The weight of the embryo or chick and residual yolk was expressed as the ratio of each to the egg weight. The organ index was expressed as the ratio of the organ weight to the embryonic weight, excluding the residual yolk.

2.3 Yolk absorption and the rupture of the shell membrane score

To the best of our knowledge, this was the first time a standard for yolk absorption and shell membrane rupture scoring was established. The egg was opened from the air chamber, and the status of the rupture of the shell membrane was photographed. Then, the embryo was immediately slaughtered and placed in a Petri dish, and the status of yolk absorption was photographed. In the end, a visual scoring standard was established. At a certain time, all the eggs to be evaluated were removed from the incubator, and the evaluation was completed within 30 min.

2.4 Rectal temperature and plasma parameters in the newly hatched chicks

After the feathers of the newly hatched chicks were dry, the rectal temperature was measured. The plasma biochemical parameters included alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), total protein (TP), albumin (ALB), globulin (GLO), total bilirubin (TBIL), blood urea nitrogen (BUN), uric acid (UA), creatinine (CRE), glucose (GLU), total cholesterol (TC), total triglyceride (TG), high-density lipoprotein cholesterol (HDL-C), and low-density lipoprotein cholesterol (LDL-C). They were detected using an automated biochemical analyzer (Hitachi, Tokyo, Japan). The plasma samples were sent to the laboratory department of Mianyang Central Hospital (Mianyang, China) to measure the levels of thyroxin (T4), free thyroid hormone (fT4), triiodothyronine (T3), and free triiodothyronine hormone (fT3) using electro-chemiluminescence immunoassay. The plasma corticosterone (CORT) level was measured using the Chicken Corticosterone ELISA Kit (Shanghai Enzyme Biotechnology Co. Ltd., Shanghai, China).

2.5 Hatching time

As shown in Figure 1A, the entire day of E21 was evenly divided into three time periods: early (incubation hours 480–488), middle (incubation hours 488–496), and late (incubation hours 496–504). The hatched chicks in each group were counted individually during the early, middle, and late periods. These periods were calculated as a percentage of the total number of the hatched chicks for each group.

2.6 Statistical analysis

The data on the embryonic or chick weight, organ indexes, and chick rectal temperature were analyzed using one-way ANOVA, and the data on the plasma parameters were analyzed using an independent sample *t*-test with SPSS 21.0 (SPSS Inc., Chicago, IL, United States). Statistical significance was considered at $p < 0.05$ and trends at $p < 0.1$. All other data were compared in absolute value.

3 Results

3.1 Embryonic development characteristics

As shown in Figure 2A, the relative weight of the embryo or chick gradually increased from E11 to D1 ($p < 0.05$). The relative weight of the residual yolk gradually decreased from E20 to D1 ($p < 0.05$, Figure 2B). As shown in Figures 2C,F, the liver and gallbladder indexes both reached the maximum on D1 ($o < 0.05$). The liver index showed no significant difference at E15, E17, E19, E20, and E21 ($p > 0.05$, Figure 2C), and the gallbladder index showed no significant difference at E19, E20, and E21 ($p > 0.05$, Figure 2F). As shown in Figure 2D, the heart index showed no significant difference at E11, E13, E15, and E17 and at E20, E21, and D1 ($p > 0.05$), but the heart index was significantly higher at E11, E13, E15, and E17 than at E21 and on D1 ($p < 0.05$). As shown in Figure 2E, the lung index showed no significant difference at E11, E13, and E15 and at E17, E19, E20, E21, and D1 ($p > 0.05$), and the lung index at E11, E13, and E15 was significantly higher than that at E17, E19, E20, E21, and D1 ($p < 0.05$).

3.2 Standard of yolk absorption and the rupture of the shell membrane score

As shown in Figure 3A, yolk absorption was divided into five representative cases, which were scored 0, 1, 2, 3, and 4. The higher the score, the more complete the yolk absorption. As shown in Figure 4A, the rupture of the shell membrane was divided into two cases: YES and NO. YES meant that the shell membrane had been ruptured, while NO meant the opposite.

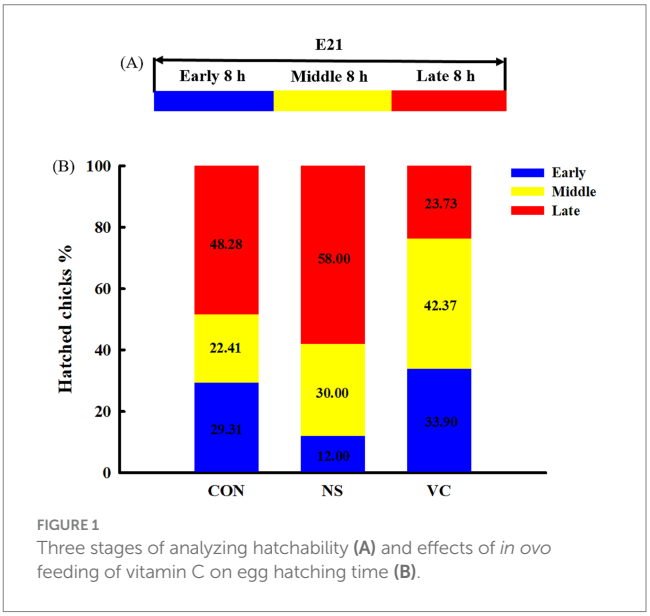

FIGURE 1
Three stages of analyzing hatchability **(A)** and effects of *in ovo* feeding of vitamin C on egg hatching time **(B)**.

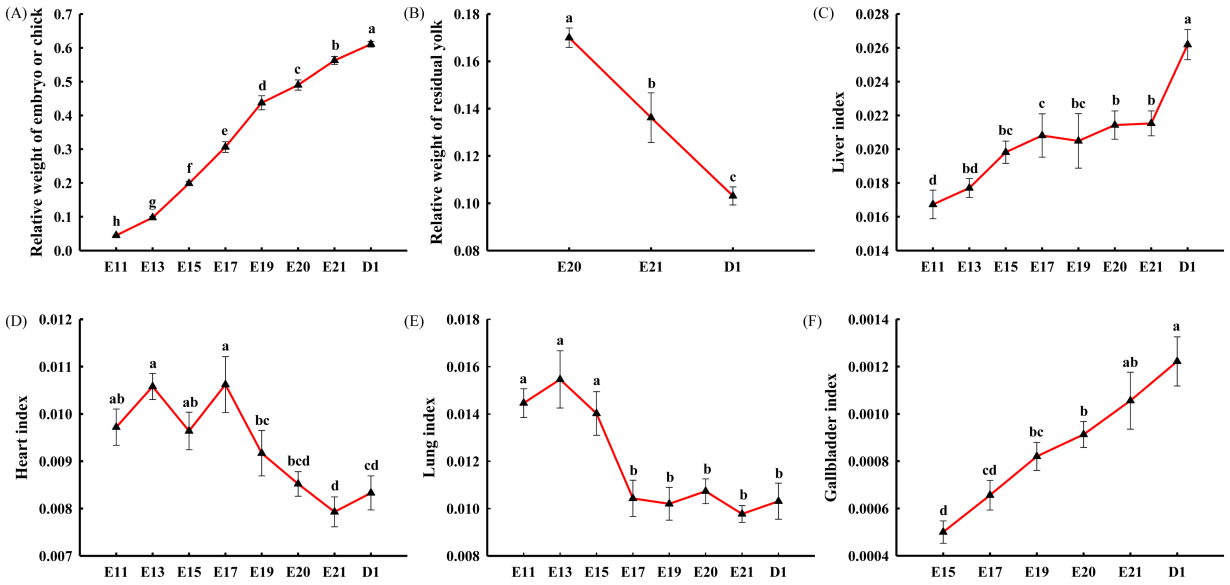

FIGURE 2
Embryonic development characteristics during incubation in the broiler chickens, including the relative weight of the embryo or chick **(A)**, the relative weight of the residual yolk **(B)**, the liver index **(C)**, the heart index **(D)**, the lung index **(E)**, and the gallbladder index **(F)**. Each triangle represents the mean ± SEM (n = 10). Triangles with different letters are significantly different ($p < 0.05$). The relative weight of the embryo or chick refers to the ratio of the weight of the embryo or chick, excluding the residual yolk, to the initial egg weight. The relative weight of the residual yolk refers to the ratio of the weight of the residual yolk to the initial egg weight. The organ index refers to the ratio of the organ weight to the embryonic or chick's weight, excluding the residual yolk.

3.3 Effect of vitamin C on the embryonic development characteristics

As shown in Table 1, IOF of VC had no significant influence on the relative weight of the embryo or chick at E13, E15, E17, E19, E20, E21, and on D1 and the relative weight of the residual yolk at E20, E21 and on D1 ($p > 0.05$). As shown in Table 2, IOF of VC had no significant influence on the organ indexes of the heart, liver, lung, and gallbladder (except at E13) at E13, E15, E17, E19, E20, E21, and on D1 ($p > 0.05$).

3.4 Effect of vitamin C on the yolk absorption score and the rupture of the SHELL membrane

As shown in Figure 3B, the proportions of a score of 4 among the CON, NS, and VC groups were 9.52, 14.29, and 23.81%, respectively; the proportions of scores 3 and 4 among the CON, NS, and VC groups were 38.09, 47.62, and 71.43%, respectively; and the proportions of scores 0, 1, and 2 among the CON, NS, and VC groups were 61.91, 52.38, and 28.57%, respectively. As shown in Figure 4B, the proportions of YES among the CON, NS, and VC groups were 33.33, 38.10, and 61.90%, respectively, while the proportions of NO among the CON, NS, and VC groups were 66.67, 61.90, and 38.10%, respectively.

3.5 Effect of vitamin C on the egg hatching time and chick rectal temperature

As shown in Figure 1B, the percentages of the early hatched chicks among the CON, NS, and VC groups were 29.31, 12.00, and 33.90%, respectively; the proportions of the early and middle hatched chicks among the CON, NS, and VC groups were 51.72, 42.00, and 38.27%, respectively; and the proportions of the late hatched chicks among the CON, NS, and VC groups were 48.28, 58.00, and 23.73%, respectively. As shown in Figure 5, the chicks' rectal temperature was lower in the VC group than in the CON and NS groups ($p < 0.05$).

3.6 Effect of vitamin C on plasma parameters

As shown in Table 3, in the VC group, the biochemical indicators of ALP, TP, ALB, GLB, TBIL, TBA, UA, and HDL-C were lower ($p < 0.05$) and ALT showed an increasing trend ($p = 0.059$), compared to the NS group,. In the VC group, the hormone level of CORT was lower ($p < 0.05$) and T4 showed a decreasing trend ($p = 0.096$), compared to the NS group,

4 Discussion

During a short period of 21 days, a fertilized egg eventually turns into a viable chick through a series of developmental procedures, including access to oxygen, diverse energy sources, and the accommodation of metabolic patterns (16). The heart, lungs, and chorioallantoic membrane are the basis for ensuring oxygen supply and gas exchange, while the liver is the center of metabolism and the basis for accommodating metabolic patterns. In addition, bile in the gallbladder improves the utilization of lipids, which are the major energy-supplying substances from the yolk. Interestingly, a

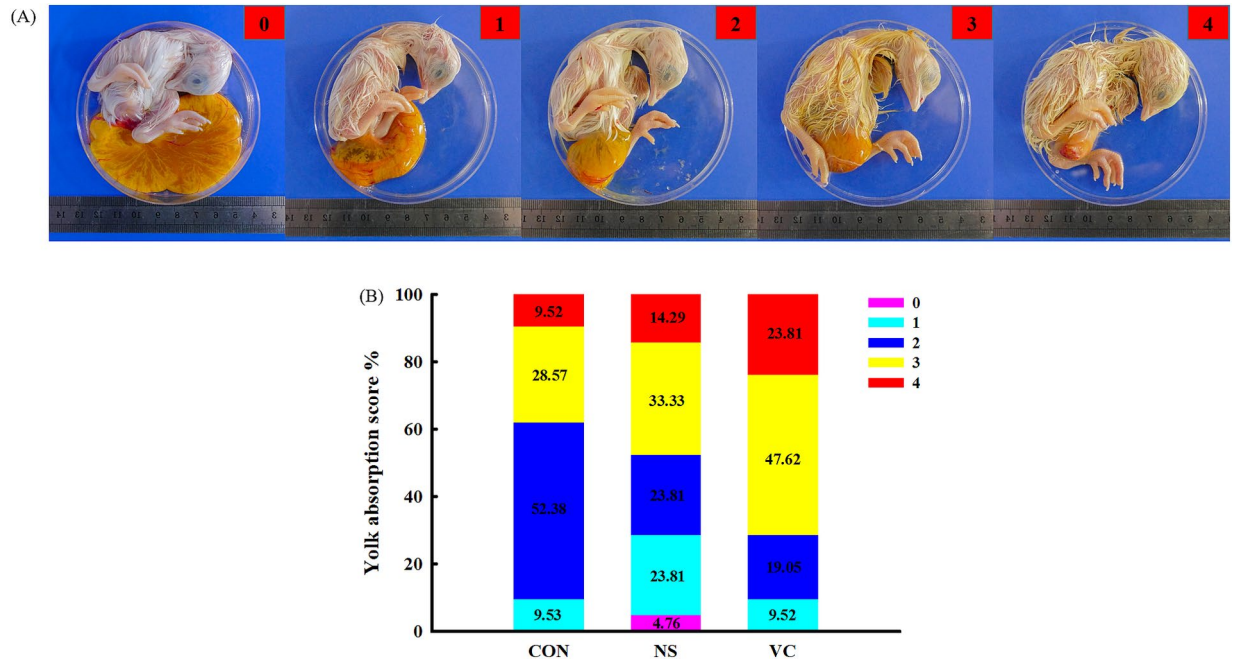

FIGURE 3
Standard of the yolk absorption score **(A)** and effects of *in ovo* feeding of vitamin C on the yolk absorption score **(B)**.

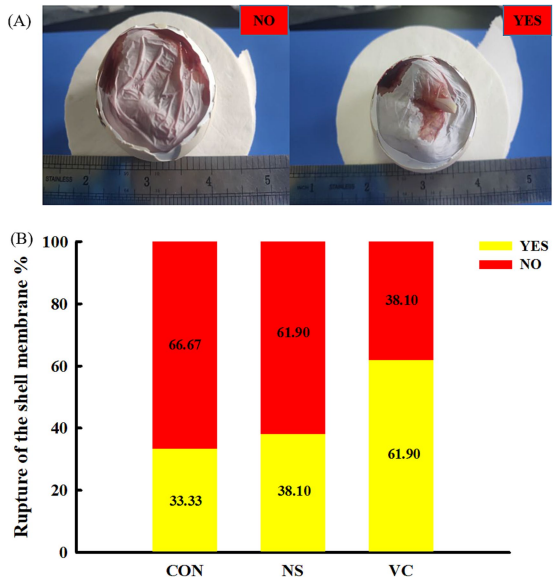

FIGURE 4
Standard of the rupture of the shell membrane score **(A)** and effects of *in ovo* feeding of vitamin C on the yolk absorption score **(B)**.

synchronized progression is observed in the organ indexes of the heart, liver, lungs, and gallbladder, as well as in the relative weight of the embryo (or chick) and residual yolk, which may together reflect embryonic developmental characteristics.

The chorioallantoic membrane forms from E4, gradually develops blood vessels, and attains mature gas exchange capacity by E11-12 (17). Meanwhile, the paired primordia of the heart begin to fuse and develop from E2 (13), and the heart adapts to mature chorioallantoic respiration by E11-12. Nevertheless, the lungs develop capillaries by E18, and pulmonary respiration occurs by E19, gradually replacing chorioallantoic respiration (18). At the same time, the heart is forced to adapt to the transition from chorioallantoic respiration to pulmonary respiration. In summary, E18 is a key time for the transition from chorioallantoic respiration to pulmonary respiration, as well as for cardiac adaptive changes. Therefore, the turning points of the heart and lung indexes were observed at E17 and E15, respectively, indicating that the transition from chorioallantoic respiration to pulmonary respiration forced adaptive changes in the heart.

The liver is structurally completed by E14 and then fully functions as the metabolic center (16), which were synchronized with the increase and stability of the liver index from E11 to E15 and from E15 to E21, respectively, in this study. From E21 to D1, a significant increase in the liver index was attributed to the rapid deposition of lipids in the liver (19). The gallbladder formed at E9 and then grew rapidly, which was consistent with the increase in the gallbladder index from E11 to D1. Bile, secreted by the gallbladder, is beneficial for the liver to utilize lipids from the yolk, which was supported by the relative weight of the residual yolk from E20 to D1. A shift in the growth rate was observed at E13, which may be related to the structural completion of the liver and bile secretion from the gallbladder, both of which together ensure the utilization of lipids as the main energy supply.

Changes in the organ indexes (the heart, liver, lungs, and gallbladder) and the relative weight of the embryo or remaining yolk are synchronized with changes in important physiological functions during embryonic development and can be used as indicators to reflect the hatching characteristics of broiler chickens. Recently, the incubation period accounts for more than 50% of an Arbor Acres broiler's productive life. Therefore, it is necessary to encourage positive

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TABLE 1 Effects of *in ovo* feeding of vitamin C at E11 on the relative weight of the embryo or chick and the residual yolk during incubation in broiler chickens.

Times	Items	Treatments			SEM	*p*-value
		CON	NS	VC		
E13	Weight of the embryo	5.76	5.60	5.86	0.095	0.553
	Relative weight of the embryo	0.098	0.094	0.099	0.0018	0.570
E15	Weight of the embryo	11.98	11.68	12.15	0.217	0.686
	Relative weight of the embryo	0.198	0.200	0.203	0.0038	0.899
E17	Weight of the embryo	18.25	19.88	18.17	0.398	0.135
	Relative weight of the embryo	0.306	0.327	0.302	0.0065	0.251
E19	Weight of the embryo	25.78	26.46	26.87	0.483	0.664
	Relative weight of the embryo	0.437	0.438	0.444	0.0078	0.931
E20	Weight of the embryo	29.16	30.46	30.18	0.352	0.294
	Relative weight of the embryo	0.490	0.456	0.507	0.0176	0.510
	Weight of the residual yolk	10.13	9.88	9.99	0.210	0.895
	Relative weight of the residual yolk	0.170	0.165	0.167	0.0030	0.808
E21	Weight of the embryo	33.644	35.312	34.065	0.355	0.136
	Relative weight of the embryo	0.563	0.582	0.570	0.0049	0.257
	Weight of the residual yolk	7.99	8.02	7.62	0.254	0.780
	Relative weight of the residual yolk	0.136	0.132	0.127	0.0040	0.680
D1	Weight of the embryo	36.65	38.04	36.39	0.427	0.253
	Relative weight of the chick	0.611	0.626	0.612	0.0052	0.463
	Weight of the residual yolk	6.20	5.54	6.27	0.201	0.282
	Relative weight of the residual yolk	0.103	0.091	0.106	0.2006	0.179

E13, embryonic age 13; D1, postnatal day 1; CON, the non-injected group; NS, the normal saline group; and VC, the vitamin C group. Relative weight of the embryo or chick, the ratio of the weight of the embryo or chick without the residual yolk to the initial egg weight. Relative weight of the residual yolk, the ratio of the weight of the residual yolk to the initial egg weight.

effects on broiler growth performance during the early growth period (20). In this study, IOF of VC had no harmful effects on the relative weight of the embryo (or chick), residual yolk, and organ indexes. In previous studies, it was found that IOF of VC had no significant influence on the weight of D1 chicks, which was the same as our results (7).

At E19, the structural development of the chicken embryo is complete. Before hatching, the embryo must complete hatching behaviors such as absorbing the remaining yolk sac into the abdominal cavity, pecking the shell membrane, and pecking the shell. Early or delayed occurrence of the above behaviors will affect the quality of chicks and the hatching time. Therefore, the hatching time and the score for yolk absorption and the rupture of the shell membrane were selected to comprehensively evaluate the effects of IOF of VC on the hatching process in broilers. In this study, the rupture of the shell membrane and yolk absorption occurred earlier, and the hatching time was correspondingly advanced in the VC group, indicating that VC accelerated the hatching process of preparation for emergence. The hatching muscle, a pair of muscles located at the back of the head, assists in the movement of the embryonic head and mouth (13) and is responsible for puncturing the shell membrane and swallowing amniotic fluid through vigorous contractions (21). The swallowing of amniotic fluid directly facilitates intestinal development (intestinal morphology, digestive enzyme activities, and nutrient transporter expression) and provides the foundation for digestion and absorption of the remaining yolk (22), which is in favor of yolk absorption. We speculated that VC may promote energy reserves in the hatching muscle to accelerate the hatching process, which needs to be further investigated.

Producing chicks with strong anti-stress ability is crucial for the poultry industry. VC has been used to mitigate heat stress by improving production performance through antioxidant modulation in chickens (23). Rectal temperature is a reliable indicator for evaluating anti-stress ability (14, 24), as are stress-related hormones, including CORT (25). CORT is a sensitive indicator in broiler chickens that reflects their physiological condition under stress. To a certain extent, environmental changes before and after hatching are stressful to newly hatched chicks. In this study, IOF of VC reduced the chicks' rectal temperature and plasma CORT levels, indicating that the chicks in the VC group possessed stronger anti-stress ability. Our previous research showed that IOF of VC could regulate embryonic endogenous protection (heat shock protein) and metabolism, which may reduce the risk associated with overheating in chicken embryos (26). In a previous study, IOF of VC reduced the rectal temperature of newly hatched chicks, but the study lacked a non-injected group (7). This study addressed the shortcomings and further confirmed the results. In addition, some studies have shown that dietary VC could reduce rectal temperature and improve heat tolerance in poultry under heat stress (27).

During the preparation for emergence, dramatic physiological and metabolic changes occur. Any intervention can markedly affect the embryonic hatching process and postnatal performance.

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TABLE 2 Effects of *in ovo* feeding of vitamin C at E11 on the embryonic or chick organ indexes of the heart, liver, lungs, and gallbladder during incubation in broiler chickens.

Times	Items	Treatments			SEM	*P*-value
		CON	NS	VC		
E13	Heart	0.011	0.011	0.011	0.0002	0.759
	Liver	0.018	0.019	0.018	0.0003	0.162
	Lungs	0.015	0.015	0.015	0.0005	0.882
E15	Heart	0.010	0.010	0.010	0.0002	0.733
	Liver	0.020	0.018	0.020	0.0004	0.830
	Lungs	0.014	0.014	0.014	0.0004	0.942
	Gallbladder	0.0005	0.0005	0.0005	0.00002	0.104
E17	Heart	0.011	0.010	0.011	0.0002	0.786
	Liver	0.021	0.022	0.022	0.0005	0.534
	Lungs	0.010	0.011	0.012	0.0003	0.222
	Gallbladder	0.0007	0.0007	0.0007	0.00003	0.992
E19	Heart	0.009	0.009	0.009	0.0002	0.289
	Liver	0.020	0.021	0.022	0.0007	0.728
	Lungs	0.010	0.012	0.011	0.0004	0.462
	Gallbladder	0.0008	0.0009	0.0008	0.00003	0.577
E20	Heart	0.009	0.008	0.009	0.0002	0.820
	Liver	0.021	0.022	0.021	0.0004	0.970
	Lungs	0.011	0.010	0.010	0.0003	0.600
	Gallbladder	0.0009	0.0010	0.0011	0.00005	0.419
E21	Heart	0.008	0.007	0.008	0.0002	0.452
	Liver	0.022	0.020	0.020	0.0004	0.255
	Lungs	0.010	0.009	0.009	0.0003	0.576
	Gallbladder	0.0011	0.0010	0.0009	0.00005	0.528
D1	Heart	0.008	0.008	0.008	0.0002	0.387
	Liver	0.026	0.024	0.024	0.0005	0.062
	Lungs	0.010	0.010	0.010	0.0004	0.816
	Gallbladder	0.0012	0.0012	0.0014	0.00007	0.465

E13, embryonic age 13; D1, postnatal day 1; CON, the non-injected group; NS, the normal saline group; and VC, the vitamin C group. Organ index, the ratio of the organ weight to the embryonic or chick's weight without the residual yolk.

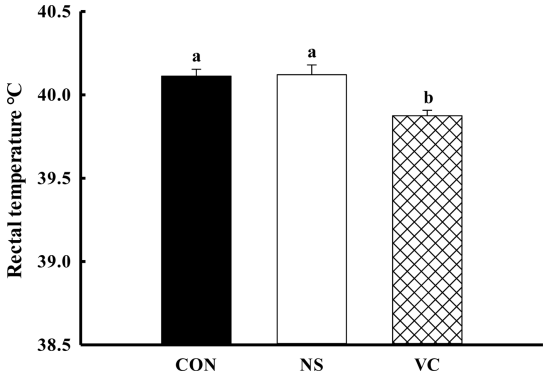

FIGURE 5
Effects of *in ovo* feeding of vitamin C on rectal temperature in the newly hatched chicks.

TABLE 3 Effects of *in ovo* feeding of vitamin C at E11 on plasma parameters in the newly hatched broiler chicks.

Items	Treatments		SEM	*P*-value
	NS	VC		
ALT (U/L)	1.11	2.01	0.436	0.059
AST (U/L)	118.93	108.33	15.550	0.510
ALP (U/L)	425.63	270.50	50.310	0.008
TP (g/L)	16.71	11.80	1.086	<0.001
ALB (g/L)	6.29	4.36	0.507	0.002
GLB (g/L)	10.43	7.44	0.621	<0.001
TBIL (μmol/L)	61.01	40.95	5.106	0.002
TBA (μmol/L)	24.09	14.80	3.191	0.011
BUN (mmol/L)	3.37	3.20	0.270	0.549
UA (μmol/L)	218.75	119.25	37.436	0.019
CRE (μmol/L)	9.58	9.96	1.131	0.737
GLU (mmol/L)	11.16	11.10	0.710	0.928
TC (mmol/L)	8.07	7.05	0.603	0.110
TG (mmol/L)	1.94	1.85	0.203	0.677
HDL-C (mmol/L)	3.40	2.15	0.228	<0.001
LDL-C (mmol/L)	2.13	2.29	0.202	0.434
T4 (nmmol/L)	12.05	9.68	1.291	0.096
fT4 (pmmol/L)	8.70	6.49	1.274	0.113
T3 (nmmol/L)	4.00	4.10	0.335	0.760
fT3 (pmmol/L)	21.69	22.72	1.941	0.609
CORT (ng/mL)	29.39	19.35	3.914	0.030

CON, the control group; NS, the normal saline group; VC, the vitamin C group; ALT, alanine aminotransferase; AST, aspartate aminotransferase; ALP, alkaline phosphatase; TP, total protein; ALB, albumin; GLB, globulin; TBIL, total bilirubin; TBA, total bile acid; BUN, blood urea nitrogen; UA, uric acid; CRE, creatinine; GLU, glucose; TC, total cholesterol; TG, total triglyceride; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; T4, total thyroid hormone; fT4, free thyroid hormone; T3, total triiodothyronine hormone; fT3, free triiodothyronine hormone; and CORT, corticosterone.

In this study, the CON group was set up to confirm the effects of IOF of VC on embryonic development, the hatching process, and the chicks' rectal temperature, while the NS group served as the strictly biological control group for the VC group. Therefore, the plasma biochemical indicators and hormone levels, sensitively reflecting embryonic physiology and metabolism, were only detected between the NS and VC groups.

It is noteworthy that the liver of newly hatched chicks was rich in lipids (28). Lipids in plasma are mainly derived from egg yolk, which is mainly composed of TG, phospholipids, and cholesterol. IOF of VC decreased the plasma HDL-C levels, implying a reduced transport of cholesterol from the blood to the liver. In addition, IOF of VC decreased the plasma levels of ALP, TBIL, and TBA, indicating that the function of the liver was in better status in the VC group. In summary, IOF of VC may be conducive for the liver to manage the physiological characteristics of rich lipids. The decreased level of plasma UA indicated that VC reduced protein catabolism. Combined with the decreased levels of plasma TP, ALB, and GLB, we speculated that VC promoted protein anabolism. Plasma GLU is mainly regulated by liver glycogen, which is derived from gluconeogenesis from glycogenic amino acids and glycerol during late incubation (29).

Based on the reduced protein catabolism, we speculated that VC may promote gluconeogenesis from glycerol in the liver, which may be related to improved liver function.

Thyroid hormones including T3, fT3, T4, and fT4 regulate physiology and metabolism in birds. In addition, thyroid hormones vary dramatically during late incubation and after hatching (30). These hormones play an important role in the thermoregulation of broiler embryos and broiler chickens, involving metabolic heat production (31). In this study, IOF of VC showed a decreasing trend in the plasma T4 level, indicating that the D1 chicks had lower metabolic heat production, which was supported by the lower rectal temperature in the VC group.

5 Conclusion

The rupture of the shell membrane and yolk absorption occurred earlier, and correspondingly, the hatching time was advanced in IOF of VC (3 mg/egg), indicating that VC accelerated the hatching process. In addition, IOF of VC improved the anti-stress ability of the D1 chicks, as evidenced by lower rectal temperatures and plasma CORT levels. These effects may be related to the regulation of body energy metabolism.

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 approved by Institutional Animal Care and Use Committee of Southwest University of Science and Technology. The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

SD: Conceptualization, Data curation, Writing – original draft, Writing – review & editing. JZ: Data curation, Software, Writing – original draft. XA: Data curation, Writing – review & editing. YZ: Conceptualization, Methodology, Writing – original draft, Writing – review & editing.

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Conflict of interest

SD, JZ, and XA were employed by Techlex Food Co., Ltd. YZ was employed by Shanxi Dayu Bioengineering Co., Ltd.

Generative AI statement

The authors declare that no Gen AI was used in the creation of this manuscript.

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The biological function of *Atractylodes lancea* and its application in animal husbandry: a review

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Atractylodes lancea, is a herbaceous plant of the Asteraceae family which is a traditional Chinese herbal medicine. It is often used for dehumidification, antiemetics, spleen strengthening and antipyretic effects. *Atractylodes lancea* is rich in various bio-active substances and has many biological functions, for instance anti-inflammatory, antioxidant and antiviral effects. Therefore, it is widely used in animal production, such as relieving heat stress, protecting intestinal health and regulating immunity. In recent years, it has received widespread attention in green cultivation. This article reviews the biological functions of *Atractylodes lancea* and looks forward to its application prospects in animal husbandry, in order to provide a theoretical basis for *Atractylodes lancea* to become a new feed additive in animal production.

KEYWORDS

***Atractylodes lancea*, biological functions, application, animal husbandry, green cultivation**

1 Introduction

Atractylodes lancea (*A. lancea*) is a perennial herb with a light aroma. It is often used for dehumidification, antiemetics, spleen strengthening and antipyretic effects (1–3), as well as relieve pain and diarrhea (4). In recent years, it is widely used to treat vomiting (5) and heatstroke (6), which is one of the traditional Chinese herbal medicine in China. The rhizome of *A. lancea* has been used widely in many countries for various indications. This compound is called "Cangzhu" in China, "Khod-Kha-Mao" in Thailand, and "So-jutsu" in Japan. There are many species of *A. lancea*, which are widely distributed around the world. Studies have found that *A. lancea* has many biological functions, including anti-inflammatory, antioxidant, antiviral, antibacterial, analgesic, so that it can be used for improving gut health, immunity and growth performance of animals (7, 8). Nowadays, with the continuous development and research of new alternative antibiotic products, *A. lancea* and its extracts have been widely used in animal husbandry due to its low cost, high efficiency and low toxicity. This article reviews the main active ingredients and biological functions of *A. lancea*, and looks forward to its application prospects in livestock and poultry production, in order to provide a reference for the efficient use of *A. lancea* in green and healthy cultivation.

2 The main biological compounds and their structures of *A. lancea*

lancea has a variety of natural bio-active compounds, including sesquiterpenes, enynes, aromatics, polysaccharides, flavonoids, phenols and organic acids (9). The main components of *A. lancea* are sesquiterpenes, including atractylodin, atractylone, β-eudesmol, atractylodes polysaccharides, and atractylenolide (10). The content of active ingredients in *A. lancea* was analyzed by gas chromatography-mass spectrometry (GC-MS). The results showed that the content was as follows: atractylodin (6.22%), hinesol (3.52%), atractylone (1.32%), β-eudesmol (0.81%), and atractylol (0.15%) (11). Wang et al. used a variety of methods to separate the volatile oil of *A. lancea* and found that the contents of atractylodin, atractylol, and atractylone were high, while the contents of atractylenolide and atractylodes polysaccharides were low. At the same time, it was confirmed that *A. lancea* has good pharmacological activities (12). The main bio-active compounds of *A. lancea* and their structures are shown in Figure 1.

3 The biological functions of *A. lancea*

3.1 Anti-inflammatory

Inflammatory response is a complex physiological response of the immune system to external stimuli, which is regulated by a variety of inflammatory mediators. Appropriate amount of inflammatory factors plays an important role in maintaining the normal physiological functions of animals, but when animals are infected by viruses or bacteria, a large amount of inflammatory factors will be deposited in the body, leading to serious inflammatory damage (13–15). Atractylenolide I and atractylenolide III can reduce the expression level of tumor necrosis factor-α (TNF-α) and the production of nitric oxide (NO) in animal serum. Atractylenolide I showed a more effective inhibitory effect on the production of TNF-α and NO in peritoneal macrophages activated by lipopolysaccharide (LPS) than atractylenolide III (16). Both nuclear factor-κB (NF-κB) and mitogen-activated protein kinase (MAPK) signaling pathways can regulate the release of inflammatory factors in animals. The study by Jeong et al. (17) showed that atractylenolide I and Atractylenolide III could inhibit the phosphorylation of p38 MAPK, c-Jun N-terminal kinase (JNK), and inhibitor of nuclear factor-κB (IκB) in LPS induced mouse inflammatory macrophages, promote the phosphorylation of extracellular signal-regulated kinase (ERK), block the translocation of NF-κB to the nucleus, and significantly reduce the expression of pro-inflammatory factors such as TNF-α, interleukin-6 (IL-6), and interleukin-1β (IL-1β). In addition, atractylenolide II can reduce the release of inflammatory factors such as NO, TNF-α, and IL-6 in mouse macrophages induced by LPS, but the effect is not significant (18). In summary, atractylenolide, as the most effective anti-inflammatory component in *Atractylodes lancea*, can reduce the expression level of inflammatory factors and alleviate inflammatory damage by regulating the MAPK and NF-κB signaling pathways, but the inflammatory regulation mechanism of other compounds still needs further study.

3.2 Antioxidant

Reactive oxygen species (ROS) are extremely bio-active materials. Excessive levels of ROS in livestock and poultry can cause oxidative stress (19). The phenolic acids and flavonoids contained in *Atractylodes lancea* have metal chelating and free radical scavenging functions, inhibiting the production of ROS (20). The Nrf2-Keap1 signaling pathway plays a pivotal role in the antioxidant response of animals and increase the activity of antioxidant enzymes, among which Nrf2 is the main effector (21). Study had shown that Atractylodes polysaccharide II can increase the activity of glutathione peroxidase (GSH-Px) and superoxide dismutase (SOD) in the tissue of mouse liver, significantly reduce the activity of nitric oxide synthase (NOS) and the content of NO and malondialdehyde (MDA) (22). It can be seen that *A. lancea* polysaccharide has good antioxidant activity. On the one hand, *A. lancea* polysaccharide balances the antioxidant system in animals by inhibiting the production of ROS; On the other hand, *A. lancea* polysaccharide can regulate the Nrf2-Keap1 signaling pathway, enhance the activity of antioxidant enzymes SOD and GSH-Px, and improve the ability of animals to clear ROS.

3.3 Anti-viral

Viral infections such as swine flu and avian flu are extremely harmful to livestock and poultry production. The clinical manifestations include diarrhea, cough, fever, and lameness. Mild cases can cause breathing and movement difficulties, while severe cases can lead to large-scale deaths, seriously affecting the economic benefits of the breeding industry. *A. lancea* can act on the cell surface to change the protein receptor structure, significantly block the adsorption and penetration of swine influenza virus into cells. Study has shown that *A. lancea* can significantly inhibit the proliferation of swine influenza virus in cells and has a direct effect of inactivating virus (23, 24). In addition, atractylone, as the main antiviral ingredient in *A. lancea*, can block the adsorption and replication of avian influenza virus (25). Toll-like receptor 7 (TLR7) is widely present in immune cells, epithelial cells and nerve cells. When livestock and poultry are invaded by pathogens, TLR7 will immediately transfer to the cell membrane, identify the pathogens, aggregate specific proteins to activate regulatory factors such as IκB, MAPK and interferon (IFN), initiate specific immune responses, and participate in the antiviral process. Atractylone can regulate TLR7 receptors, inhibit the activation of MAPK and NF-κB signaling pathways, relieve epidemic diarrhea and respiratory diseases caused by influenza A virus infection. Chen et al. (26) found that continuous treatment with 10, 20, and 40 mg/kg atractylone for 5 days could alleviate influenza A virus-induced lung injury in mice, significantly reduce serum TNF-α, IL-6, and IL-1β levels, meanwhile increase IFN-β levels, indicating that atractylone can promote IFN-β production by activating the TLR7 signaling pathway, interfere with viral replication, and recruit immune cells to activate specific immune responses and eliminate viruses. Therefore, *A. lancea* has important application potential in livestock and poultry production because of its antiviral effects.

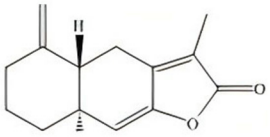
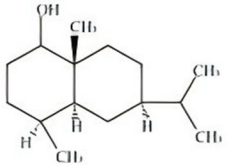
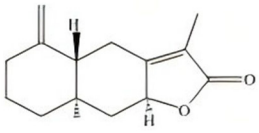
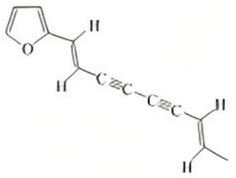
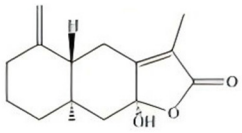
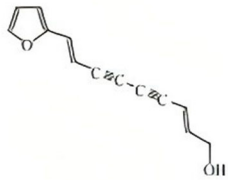
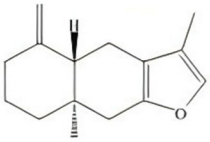
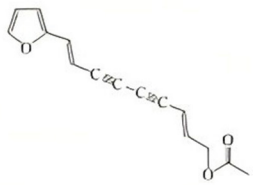
Chemical names	Forumula	Molecular structure	Chemical names	Forumula	Molecular structure
Atractylenolide Ⅰ	$C_{14}H_{15}O_2$		β-eudesmol	$C_{15}H_{28}O$	
Atractylenolide Ⅱ	$C_{14}H_{17}O_2$		Atractylodin	$C_{13}H_{10}O$	
Atractylenolide Ⅲ	$C_{14}H_{17}O_3$		Atractyoldinol	$C_{13}H_{10}O_2$	
Atractylone	$C_{14}H_{17}O$		Acetyla tractlodinol	$C_{15}H_{12}O_3$	

FIGURE 1
The main bio-active compounds of *A. lancea* and their structures.

3.4 Other functions

A. lancea also has anti-tumor, liver protection, diuretic, glucose metabolism, and lipid metabolism regulating functions. Studies have shown that *A. lancea* polysaccharides have anti-tumor effects, which can activate macrophages through the Toll-like receptor 4 (TLR4) signaling pathway, reduce the expression of B cell lymphoma-2 (Bcl-2), increase the expression of pro-apoptotic factors such as Bcl-2-associated X protein (Bax) and cysteine aspartate proteinase-9 (Caspas-9), finally promote tumor cell apoptosis (27–29). Additionally, *A. lancea* polysaccharides can prevent liver damage caused by harmful chemicals and toxins. Han et al. (22) showed that *A. lancea* polysaccharide can reduce the expression levels of AST, ALT, and MDA in liver, increase the activity of SOD and GSH-Px, and alleviate LPS-induced liver inflammation in mice by inhibiting the NF-κB signaling pathway. It is reported that *A. lancea* has a diuretic effect. Study has found that intravenous injection or oral administration of 1.0 g/kg *A. lancea* solution can significantly increase the urine output of mice (27). In addition, the active ingredients in *A. lancea* can improve glucose uptake, inhibit fat production, and regulate lipid metabolism in animals (30). The regulatory mechanism of *A. lancea* on MAPK, NF-κB, Toll-like receptor (TLR) and Nrf2-Keap1 signaling pathways is shown in Figure 2. The key functions and associated mechanisms of *Atractylodes lancea* was showed in Table 1.

4 Application prospects of *A. lancea* in livestock and poultry production

4.1 Improving productive performance

A. lancea is rich in natural active substances, which have multiple functions such as improving immunity, resisting stress and promoting digestion. It has great potential in improving the production performance of livestock and poultry. Li et al. (31) found that adding 3, 6, and 9 g/kg of *A. lancea* polysaccharide to the diet could significantly improve the growth performance of early weaned piglets. On the one hand, *A. lancea* polysaccharides can play an antioxidant role and alleviate the adverse effects of stress on growth performance (32). On the other hand, *A. lancea* polysaccharide can stimulate lymphocyte proliferation and antibody production in early weaned piglets, improve disease resistance and reduce diarrhea rate (33). In addition, *A. lancea* also plays an important role in improving the productive performance of poultry and ruminants. Study has found that adding *A. lancea* polysaccharides to laying hen diets can significantly increase egg

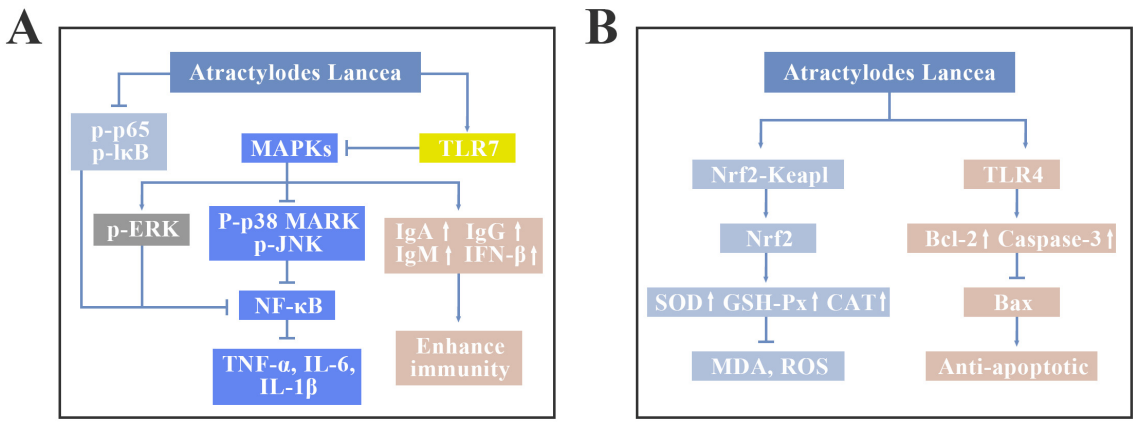


FIGURE 2
The regulatory mechanism of *A. lancea* on MAPK, NF-κB, Toll-like receptor (TLR) and Nrf2-Keap1 signaling pathways. **(A)** MAPK, NF-κB and TLR7 signaling pathways. **(B)** Nrf2-Keap and TLR4 signaling pathways.

TABLE 1 The key functions and associated mechanisms of *Atractylodes lancea*.

Biological functions	Active ingredients	Relative mechanisms	References
Anti-inflammatory	Atractylenolide I	TNF-α, NO, IL-6, IL-1β↓	(16)
	Atractylenolide III	p38 MAPK, JNK, IκB↓	(17)
			(18)
Antioxidant	Atractylodes polysaccharide II	GSH-Px, SOD↑	(22)
		NOS, NO, MDA↓	(20)
Anti-viral	Atractylone	MAPK and NF-κB signaling pathways↓	(25)
		TNF-α, IL-6, IL-1β↓	(26)
Anti-tumor	Atractylodes polysaccharides	Bcl-2↓	(27–29)
		Bax, Caspas-9↑	
Liver protection	Atractylodes polysaccharides	AST, ALT, and MDA↓	(27)
		SOD, GSH-Px↑	

weight, egg production rate, and feed conversion rate, reduce mortality of laying hens (34). In the ruminants, studies have shown that adding 0.75% of *A. lancea* to the diet can improve the fermentation capacity, increase the efficiency of rumen microorganisms in degrading substances such as protein and cellulose, and promote the synthesis of bacterial protein in the rumen (35). It can be seen that *A. lancea* has good application value and research potential in improving the production performance of livestock and poultry, but its specific addition amount needs further exploration.

4.2 Immune regulation

After weaning, piglets lose the protection of maternal antibodies and their immune system is not fully developed, so they are easy to meet with problems such as poor disease resistance, growth retardation and diarrhea. Li et al. (36) added 0.3% purified *A.lancea* polysaccharide, 0.6% crude polysaccharide and 0.6% crude *A. lancea* polysaccharide to the diet of weaned piglets. The results showed that all three polysaccharides could increase the antibody content in serum, promote lymphocyte proliferation and improve immunity of weaned piglets. Wang et al. (37) found that adding 0.1, 0.2, and 0.3% fermented *A. lancea* to the diet of early weaned piglets could increase the levels of immunoglobulin A (IgA), immunoglobulin G (IgG) and immunoglobulin M (IgM) as well as TP and ALB in the serum, among which 0.2% fermented *A. lancea* had the most significant effect. Interleukin-1(IL-1) and interleukin-2 (IL-2) are cytokines produced by activated T cells, which can stimulate the proliferation and differentiation of immune cells and enhance animal immunity. The experimental results of Xu et al. (33) showed that adding an appropriate amount of *A. lancea* polysaccharide to the diet of weaned piglets can promote lymphocyte proliferation, increase the levels of antibodies such as IgA and IgG in serum and release cytokines such as IL-1 and IL-2, thereby improving the immunity of weaned piglets. *A. lancea* can significantly increase the spleen and thymus index of livestock and poultry, and it has a positive effect on improving immunity and intestinal health. Li et al. (38) found that *A. lancea* polysaccharide can alleviate cyclophosphamide-induced immune

organ damage in geese. In ruminants, *A. lancea* polysaccharides can induce the proliferation of bovine mammary lymphocytes and activate immune cells (39). In summary, *A. lancea* can be used as an immunomodulator and has important application potential in livestock and poultry production, but its specific mechanism of regulating immunity needs further study.

4.3 Improve intestinal health

4.3.1 Intestinal barrier

Intestinal health is an important factor affecting the production performance of livestock and poultry, it is also an important indicator for assessing animal welfare. Bose et al. (40) found that fermented *A. lancea* polysaccharide can alleviate LPS-induced intestinal epithelial cell damage and reduce intestinal mucosal permeability through *in vitro* experiments. Shi et al. (41) found that the *A. lancea* can promote the integrity of the intestinal mucosal barrier by inhibiting the phosphorylation of p38 and MAPK signaling pathways and increasing the mRNA expression of *ZO-1*, *Claudin-1*, and *Occludin*. This indicates that *A. lancea* protects the intestinal barrier of animals by reducing the level of inflammatory factors. In addition, adding an appropriate amount of *A. lancea* polysaccharide to the LPS-induced enteritis model can increase the protein level of ZO-1 and Occludin, and alleviate intestinal inflammation in goose (42). In terms of intestinal microbes, *A. lancea* polysaccharides can reduce the relative abundance of *Escherichia coli* in weaned piglets while increasing the level of *Lactobacillus* (36). In addition, Wang et al. (43) reported that *A. lancea* polysaccharides can regulate the structure of intestinal microbes, promote the colonization of probiotics in the intestine, and reduce the abundance of harmful bacteria in mice. Studies on ruminants have shown that adding *A. lancea* to the diet can increase the activity of rumen microbiota, changing the structure of rumen microbes, and promoting rumen fermentation (44, 45). It can be seen that *A. lancea* plays a regulatory role in the intestinal health of animals, but its role in affecting the composition and activity of intestinal microbes still needs further study.

4.3.2 Colon health

Colitis often occurs in piglets from 4 to 16 weeks of age after weaning, leading to increasing intestinal mucosal permeability, diarrhea, and reduced the growth performance of piglets (46). Studies have found that *A. lancea* can increase the content of tight junction proteins, thereby reducing diarrhea caused by colitis in mice (3, 47, 48). The mechanism of *A. lancea* in relieving colitis is as follows: on the one hand, *A. lancea* can promote the secretion of mucin by goblet cells, increase the content of ZO-1 and Occludin, reduce intestinal mucosal permeability, and restore the normal physiological function of colon in piglets; On the other hand, *A. lancea* inhibits the phosphorylation of MAPK and NF-κB signaling pathways in the intestine, reduces the expression of inflammatory factors such as TNF-α, IL-6, and IL-1β, alleviates the absorption disorder of sodium ions and chloride ions disturbed by inflammatory factors, improves the water re-absorption capacity in the colon, and reduces diarrhea in piglets. IL-6, which is a reference indicator of colitis, can increase the permeability of intestinal mucosa (49). Studies have shown that *A. lancea* can inhibit the release of TNF-α and IL-6, up-regulate the expression of autophagy genes, and significantly alleviate colon damage in mice (3, 50, 51). Since colitis is a disease caused by immune system disorders, it is speculated that *A. lancea* can maintain the homeostasis of the internal environment by activating the cell autophagy pathway and ultimately improve colitis.

4.4 Relief of heat stress

Heat stress refers to the sum of non-specific physiological responses made by the body to any requirements imposed by the thermal environment at high ambient temperatures (52). In general, there is an isothermal zone in homeothermic animals, and when the ambient temperature is in the range of the isothermal zone, the animal can maintain normal temperature through body temperature regulation; When the ambient temperature is higher than the upper limit of the isothermal zone, the animal will be subjected to heat stress (64). When heat stress occurs, animals typically show increased breathing, increased heart rate, and impaired electrolyte balance (53). Therefore, heat stress can effect the growth performance and reproductive performance. Under the influence of heat stress, animals' appetite decreases, resulting in a decrease in feed intake, which seriously affects production performance (54). Studies have shown that heat stress can lead to oxidative stress, adding *A. lancea* to pig diets can increase the activity of antioxidant enzymes in serum, reduce MDA and ROS levels, and thus alleviate heat stress (55, 56). *A. lancea* can terminate lipid peroxidation by removing ROS and hydrogen peroxide, improve ROS-induced intestinal epithelial cell shedding, and restore the digestive and absorptive functions of the intestine. Xu et al. found that adding *A. lancea* polysaccharides to broiler diets can significantly improve the antioxidant capacity, enhance immunity, and alleviate the damage caused by heat stress (57). In addition, *A. lancea* also has a good effect in alleviating heat stress in ruminants. It has been reported that under heat stress conditions, adding *A. lancea* to cattle diets can significantly improve immunity and antioxidant capacity, promote rumen digestion and absorption of nutrients in the feed, and improve production performance (57). It can be seen that *A. lancea* plays an important role in alleviating heat stress in animals, but its effective components and optimal additive dosage need further study.

4.5 Other application prospects

4.5.1 Antimicrobial agents

Studies have shown that *A. lancea* has antibacterial effects on a variety of microbes, including *Escherichia coli*, *Candida albicans* and *Staphylococcus aureus* (2, 58). Peng et al. reported that 5–40 mg/mL Atractyoldinol had a significant inhibitory effect on *Staphylococcus aureus*, *Escherichia coli,* and *Bacillus subtilis* (59). The antibacterial mechanism of *A. lancea* is that on the one hand, *A. lancea* can destroy the protein structure in the bacterial cell *membrane*, causing the cell contents to flow out; On the

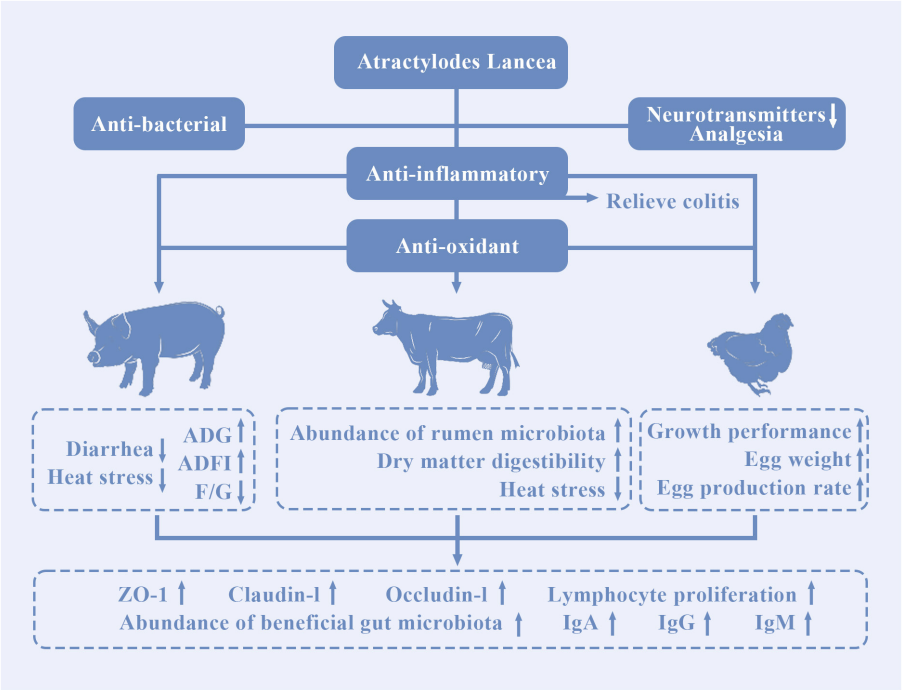


FIGURE 3
The application of *A. lancea* in livestock and poultry production.

other hand, adding *A. lancea* to animal feed as a feed additive can directly remove aflatoxin and improve the digestibility of nutrients in the feed. It can be seen that *A. lancea* has important application value in livestock and poultry production as an antibacterial agent.

4.5.2 New type of analgesic agents

Ohara et al. found that atractyoldinol and β-eudesmol have analgesic effects, which can inhibit the function of the nervous system, and reduce the sensitivity of animals to external stimuli (60). Atractylodesinol is used as an analgesic in livestock and poultry production. On the one hand, atractylodesinol can inhibit the release of neurotransmitters (61, 62); On the other hand, atractyoldinol can block acetylcholine receptor channels, weaken nerve signal transmission, and relieve pain caused by production processes such as sow farrowing, piglet castration and rectal prolapse suture (63). Therefore, atractyoldinol has a good analgesic effect which can be used as a new analgesic. However, it is currently rarely used in livestock and poultry, the specific dosage needs further experimental research to explore. The application of *A. lancea* in livestock and poultry production is shown in Figure 3.

5 Conclusion and perspectives

Under the background of "total ban on antibiotics," optimizing livestock and poultry feed formula and finding new green feed additives have become research hotspots. *A. lancea* can inhibit the phosphorylation of MAPK and NF-κB signaling pathways, reduce the level of inflammatory factors, and alleviate the inflammatory response of livestock and poultry; At the same time, *A. lancea* can reduce the level of ROS and oxidative stress damage in animals by regulating the Nrf2-Keap1 signaling pathway; *A. lancea* can also inhibit the TLR signaling pathway to exert antiviral function. Adding *A. lancea* to animal diet can improve their growth performance and immunity, improve intestinal health and relieve heat stress. In addition, *A. lancea* can also be used as an antibacterial agent and analgesic, which has important application prospects in livestock and poultry production. However, the production process of *A. lancea* still needs to be optimized. Therefore, in the future, we should further develop *A. lancea* as a feed additive, promote its application in livestock and poultry production, explore the appropriate amount of addition at different growth stages, so as to promote the development of green ecological farming.

Author contributions

YG: Funding acquisition, Writing – original draft. DoW: Writing – review & editing, Validation, Software. XM: Software, Writing – review & editing. JL: Supervision, Writing – review & editing. DiW: Supervision, Writing – review & editing. BC: Supervision, Writing – review & editing. XY: Supervision, Writing – review & editing. HL: Resources, Software, Writing – original draft.

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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.

Generative AI statement

The author(s) declare that no Gen AI was used in the creation of this manuscript.

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Dietary Fe-Gly supplementation attenuates enterotoxigenic *Escherichia coli* (ETEC)-induced inflammation response and intestinal barrier dysfunction in piglets

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Iron in the animal gut that is not utilized by the host can be directly utilized by microorganisms, particularly harmful ones. Organic iron (such as Fe-Gly) has high digestive and absorption efficiency in the body. It is currently unclear whether it can reduce the utilization of iron by ETEC, thereby mitigating the harm caused by ETEC infections. This experiment mainly studies the effects of adding Fe-Gly to the diet on the growth performance, iron nutritional status, and intestinal morphology of weaned piglets infected with ETEC. The study found that adding 50 mg of Fe-Gly to the diet significantly increased ADFI and ADG by 30.6 and 35.3%, respectively ($p < 0.05$), and alleviated the issues of diarrhea and reduced growth performance caused by ETEC infection. The diarrhea rate decreased by 40% (from 31.25 to 18.75%). In addition to protecting the health of piglets, adding Fe-Gly can also increase the TIBC level in piglet serum ($p < 0.05$), enhancing their ability to bind and transport iron. From the gene expression results and tissue section results, adding Fe-Gly can also alleviate the damage to the jejunum caused by ETEC challenge to some extent ($p < 0.05$). In conclusion, adding 50 mg of Fe-Gly can meet the daily needs of piglets, improve iron utilization efficiency, and reduce the residual iron in the intestines. This decreases the iron available for pathogenic microorganisms in the gut, thereby inhibiting the proliferation of intestinal pathogens and ensuring the intestinal health of piglets.

KEYWORDS

iron, piglets, enterotoxigenic *Escherichia coli* (ETEC), inflammation response, intestinal barrier

1 Introduction

Iron is an important nutrient for piglets, iron deficiency seriously affects the normal growth of piglets, resulting in loss of appetite, slow growth, and many other undesirable symptoms. Additionally, iron deficiency can reduce the piglet's ability to resist disease, reduce the piglet's disease resistance by decreasing specific immune cell numbers, thereby

compromising cellular immunity, and affecting the humoral and non-specific immunity (1). Only 5–20% of the iron ingested through feed is absorbed by the duodenum. About 80% of the ingested iron is retained in the intestinal lumen for microbial absorption and use (2). This is because iron is a protein cofactor involved in key metabolic pathways, and the replication and growth of almost all bacteria in the intestinal microbiota depend on this unabsorbed iron in the intestinal lumen (3, 4). Thus, it is clear that iron is closely related to microbial proliferation, and increasing the efficiency of the body's absorption of ingested iron, reducing the unabsorbed iron in the intestinal lumen may be an effective antimicrobial strategy to benefit piglet health.

Currently, ferrous sulfate ($FeSO_4$) is the most commonly used form of iron in feed. However, inorganic iron has several disadvantages, including chemically unstable, less absorption efficiently, and a tendency to accelerate the oxidation and rancidity of lipids in the feed. Ding et al. found that excessive addition of $FeSO_4$ in the diet significantly reduced the villus height of the duodenum, jejunum, and ileum in weaned piglets, damaging the intestinal morphology. Furthermore, excessive $FeSO_4$ significantly inhibited the expression of tight junction proteins in the duodenum, increased intestinal permeability, and caused mitochondrial swelling in the duodenum, thereby impairing the repair and regeneration capacity of the intestinal mucosa (5). Therefore, when using inorganic iron as an iron source supplement for piglets, the amount added tends to be much higher than the actual requirement, which not only raises the cost of feed production, but also increases the source of iron available to microorganisms in the gut and raises the risk of intestinal disease. In addition, the iron that is eliminated from the body with feces can also pollute the environment. In recent years, many studies have found that amino acid chelated iron is structurally stable, does not form ionic iron, and reduces the effects of various anti-nutrients (6). Iron supplemented in the form of amino acid chelated iron can be directly absorbed by the body and transported to specific sites to perform its functions, reducing intermediate processes and thus improving bioavailability efficiency. Some studies have shown that the absorption rate of amino acid chelated iron is 2–4 times higher than that of $FeSO_4$, and that Fe-Gly enters the cellular mucosa in a chelated form (7–10). Ma et al. found that in the Caco-2 cell model, the Fe-Gly translocation rate across the cell monolayer was found to be significantly higher than that of $FeSO_4$ (11). Iron glycinate (Fe-Gly), as a kind of amino acid chelated iron, has the advantages of amino acid chelated iron, and is a kind of efficient iron supplement additive (12). Therefore, we hypothesize that the addition of $FeSO_4$ and Fe-Gly at equivalent doses may significantly increase iron absorption and reduce iron levels in the cecum and colon, thereby inhibiting the proliferation of harmful microorganisms. This experiment uses $FeSO_4$ as a control and aims to investigate the effects of low-dose Fe-Gly addition on the production performance of weaned piglets, as well as its intervention effects on the intestinal damage and disorders caused by ETEC infection.

2 Materials and methods

The animal experiment in this study was carried out after approved by the Animal Care and Use Committee of Sichuan Agricultural University (Chengdu, China, No. 20210111).

2.1 Experimental materials

Fe-Gly, $FeSO_4$, and other trace elements were purchased from Hunan Depon Biotechnology Co. Feed ingredients: corn, wheat bran, fish meal, rock flour, calcium bicarbonate, sodium chloride, synthetic amino acids, choline chloride (from Sichuan Agricultural University Teaching and Research Base); peeled soybeans, expanded soybeans, soybean oil, sucrose, glucose, enzyme preparation (purchased from Sichuan Chiyang Agricultural and Animal Husbandry Co., Ltd).

2.2 Animal experiments

In this experiment, 32 weaned piglets with an average body weight of 7.08 kg at 21 days of age were selected. After 3 days of pre-feeding, they were weighted and divided into 4 treatment groups using randomized block group design, with 8 replicates each, 1 piglet in each replicate (Table 1). The piglets were fed diets containing 100 mg/kg of $FeSO_4$ and 50 mg/kg of Fe-Gly, respectively. The amount of $FeSO_4$ added is based on the standards set by the NRC (2012), while the Fe-Gly reference is derived from previous studies (9). The test period lasted 21 days, during which the piglets were fed and watered libitum. At the end of the test, the piglets were challenged with ETEC by oral gavage, while the control group received the same volume of LB medium. The piglets were observed for diarrhea after the challenge, and sampling was performed when diarrhea was evident in the two treated groups.

2.3 Piglet test diets

The diets in this trial were configured with reference to the NRC (2012) nutritional requirements for piglets in the 5–11 kg stage (Table 2).

2.4 Breeding management

The experiment was conducted at the teaching and research base of the Institute of Animal Nutrition, Sichuan Agricultural University, and the pen was fully disinfected before the start of the experiment. During the test period, we kept the barn ventilated, controlled the temperature of the experimental enclosure at 26–28°C, cleaned the enclosure every day and disinfected it accordingly, fed the piglets three times a day (at 8:00, 14:00 and 20:00), and inspected the piglets every 2 h to observe the feeding situation, and ensured that the residue in the trough of the piglets was not less than 1/4 of the trough's volume.

TABLE 1 Experimental design.

Treatment	Diets	Number of piglets
T1	$FeSO_4$ (100 mg Fe/Kg)	8
T2	Fe-Gly (50 mg Fe/Kg)	8
T3	$FeSO_4$ (100 mg Fe/Kg) + ETEC	8
T4	Fe-Gly (50 mg Fe/Kg) + ETEC	8

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TABLE 2 Base diet formulation and nutritional levels (%).

Ingredient	Proportion (%)	Calculated nutrient levels	
Corn (CP7.8%)	66.100	CP (%)	17.00
Bran (CP13.6%)	4.100	EE (%)	5.85
Fishmeal (CP68%)	4.500	CF (%)	2.65
Pulped soybean meal (CP46%)	8.200	ASH (%)	5.10
Puffed soya bean (CP36.5%)	9.000	Ca (%)	0.67
Soybean oil	1.500	P (%)	0.56
Fructose	2.000	N-Phy-P (%)	0.36
Glucose	1.000	Lys (%)	1.37
Talcum powder (38%)	0.600	Met (%)	0.50
Calcium hydrogen phosphate	0.800	Cys (%)	0.25
NaCl	0.450	Thr (%)	1.00
L-Lysine hydrochloride	0.670	Trp (%)	0.28
DL-Methionine (99%)	0.210	Arg (%)	1.19
L-Threonine (98.5%)	0.405	Ile (%)	0.60
Tryptophan (98.5%)	0.105	Val (%)	0.75
Choline chloride (50%)	0.150	DE (Macl/kg)	3.48
Phytase 5000	0.020	ME (Macl/kg)	3.20
Antioxidants (30%)	0.020	NE (Macl/kg)	2.45
Mildew inhibitor	0.070		
Multivitamin[1]	0.050		
Complex polymetallic[2]	0.050		
Total	100.00		

[1]The premix provides: 4 mg VB1; 8 mg VB2; 6 mg VB12; 3000 IU VD3; 60 IU VE; 4 mg VK3; 0.3 mg Biotin; 50 mg Niacinamide; 30 mg Calcium D-Pantothenate; and 2 mg Folic Acid per kg of ration.

[2]The mineral premix provides per kg of full-price feed: 100 mg Fe ($FeSO_4$ H_2O) in the control group; 50 mg Fe (Fe[C2H4O2N]2) in the Fe-Gly group; 5.5 mg Cu ((C2H4NO2)2Cu); 3.5 mg Mn; 90 mg Zn (C4H8N2O4Zn); 0.14 mg I (Ca (IO3)2); 0.275 mg Se (Na2SeO3).

2.5 Sample collection and processing

2.5.1 Blood sample collection

On the third day after the ETEC infection, blood was collected from the heart of piglets. 1 mL of anticoagulated blood (sodium heparin vacuum blood collection tubes were used for the whole blood analyzer) was collected, and the remaining whole blood was packed in normal vacuum blood collection tubes, centrifuged at 3000 r/min for 15 min to obtain the serum, and the serum samples were stored in separate packages at −20°C until analysis.

2.5.2 Tissue sample collection

After blood collection, all piglets were injected with anesthetics (hypospray), bled to death, and the abdominal cavity of the piglets was quickly dissected to isolate the organs and weigh and measure the lengths of the individual intestinal segments. The duodenum, jejunum, liver, spleen and kidneys were collected, and part of them were preserved in 4% paraformaldehyde solution for subsequent HE, and part of them were frozen in liquid nitrogen and promptly transferred to a −80°C refrigerator for gene and protein analyses; the cecum and colon coeliacs were collected, frozen in liquid nitrogen and promptly transferred to a −80°C refrigerator for preservation.

2.6 Growth performance

Piglets were weighed on an empty stomach at 8:00 a.m. on trial days 0, 22 and the day of sampling, and the amount of feed added to each pen each day was recorded, and residual and lost feed in each pen was settled weekly to determine piglet performance at all stages and throughout the whole period of the trial, including the average daily gain (ADG), average daily feed intake (ADFI), and feed gain ratio (F/G).

Average daily weight gain (g/d) = average weight gain (g)/number of feeding days (d).

Average daily feed intake (g/d) = average feed intake (g)/number of feeding days (d).

Feed gain ratio (F/G) = total feed consumption during the test period (g)/piglet weight gain during the test period (g).

2.7 Assessment of diarrhea

Piglets were observed and scored for defecation at 8:00, 12:00 and 16:00 every day during the experiment (Table 3). In simple terms: 0 = Normal (Formed or granular), 1 = Mild (soft manure, can be shaped), 2 = Moderately (thick, unformed, no separation of feces and water), 3 = Seriousness (Liquid, unformed, with separation of feces and water) and score $\geq$ 2 was considered diarrhea.

Diarrhea rate (%) = (days of piglet diarrhea/test days) × 100.

Diarrhea index = sum of diarrhea scores/(number of pigs per pen × number of days of scoring × number of scores per day).

2.8 Morphological analysis of the intestinal tract

The jejunal tissue samples fixed in 4% paraformaldehyde were stained with Periodic acid-schif (PAS) after ethanol gradient dehydration, xylene transparency, and immersion wax embedded sections. After sealing the sections, 10 fields of view with intact villi and straight orientation were selected under an Eclipse Ci-L (Nikon, Japan) photomicroscope, and the jejunal villus height and Crypt depth was measured using ImageJ image processing and analysis software.

2.9 Determination of non-heme iron in liver, spleen, kidney and each intestinal segment

About 30 mg of tissue was taken and mixed with 1 mL of tissue digestion solution (the formula was a mixture of 3 mol/L hydrochloric acid and 0.61 mol/L trichloroacetic acid, which was dissolved and then fixed to 1 L with deionized water), ensuring that the tip was not contaminated with iron. Subsequently, the tissue was digested at 65°C for not less than 50 h, with shaking 3 times, each time for not less than 10 min, to ensure complete digestion. After digestion was completed,

the tissue digest was fixed to 1.5 mL, centrifuged at 10,000 g for 10 min, and 10 μL was pipetted into a 96-well plate, and 200 μL of iron color working solution was added to each well and the color was developed at room temperature for 10 min. Finally, the absorbance value was read using an enzyme marker at 535 nm (13).

2.10 Routine blood indicators

Red blood cell (RBC), hemoglobin concentration (HGB), erythrocyte pressure volume (HCV), mean corpuscular volume (MCV), and mean corpuscular hemoglobin content (MCHC) were determined using a fully automated hematology analyzer (Hitachi, Japan).

2.11 Serum iron measurement

Serum iron (Serum iron), Unsaturated Iron Binding Capacity (UIBC), Total Iron Binding Capacity (TIBC), and Iron Saturation (TF%) were measured using the Serum Iron Kit (Pointe Scientific™ Iron Standard Reagent) (13).

2.12 Statistical analysis

The experimental data were organized and analyzed using Excel for statistical purposes. Statistical analyses were performed using IBM SPSS 27 software, where pre-infection data were analyzed by one-way ANOVA followed by Tukey multiple comparison test; post-infection data were analyzed using two-way ANOVA in a general linear model to assess main effects and interactions. All data are presented as mean ± SEM. Statistical significance was set at $p < 0.05$, and $0.05 < p < 0.10$ was considered as a trend. Graphs were generated using GraphPad Prism 10.1.2 software.

3 Results

3.1 The effect of Fe-Gly addition on the growth performance and diarrhea in piglets

Table 4 showed that during 1–21 d of the experiment, piglets in the $FeSO_4$ supplementation group exhibited significantly higher ADFI and ADG compared to the control group ($p < 0.05$). As depicted in Table 5, ETEC gavage significantly increased the rate of piglet diarrhea ($p < 0.05$), while the addition of Fe-Gly group effectively alleviated the diarrhea in the infected group, and the addition of Fe-Gly reduced the rate of diarrhea by 12.5% and the diarrhea index by 43.2% compared with the control group.

3.2 The effect of Fe-Gly addition on the organ index of piglets

Table 6 showed that there was no significant difference in the organ indexes of piglets in each group except for the spleen; there was a tendency for an increase in the spleen indexes of piglets in the Fe-Gly added group compared with that of the $FeSO_4$ added group ($p = 0.051$); and there was a certain decrease in the spleen indexes of the ETEC infected compared with that of the uninfected group in the Fe-Gly added group ($p = 0.091$).

TABLE 3 Scoring criteria for the degree of diarrhea in piglets.

Fecal appearance	Diarrhea level	Diarrhea score
Formed or granular	Normal	0
Soft manure, can be shaped	Mild	1
Thick, unformed, no separation of feces and water	Moderately	2
Liquid, unformed, with separation of feces and water	Seriousness	3

TABLE 4 Effect of adding Fe-Gly in 1-21d on piglet performance.

Items	Treatment		*p*-values
	100 mg $FeSO_4$	50 mg Fe-Gly	
Initial BW Kg	7.05 ± 0.14	7.11 ± 0.13	0.724
21d BW Kg	11.13 ± 0.45	12.64 ± 0.63	0.061
ADFI (Kg/d)	297.8 ± 21.2	389.0 ± 35.3	0.035
ADG (Kg/d)	185.6 ± 17.3	251.2 ± 24.8	0.038
F/G	1.69 ± 0.08	1.59 ± 0.05	0.285
Diarrhea rate (%)	6.82 ± 2.68	4.83 ± 1.63	0.532
Diarrhea index	0.22 ± 0.08	0.17 ± 0.06	0.655

3.3 The effect of Fe-Gly addition on the morphology of the jejunum in piglets

Figure 1 demonstrated that there was no significant difference in villus height in the Fe-Gly added group compared to the control group ($FeSO_4$ added group) in the absence of infection (Figures 1A,D,E). Significant crumpling of the intestinal villi occurred in piglets in the group with 100 mg of added $FeSO_4$ after infected ($p < 0.05$), whereas there was no significant change in the intestinal villi in the group with 50 mg of added Fe-Gly (Figures 1A–G). Crypt depth did not change significantly between treatments, but the villus-crypt ratio showed similar differences to villus height (Figures 1B–G). This suggests that the addition of 50 mg of Fe-Gly can alleviate to some extent the damage to the intestinal tract of piglets due to ETEC infection.

3.4 The effect of Fe-Gly addition on iron metabolism in the body

Figure 2 illustrated that in the absence of infection treatment, compared with the control group ($FeSO_4$ group), there were no significant differences in blood indices such as RBC, HGB, HCT, MCV, MCH, MCHC, serum iron, UIBC, TIBC, and TF (Figures 2A–J). However, in the Fe-Gly-added group, splenic

TABLE 5 Effects of added Fe-Gly on piglet performance in 1–24 d.

Items	Treatment				*p*-values		
	100 mg $FeSO_4$	50 mg Fe-Gly	100 mg $FeSO_4$ + ETEC	50 mg Fe-Gly + ETEC	P_1	P_2	P_3
Initial BW Kg	7.03 ± 0.23	7.24 ± 0.19	7.06 ± 0.16	6.98 ± 0.18	0.729	0.559	0.445
24d BW Kg	11.67 ± 0.68	13.19 ± 0.97	11.67 ± 0.69	13.4 ± 0.97	0.064	0.905	0.898
22d–24d (Post challenge)							
ADFI (g/d)	386.4 ± 36.4	501.6 ± 94.6	390.9 ± 41.9	494.5 ± 55.4	0.086	0.983	0.926
ADG (g/d)	267.5 ± 64.9	301.9 ± 64.1	273.1 ± 43.4	351.3 ± 29.8	0.367	0.658	0.724
Diarrhea rate %	0.00 ± 0.00^b	0.00 ± 0.00^b	31.25 ± 13.15^a	18.75 ± 13.15^{ab}	0.481	0.010	0.481
Diarrhea index	0.00 ± 0.00^b	0.00 ± 0.00^b	0.88 ± 0.39^a	0.50 ± 0.34^{ab}	0.446	0.010	0.446

P_1 is the *P*-value comparing the addition of $FeSO_4$ with Fe-Gly, indicating the effect produced by the addition of different iron sources; P_2 is the *p*-value comparing the ETEC infected group with the uninfected group, indicating the effect produced by the infection by ETEC. P_3 is the *p*-value comparing the effect produced by the infection by ETEC in the interaction of the infection by ETEC with the different iron sources. The following is the same.

TABLE 6 Effect of Fe-Gly addition on piglet organ indexes.

Items	Treatment				*p*-values		
	100 mg $FeSO_4$	50 mg Fe-Gly	100 mg $FeSO_4$ + ETEC	50 mg Fe-Gly + ETEC	P_1	P_2	P_3
Heart index (%)	0.49 ± 0.02	0.49 ± 0.04	0.54 ± 0.02	0.53 ± 0.02	0.714	0.093	0.952
Liver index (%)	2.76 ± 0.11	2.58 ± 0.08	2.51 ± 0.14	2.6 ± 0.14	0.701	0.351	0.269
Spleen index (%)	0.21 ± 0.02^b	0.28 ± 0.03^a	0.21 ± 0.01^b	0.22 ± 0.02^b	0.051	0.091	0.114
Lung index (%)	1.26 ± 0.06	1.19 ± 0.02	1.24 ± 0.05	1.29 ± 0.03	0.794	0.379	0.154
Kindey index (%)	0.50 ± 0.02	0.55 ± 0.01	0.49 ± 0.03	0.52 ± 0.02	0.098	0.483	0.645

In the row for spleen index, different lowercase letters indicate significant differences between the treatment groups ($P < 0.05$).

$CD71^+TER119^-$ cells were significantly increased, and $CD71^-TER119^+$ cells were significantly reduced ($p < 0.05$, Figures 2K,L). After the infection, UIBC was significantly higher in the group with 50 mg of Fe-Gly ($p < 0.05$), whereas there was no significant change in the group with 100 mg of $FeSO_4$ (Figure 2H). In the Fe-Gly group, there was a significant increase in splenic $CD71^-TER119^+$ cells ($p < 0.05$, Figure 2H).

3.5 Effect of adding Fe-Gly on the iron content of various tissues in piglets

Figure 3 revealed that the addition of Fe-Gly decreased the iron content in the liver and kidney compared with the control group, while there was no significant effect on the iron content in other tissues. In the toxicity group, the addition of Fe-Gly can increase the iron content in the duodenum, jejunum, ileum and colon to a certain extent, indicating that the addition of Fe-Gly in the ration can alleviate the damage caused by ETEC, and still effective intake of iron sources to meet the body's needs.

3.6 Expression of genes related to duodenal iron metabolism

Figure 4 demonstrated that in the absence of infection treatment, compared with the control group (the group with added $FeSO_4$), in the group with added Fe-Gly, the expression of duodenal *DMT1* gene tended to be elevated ($p = 0.08$, Figure 4A), while the rest of the genes did not show significant differences (Figures 4A–F). After the infected treatment, the expression of duodenal *TFR1* gene tended to decrease by the addition of $FeSO_4$ group ($p = 0.08$, Figure 4B); the addition of Fe-Gly group showed a significant decrease of duodenal *DMT1* gene ($p < 0.01$, Figure 4A), the expression of *FPN* gene was significantly decreased ($p < 0.05$, Figure 4C), and the expression of *FTL and FTH* gene expression tended to decrease (Figures 5E,F). Under infected treatment, compared with the control group, the addition of Fe-Gly group showed a highly significant decrease in the expression of *FTL* of duodenal genes ($p < 0.001$, Figure 4E), and there was a tendency for a decrease in the expression of *FPN*, *HAMP*, and *FTH* genes, but there was no significant difference (Figures 4C,D,F).

3.7 Expression of genes related to hepatic iron metabolism

Figure 5 indicated that in the case of no infected treatment, compared with the control group (the group with added $FeSO_4$), in the group with added Fe-Gly, the expression of hepatic *FPN* gene was significantly elevated (Figure 5C), and the expression of *TFR1* gene tended to be elevated ($p = 0.063$, Figure 5B). After the infection, in the Fe-Gly addition group, the expression of hepatic *FTL* gene was significantly decreased ($p < 0.05$, Figure 5E), the expression of HAMP gene tended to decrease ($p = 0.050$, Figure 5D), and the expression of *FTH* gene had a tendency to decrease ($p = 0.051$, Figure 5F). In the $FeSO_4$ addition group, the expression of hepatic genes did not show

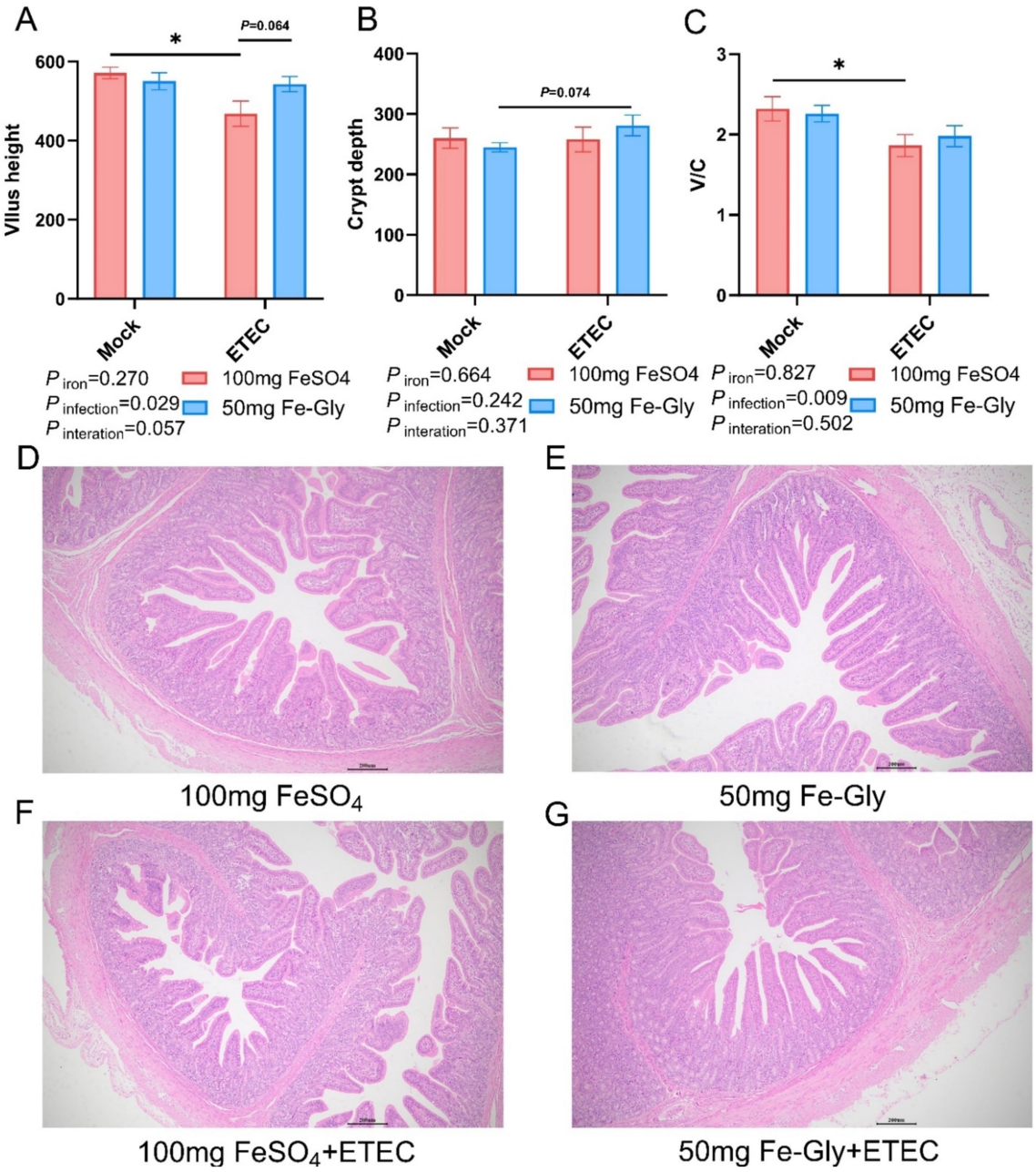

FIGURE 1
Effect of Fe-Gly addition on the structural morphology of jejunum in piglets. **(A)** Jejunal villus height, $n = 8$; **(B)** Jejunal crypt depth, $n = 8$; **(C)** Jejunal villus to crypt ratio, $n = 8$; **(D–G)** Jejunal pathological sections. Statistical significance is indicated as follows: $^*P < 0.05$.

any significant change in all groups (Figures 5A–F). These results suggest that the addition of Fe-Gly can partially modify the effect of ETEC infection on hepatic iron metabolism.

3.8 Expression of genes related to jejunal iron metabolism

Figure 6 revealed that the expression of jejunal *FPN* gene was significantly higher in the Fe-Gly group compared with the control group ($FeSO_4$ group) in the absence of infection ($p < 0.05$, Figure 6C). The addition of Fe-Gly group enhanced the expression of the jejunal *DMT1* and *HAMP* genes in the absence of infection compared with the control ($FeSO_4$ group) (Figures 6A,F); the expression of jejunal *FPN* gene was significantly lower in the 100 mg of $FeSO_4$ group after infected ($p < 0.05$, Figure 6C); the expression of jejunal *FPN* gene was significantly lower in the 100 mg of $FeSO_4$ group compared with the control ($FeSO_4$ group) in the presence of infected. After the addition of 100 mg of $FeSO_4$, the expression of jejunal *FPN* gene was significantly reduced ($p < 0.05$, Figure 6C). Under infection conditions, the addition of Fe-Gly group showed a tendency to reduce the expression of jejunal *FTL* gene compared with the control group ($p = 0.076$, Figure 6D). For the expression of jejunal *TFR1* and *FTH* genes, there were no significant differences among treatments (Figures 6B,E).

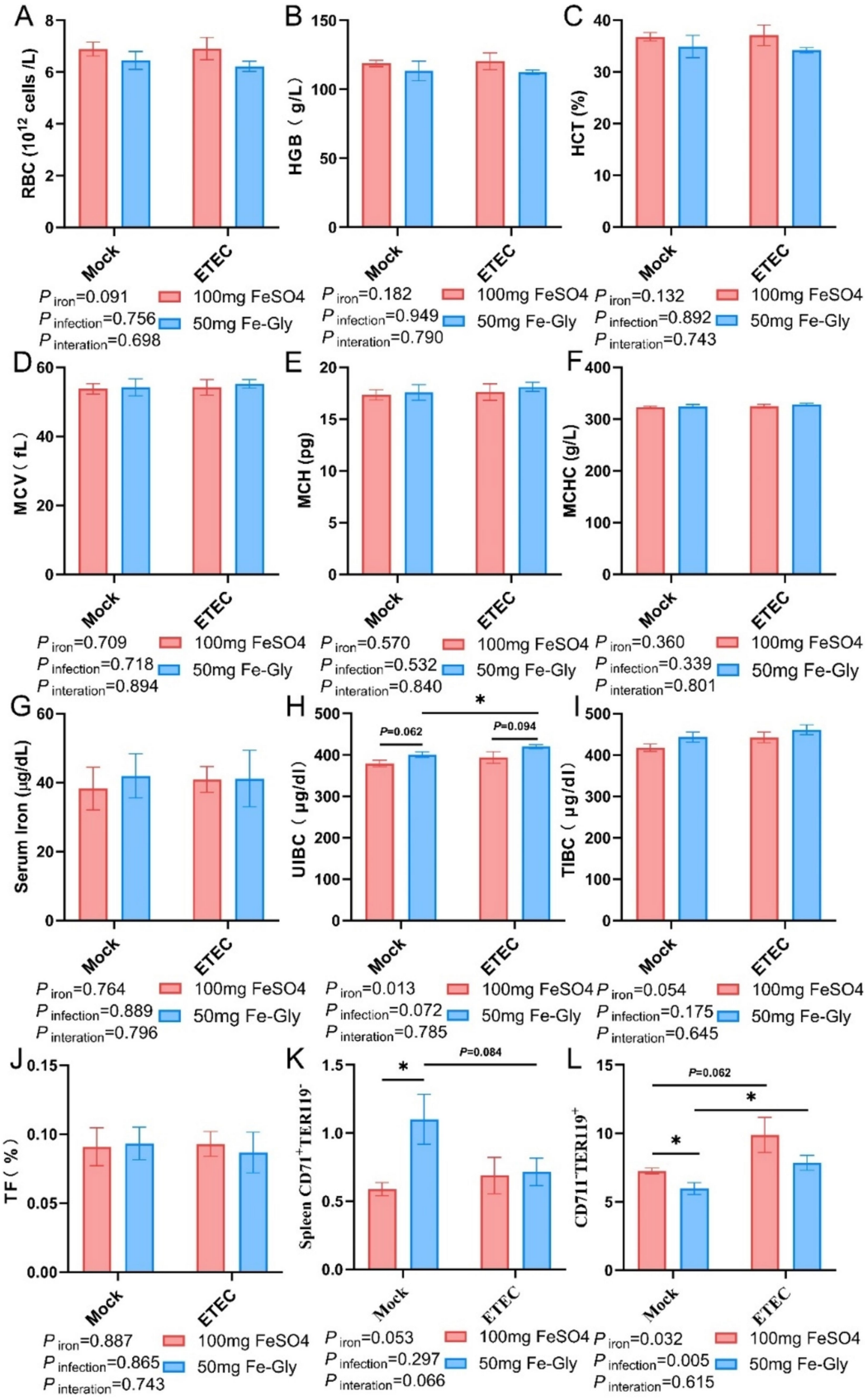

FIGURE 2
Effect of Fe-Gly addition on hematological and serum iron levels in piglets. **(A)** Erythrocyte number, n = 8; **(B)** Hemoglobin, n = 8; **(C)** Erythrocyte pressure area, n = 8; **(D)** Mean erythrocyte volume, n = 8; **(E)** Mean erythrocyte hemoglobin content, n = 8; **(F)** Mean erythrocyte hemoglobin concentration, n = 8; **(G)** Serum iron, n = 8; **(H)** Unsaturated iron-binding capacity, n = 8; **(I)** Total iron-binding capacity, n = 8; **(J)** Iron saturation, n = 8; **(K–L)** Erythroid Progenitor Cells in the spleen, n = 8. Statistical significance is indicated as follows: $^*P < 0.05$.

3.9 Expression of jejunal inflammatory factor-related genes

Figure 7 demonstrated that regarding the expression of jejunal *IL-6* gene, there was no significant difference between the treatment groups, but in both control and infected groups, the addition of Fe-Gly decreased the expression of jejunal *IL-6* gene compared to the addition of $FeSO_4$ (Figure 7A). After infection, the addition of $FeSO_4$ significantly increased in the expression of jejunal *IL-1β* gene ($p < 0.05$, Figure 7C); in the infected treated group, the addition of Fe-Gly significantly increased in the expression of jejunal *IL-1β* gene compared with the control group ($p < 0.05$, Figure 7C). These results suggest that the addition of 50 mg of Fe-Gly could alleviate the inflammatory response induced by ETEC infection on the organism to a certain extent.

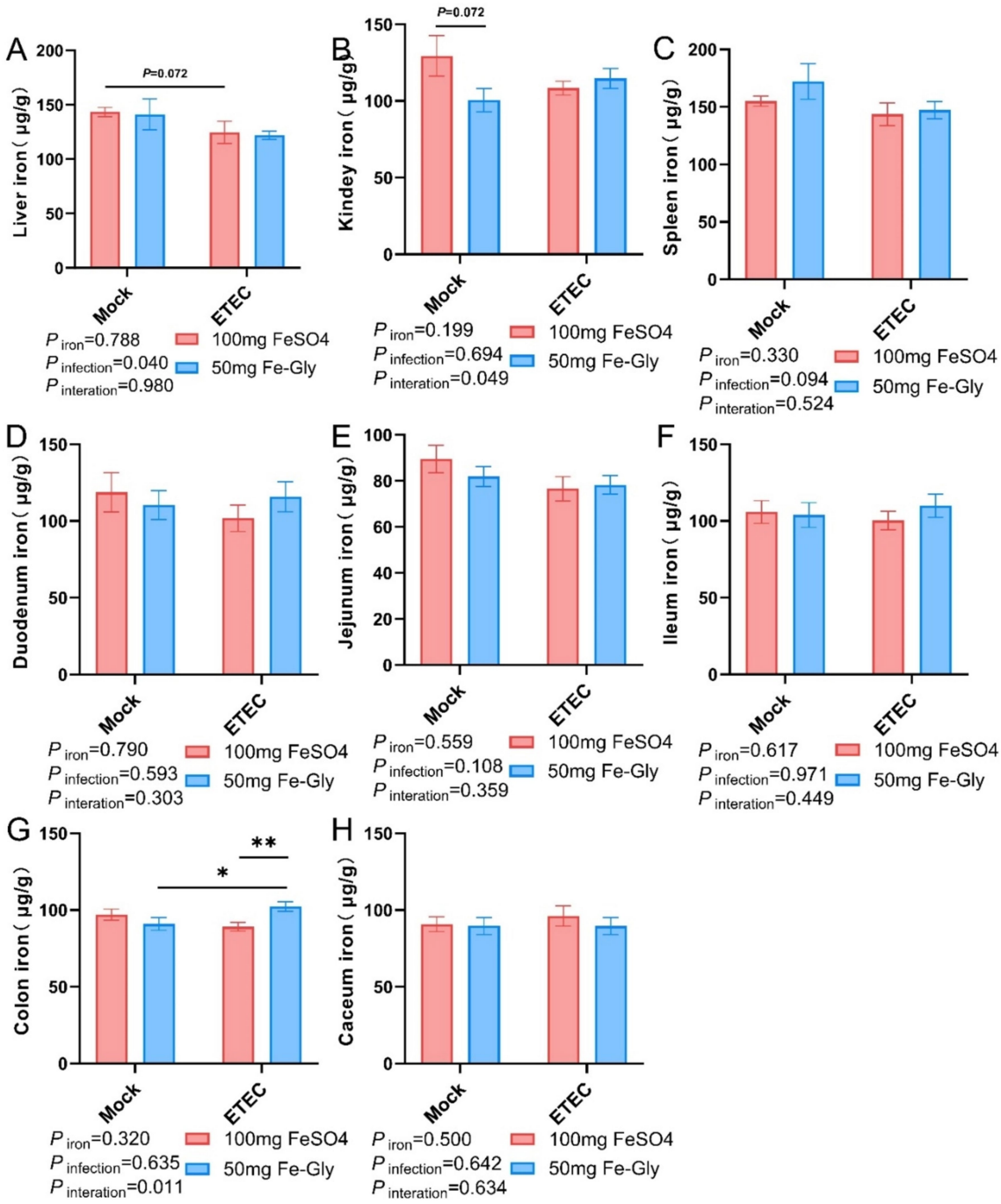

FIGURE 3
Effect of Fe-Gly addition on iron content of piglets in various tissues. **(A)** Hepatic non-heme iron levels, $n = 8$; **(B)** Renal non-heme iron levels, $n = 8$; **(C)** Splenic non-heme iron levels, $n = 8$; **(D)** Duodenal non-heme iron levels, $n = 8$; **(E)** Jejunal non-heme iron levels, $n = 8$; **(F)** Ileal non-heme iron levels, $n = 8$; **(G)** Colonic non-heme iron levels, $n = 8$; and **(H)** Cecum non-heme iron levels, $n = 8$. Statistical significance is indicated as follows: $^*P < 0.05$, $^{**}P < 0.01$.

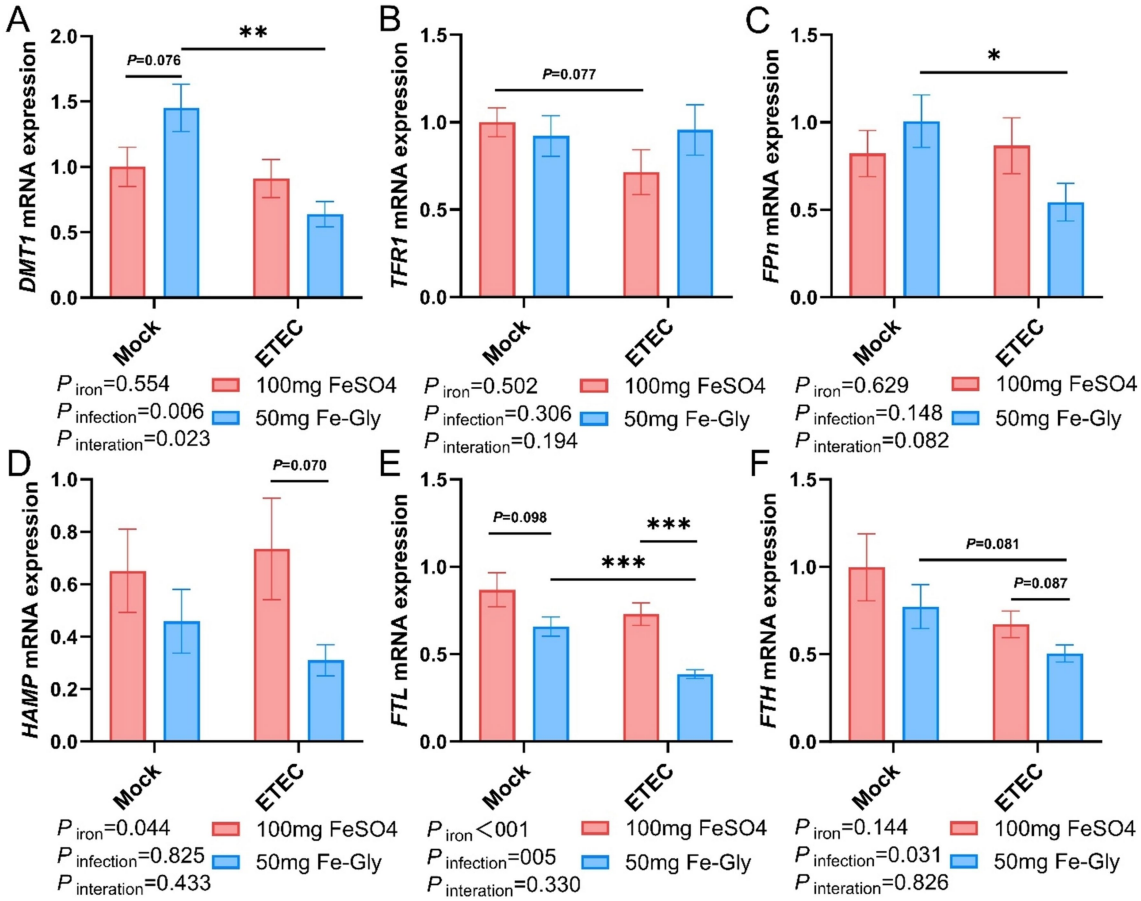

FIGURE 4
Effect of Fe-Gly addition on the expression of genes related to iron metabolism in the duodenum of piglets. **(A)** Divalent metal transporter 1 (*DMT1*), $n = 8$; **(B)** Transferrin receptor 1 (*TFR1*), $n = 8$; **(C)** Ferroportin (*Fpn*), $n = 8$; **(D)** Hepcidin antimicrobial peptide (*HAMP*), $n = 8$; **(E)** Ferritin light chain (*FTL*), $n = 8$; **(F)** Ferritin heavy chain (*FTH*), $n = 8$. Statistical significance is indicated as follows: $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

4 Discussion

The weaning stage of piglets is often accompanied by changes in the environment and dietary structure, leading to a series of stress reactions (14). Sow milk has low iron content, and piglets can obtain less iron from their mother. Additionally, the liver iron storage of newborn piglets is low, which cannot meet the demand for rapid growth during the early stage (15). Therefore, exogenous iron supplementation is usually performed for newborn piglets, while adding easily absorbed iron to the feed is the primary method to prevent iron deficiency in weaned piglets (16, 17). Li Jiangtao et al. found that the effect of adding 50 mg/kg of Fe-Gly to the diet was better than that of adding 150 mg/kg of $FeSO_4$ in terms of AGD, ADFI, and F/G, with a decreasing trend observed as the amount of Fe-Gly increased (18). Feng et al. reported that the addition of Fe-Gly at a level of 90 mg/kg in diets was superior than the addition of 120 mg/Kg of $FeSO_4$ (9). Ma et al. demonstrated that Fe-Gly in the diet significantly increased ADFI and ADG while significantly reducing early diarrhea in piglets (19). Sun Limei et al. found that the supplementation of small peptide chelated iron in the ration could increase iron storage in animals and improve intestinal barrier function and immune function (20). We obtained similar results regardless of whether ETEC infection was present or absent. In this study, the addition of 50 mg of Fe-Gly significantly increased ADFI and ADG, showing no difference compared to the 100 mg/kg $FeSO_4$ addition group (Table 4). The inclusion of Fe-Gly in the ration improved piglet growth performance and reduced the incidence of diarrhea, which is completely consistent with previous research. Furthermore, the addition of 100 mg of $FeSO_4$ and 50 mg of Fe-Gly to the diets had no significant effect on iron deposition in the tissues (Figure 3). These results further demonstrate that adding Fe-Gly to the diet can effectively enhance the growth performance of weaned piglets. In addition, the high bioavailability of Fe-Gly can significantly reduce iron excretion, thereby decreasing environmental pollution.

Iron in food is primarily absorbed into intestinal cells in the duodenum via DMT1, and then pumped into the bloodstream by *FPn*, where it is transported to the liver for storage (21). Our research found that the addition of 50 mg of Fe-Gly to the feed significantly increased the gene expression of *DMT1* in the duodenum under non-infected conditions (Figure 4). This result indicates that Fe-Gly is more easily absorbed in the body compared to $FeSO_4$, and the increase in serum iron and TIBC further supports this notion (Figure 2). This is consistent with previous reports that the absorption efficiency of Fe-Gly in the intestine is significantly higher than that of inorganic iron (19). This explains why the addition of 50 mg/kg of Fe-Gly can achieve effects equivalent to

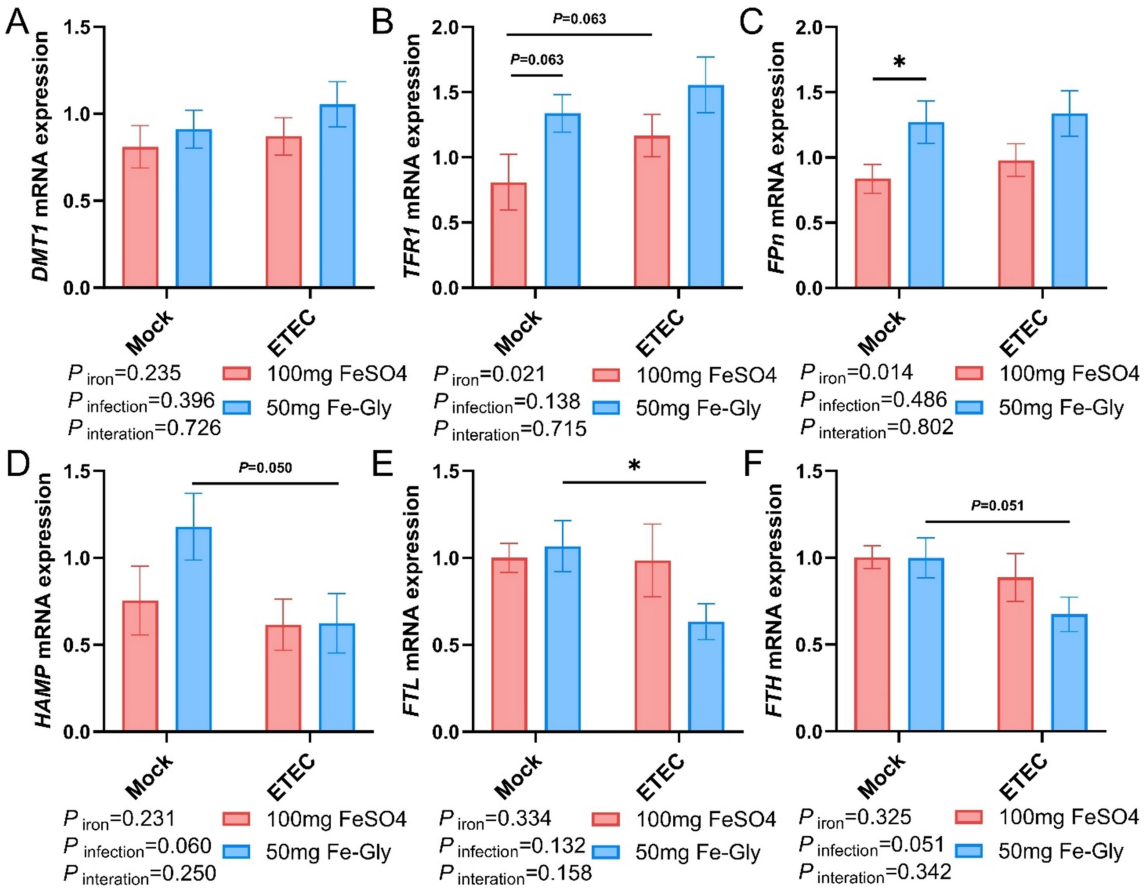

FIGURE 5
Effect of Fe-Gly addition on the expression of iron metabolism-related genes in piglets' liver. **(A)** Divalent metal transporter 1 (*DMT1*), $n = 8$; **(B)** Transferrin receptor 1 (*TFR1*), $n = 8$; **(C)** Ferroportin (*Fpn*), $n = 8$; **(D)** Hepcidin antimicrobial peptide (*HAMP*), $n = 8$; **(E)** Ferritin light chain (*FTL*), $n = 8$; **(F)** Ferritin heavy chain (*FTH*), $n = 8$. Statistical significance is indicated as follows: $^*P < 0.05$.

100 mg/kg of $FeSO_4$, and even better results. Typically, unabsorbed iron in the duodenum enters the hindgut along with the chyme (22). Since microorganisms have a much greater capacity to utilize iron than the host itself, the accumulation of iron in the hindgut will significantly reduce the abundance of harmful bacteria while promoting the growth of pathogenic bacteria, thereby increasing intestinal inflammation (23, 24). For example, in ETEC, siderophores are transported across the double membrane envelope via a gating mechanism that links the inner and outer membranes. This mechanism ensures that siderophores can effectively capture iron from the external environment and transport it into the cell to meet the physiological needs of the cell. In this way, bacteria can survive and reproduce under conditions of iron scarcity, while also demonstrating the complex adaptive mechanisms of microorganisms in iron acquisition (23). Additionally, this can also enhance the infection of pathogenic microorganisms, leading to serious diarrhea in piglets (25). The results of this study found that, the addition of 50 mg of Fe-Gly was effective in decreasing the levels of iron in the cecum and colonic. Notably, compared to the control group, ETEC infection significantly increased the incidence of diarrhea in piglets and proinflammatory cytokine *IL-1β* gene expression of jejunum (Figure 7). In contrast to the $FeSO_4$ addition group, the feed supplemented with Fe-Gly effectively reduced the incidence of diarrhea in piglets and *IL-1β* gene expression of jejunum. This demonstrates that adding a lower dose of Fe-Gly to the feed can effectively alleviate diarrhea in piglets and intestinal inflammation caused by ETEC infection without affecting their growth performance. This may be related to the reduced dosage of Fe-Gly, the lower iron content in the intestine, and the high absorption efficiency of Fe-Gly.

The intestinal tract is the most important digestive organ in the organism, playing a crucial role in maintaining the homeostasis of the organism (26). Among them, the morphology and structure of the small intestine, as well as its functional integrity, play an important role in the maintain the health of the animal organism (27). Intestinal villi in the small intestine's wrinkled wall greatly increase the area for nutrient absorption, and their height and health determine the efficiency of digestion and absorption in animals (28). Increased villus height indicates stronger absorption function, while decreased height signifies fewer absorption-related cells, weakening this function (29). Previous studies have shown that ETEC infection in weaned piglets can lead to crumpling of small intestinal villi, a decrease in villus height, a significant increase in crypt depth, and changes in intestinal morphology, which in turn affect the body's nutrient absorption efficiency (30, 31). Studies have found that Fe-Gly can improve gut barrier

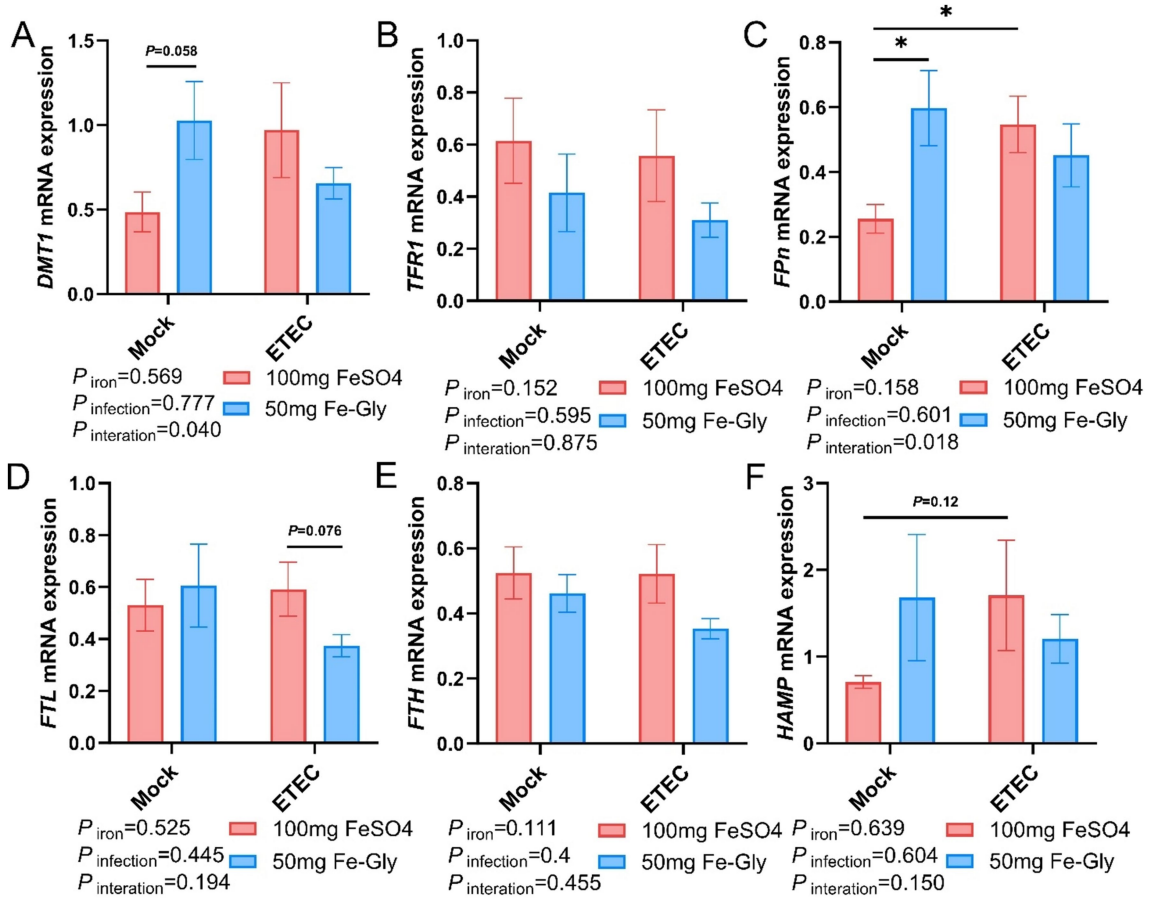

FIGURE 6
Effect of Fe-Gly addition on the expression of genes related to iron metabolism in the jejunum of piglets. **(A)** Divalent metal transporter 1 (*DMT1*), $n = 8$; **(B)** Transferrin receptor 1 (*TFR1*), $n = 8$; **(C)** Ferroportin (*Fpn*), $n = 8$; $n = 8$; **(D)** Ferritin light chain (*FTL*), $n = 8$; **(E)** Ferritin heavy chain (*FTH*), $n = 8$, **(F)** Hepcidin antimicrobial peptide (*HAMP*). Statistical significance is indicated as follows: $^*P < 0.05$.

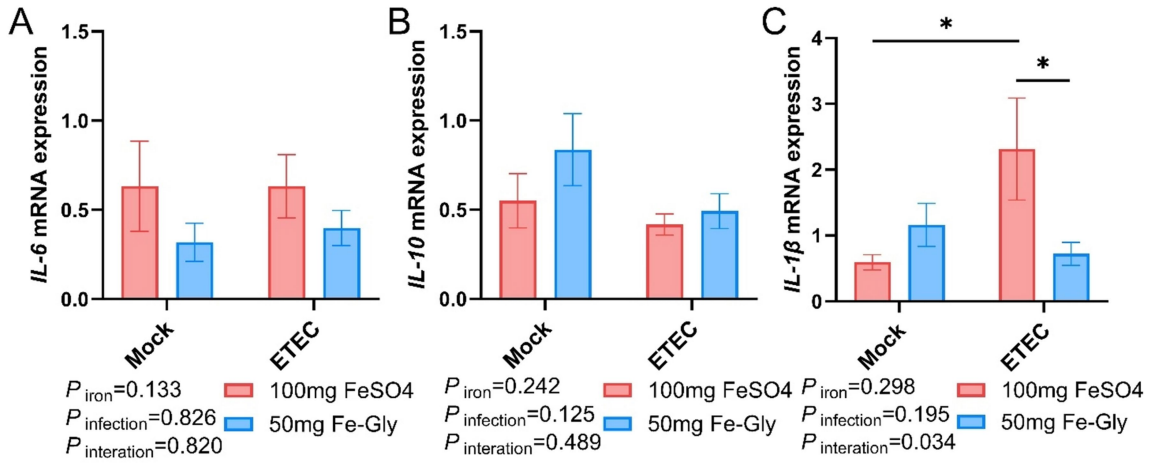

FIGURE 7
Effect of Fe-Gly addition on jejunal inflammation gene expression in piglets. **(A)** Interleukin 6 (*IL-6*), $n = 8$; **(B)** Interleukin 10 (*IL-10*), $n = 8$; **(C)** Interleukin 1β (*IL-1β*), $n = 8$. Statistical significance is indicated as follows: $^*P < 0.05$.

function by regulating the composition of the gut microbiome, increasing the relative abundance of beneficial bacteria, and decreasing the relative abundance of potentially pathogenic bacteria (32). After ETEC infection, piglets in the $FeSO_4$ group showed a significant decrease in villus height and no significant change in crypt depth, resulting in a reduced villus-crypt ratio. In contrast,

there were no significant changes in villus height and crypt depth in the Fe-Gly group. This may be due to the low dosage of Fe-Gly added, which does not produce ionic iron during the absorption process. Consequently, absorption efficiency was significantly improved, reducing the iron available to the intestinal microorganisms, inhibiting the proliferation of ETEC, and thereby mitigating intestinal damage and promoting intestinal integrity of the piglets.

5 Conclusion

Adding 50 mg Fe-Gly to the diet can significantly increase ADFI and ADG, thereby promoting piglet performance to a certain extent and alleviating issues related to piglet diarrhea and reduced performance caused by ETEC infection. Furthermore, the addition of Fe-Gly can also increase the level of TIBC in the serum of the piglets, enhancing their ability to bind and transport iron. Fe-Gly can also mitigate the damage to the jejunal segment caused by ETEC infection to some extent. In summary, adding 50 mg of Fe-Gly not only meets the daily iron requirements of piglets but also improves the efficiency of iron utilization. This addition reduces residual pathogenic ETEC iron in the intestine, thereby decreasing the availability of iron to pathogenic microorganisms, inhibiting the proliferation of intestinal pathogens, and protecting the intestinal health of piglets. Additionally, it can significantly reduce environmental pollution. By minimizing the sources of unutilized iron, the use of Fe-Gly helps to lessen the negative impact on the environment and promotes sustainable farming practices.

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 approved by the Animal Care and Use Committee of Sichuan Agricultural University (Chengdu, China, No. 20210111). The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

QG: Data curation, Writing – original draft, Formal analysis, Investigation, Methodology. YZ: Data curation, Formal analysis, Investigation, Methodology, Writing – original draft. YW: Resources, Writing – original draft. DG: Resources, Writing – original draft. JC: Investigation, Writing – original draft. CY: Investigation, Writing – original draft. HW: Methodology, Writing – original draft. DZ: Supervision, Writing – original draft. DC: Conceptualization, Funding acquisition, Methodology, Supervision, Validation, Writing – original draft, Writing – review & editing. AW: Conceptualization, Funding acquisition, Methodology, Supervision, Validation, Writing – review & editing.

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Conflict of interest

QG, YW, and DG were employed by Hunan Debon Biotechnology Co., Ltd. HW and DZ were employed by Tongwei Agricultural Development 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.

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The active roles of *Rhodotorula mucilaginosa* ZTHY2 in regulating antioxidant capacity and immune function of Leizhou black ducks

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Previous studies in mice have demonstrated that *Rhodotorula mucilaginosa* ZTHY2 can promote animal growth, enhance antioxidant and immune functions, and regulate intestinal flora in our laboratory. This study focuses on the Leizhou black duck, a local breed in Zhanjiang, to evaluate the effects of *Rhodotorula mucilaginosa* ZTHY2 on its growth, antioxidant capacity, and immune function. A total of 150 1-day-old male Leizhou black ducks, of similar size and healthy, were selected for this study and randomly assigned to five treatment groups. Each group contained three replicates with ten birds each. The control group (Control) was given a standard basal diet, while the RM group received a diet supplemented with ZTHY2 at concentrations of 2×10^7 (RM1), 2×10^8(RM2), or 2×10^9(RM3) CFU/kg, respectively. The LA group was supplemented with 2×10^9 CFU/kg of *Lactobacillus acidophilus* in addition to the basal diet. The feeding trial lasted 42 days. The analysis revealed significant improvements in the average body weight for the RM2 and RM3 groups, which were significantly higher than that of the control group ($p < 0.05$ and $p < 0.01$). Treatment with ZTHY2 induced a dose-dependent elevation in superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GSH-Px), and SOD activities, and a reduction in malondialdehyde (MDA) content in the serum at 42 days. The serum levels of complement components C3 and C4, immunoglobulin IgG, and cytokines IFN-γ, IL-2, IL-4, IL-6, and TNF-α were significantly increased in Leizhou black ducks treated with ZTHY2 at 42-days post-treatment, with the therapeutic effect becoming more pronounced as the duration of the experiment prolonged. The greatest impact was observed at a dosage of 2×10^9 CFU/kg of ZTHY2. Moreover, ZTHY2 modulated the mRNA expression profiles of these cytokines in the thymus, spleen, and bursa, thereby sustaining the balance of immune dynamics. In summary, the supplementation of *Rhodotorula mucilaginosa* ZTHY2 at a dosage of 2×10^9 CFU/kg had been found to most effectively enhance the growth performance of Leizhou black ducks by optimizing their immune function and antioxidant capacity.

KEYWORDS

***Rhodotorula mucilaginosa* ZTHY2, Leizhou black duck, antioxidant capacity, immune function, growth performance**

1 Introduction

Oxidative stress is a subject of much attention in the field of immunology because it is closely linked to the decline of immune organ and immune cell function (1, 2). Under physiological conditions, a moderate increase in the level of oxidative stress can act as a signal to activate immune cells, promote their proliferation and differentiation, and thus enhance the body's immune response to pathogens. The activation effect of moderate oxidative stress on the immune system reflects the "double-edged sword" characteristic of redox balance in organisms, that is, it is beneficial to the body within a certain range, but may turn into harmful factors beyond a certain limit (3). However, when the level of oxidative stress exceeds the body's physiological regulatory capacity, that is, when it exceeds a certain threshold, it will begin to have a negative impact on the immune system and weaken immune defenses (4). Under physiological conditions, a moderate increase in the level of oxidative stress can act as a signal to activate immune cells, promote their proliferation and differentiation, and thus enhance the body's immune response to pathogens. The activation effect of moderate oxidative stress on the immune system reflects the "double-edged sword" characteristic of redox balance in organisms, that is, it is beneficial to the body within a certain range, but may turn into harmful factors beyond a certain limit. However, when the level of oxidative stress exceeds the body's physiological regulatory capacity, that is, when it exceeds a certain threshold, it will begin to have a negative impact on the immune system and weaken immune defenses (3, 5). Therefore, maintaining a delicate balance between oxidative stress and antioxidant mechanisms is essential to maintaining the integrity and effectiveness of the immune system. This requires the body's antioxidant system, including enzymatic (such as superoxide dismutase, glutathione peroxidase, etc.) and non-enzymatic (such as vitamin C, glutathione, etc.) antioxidants, to effectively remove excess ROS and protect immune cells from oxidative damage (6, 7). Proper supplementation of antioxidants can help maintain this balance, thereby maintaining and enhancing the body's immune function.

Lactobacillus acidophilus and other probiotics such as *Bacillus subtilis* and Marine red yeast show great potential in the field of animal nutrition and health. They can not only enhance the immune response of animals, but also improve intestinal health and provide a solid foundation for the growth and development of animals (8, 9). With further research into the function of these probiotics, we are expected to discover more new applications that will benefit animal health and production. Currently, *Lactobacillus acidophilus* was identified as a probiotic in the Catalog of Feed Additives (2013) published by the Ministry of Agriculture and Rural Affairs of China. Extensive experimental data had shown that the incorporation of a *Lactobacillus acidophilus* bacterial preparation into the diet significantly enhances the integrity of the intestinal epithelial barrier, maintains the balance of the immune system, and increases the diversity of the intestinal microbiota in laying hens (9). A recent investigation demonstrated that the incorporation of 0.5g/kg of *Bacillus subtilis* into the dietary regimen of laying hens effectively suppressed intestinal inflammation, enhanced antioxidant status, and maintained duodenal barrier integrity (10). Marine red yeast is a kind of microorganism that exists naturally in the marine environment and has been widely used in animal production in recent years due to its rich nutritional value and immune-enhancing effect. Marine red yeast is rich in protein, amino acid, unsaturated fatty acid, astaxanthin, beta-carotene, polysaccharide and other nutrients (11, 12). These ingredients not only provide animals with essential nutrients, but also have the potential to enhance the body's immune function. Adding marine red yeast to laying hens' feed can improve laying performance by regulating small intestine homeostasis (8). This suggests that Marine *rhodoyeast* may also play an important role in poultry production.

Rhodotorula mucilaginosa belongs to the genus *Rhodotorula*. It has strong stress resistance, can survive in a variety of environments, and has chemoheterotrophic, thermophilic, acidophilic, facultative anaerobic characteristics (11, 13, 14). The metabolites, such as astaxanthin and polysaccharides, produced during the growth of *Rhodotorula mucilaginosa*, have the ability to regulate the expression of antioxidant enzymes and remove oxygen free radicals, showing remarkable antioxidant properties (13–15). Our early research has confirmed that *Rhodotorula mucilaginosa* ZTHY2 is safe, residual-free, and capable of producing a variety of nutritionally beneficial metabolites. These metabolites are able to promote mice growth, enhance antioxidant and immune system function, and reshape the gut microbiota, showing their potential applications in the field of animal nutrition and health; and gavage with *R. mucilaginosa* ZTHY2 at the concentration of 2×10^8 CFU/mL or 2×10^9 CFU/mL could effectively increase the content of GSH, SOD, and CAT in immunosuppressed mice, remove excess ROS and reduce MDA (11). When co-cultured with other yeasts, the antioxidant properties of *Dendrobium kumungensis* could be significantly enhanced, and the immunomodulatory effects of macrophages could be enhanced by increasing the contents of phenols, flavonoids and B vitamins (16). Given the above properties of *Rhodotorula mucilaginosa*, it has the potential to replace antibiotics for feeding purposes. As a non-toxic, residual-free and drug-resistant microbe, it can be used as a natural feed additive to improve digestibility and gut health of animals while regulating the gut microbiota. With the further research and development of the characteristics of this microorganism, its application in the future will be more extensive.

In the field of probiotic research, *Rhodotorula mucilaginosa*, as one of them, has shown remarkable effects in regulating the body's antioxidant and immune function. Studies have shown that the bacteria can protect the body from oxidative stress by enhancing the activity of antioxidant enzymes and reducing the production of free radicals. Polysaccharide EPS-1, which was extracted from the fermentation broth of *Bifidobacterium breva*, is a kind of polysaccharide rich in mannose, and its unique structural characteristics give it immunomodulatory activity. Studies have shown that EPS-1 can activate macrophages and enhance their ability to engulf and kill pathogens, thus playing a key role in the immune response (17). In aquaculture, *Rhodosaccharomyces vulgaris* as a feed additive has been shown to significantly enhance the immune response of tilapia. By adding 0.5 to 1% of *Rhodiomyces vulgaris* in the feed, it can improve the resistance of fish to disease and reduce the occurrence of disease, thereby improving the efficiency and economic benefits of breeding (18). Feeding experiments with *saccharomyces* extract showed that serum levels of key immune indicators such as immunoglobulin, IL-2 and IFN-γ were significantly increased in experimental animals. These results show that *saccharomyces* extract can effectively enhance the humoral and cellular immune response of experimental animals and improve their defense against pathogens (19). The study by Liu et al. further confirms the potential of marine

rhodosaccharomyces rubra for use in animal nutrition and health. By adding 0.1–0.5% marine red yeast to lamb milk replacer, the intestinal immunity of lambs can be significantly enhanced, the incidence of diarrhea in early weaned lambs can be reduced, and the antioxidant capacity of serum can be increased (12). These results provide a strong scientific basis for the use of Marine *rhodotorula* as a natural and safe feed additive. In conclusion, the study of probiotics not only reveals their important role in enhancing the host immune response, but also provides new ideas for the development of novel feed additives and alternative antibiotics.

The magnitude of China's meat duck industry was pivotal within the international market landscape (20). The Leizhou Black Duck possesses attributes such as succulent meat, high egg-laying capacity, and resilience to coarse feed, among others, and it represented a breed of duck with distinct local characteristics that was suitable for dual-purpose egg and meat production (21, 22). In the current context of increasing concern over antibiotic resistance (23), the development of natural substances with antioxidant properties as alternatives to antibiotics had become a global research hotspot. These natural antioxidants not only inhibit oxidation reactions and reduce the generation of free radicals but also enhance animal immune systems through various mechanisms, thereby promoting animal health and growth without the use of antibiotics. *Rhodotorula mucilaginosa* ZTHY2 possesses probiotic attributes, as a natural probiotic, it exhibited considerable potential in replacing conventional antibiotic additives within animal feed formulations (11). By maintaining the dynamic balance of immune function, ZTHY2 can improve animal immunity and enhance antioxidant function, which suggests that ZTHY2 has a good application potential in livestock production.

In this study, Leizhou black duck with local characteristics in Zhanjiang city was selected as the research object, the effects of *Rhodotorula mucilaginosa* ZTHY2 were explored on the growth performance, antioxidant capacity and immune function of the ducks. The results showed that ZTHY2 promoted the regulation of immune function and antioxidant capacity of Leizhou black ducks, and improved the growth performance of the ducks, with the best effect when the supplemental level was 2×10^9 CFU/kg. The objective was to illuminate the potential roles of ZTHY2 as a substitute feed additive and to explore additional strategies for bolstering animal immunity and enhancing breeding efficiency.

2 Materials and methods

2.1 Experimental strains of bacteria

The bacterium *Rhodotorula mucilaginosa* ZTHY2 was isolated and purified from the marine environment of the Leizhou Peninsula in China. It was currently preserved at the Chinese Typical Culture Preservation Center, with the accession number M2015296. *Lactobacillus acidophilus* (BNCC185342) was acquired from Beina Biotechnology Co., LTD. China.

2.2 Experimental animals and group design

A total of 150 healthy, similarly sized, 1-day-old male Leizhou black ducks were selected and randomly assigned to five treatment groups, each with three replicates and 10 birds per replicate. The hens in the control group were provided with a standard basal diet, whereas the RM groups received the basal diet supplemented with 2×10^7 CFU/kg, 2×10^8 CFU/kg, or 2×10^9 CFU/kg of *Rhodotorula mucilaginosa* ZTHY2, respectively, named as Control (Control group), RM1 (2×10^7 CFU/kg RM group), RM2 (2×10^8 CFU/kg RM group), and RM3 (2×10^9 CFU/kg RM group). Add dosage and quality control, refer to our previous research report in mice (The Rl, Rm, and Rh groups were treated with 0.5 mL of *R. mucilaginosa* ZTHY2 with concentrations of 2×10^7 CFU/mL, 2×10^8 CFU/mL, and 2×10^9 CFU/mL, respectively) (11). Additionally, the LA group was fed a diet supplemented with 2×10^9 CFU/kg of *Lactobacillus acidophilus*, named LA (2×10^9 CFU/kg LA group). The feeding trial lasted for a duration of 42 days. All experimental protocols were approved by the Animal Ethics Committee of Guangdong Ocean University, China (IACUC No. GDOU-LAE-2020-007). The experimental feeding method was indoor flat feeding with mixed materials. Clean the house regularly and keep ventilation to observe the health status of Leizhou black ducks.

2.3 Feeding management and basic feed

Prior to the trial, the duck housing and utensils underwent a complete cleaning and disinfection process. After proper ventilation and purification, the experiment began. It employed an indoor, horizontal feeding system with a varied diet. The Leizhou black ducks had continuous access to feed and water, while the facility's temperature, humidity, and lighting were carefully controlled. Regular cleaning maintained good ventilation. The ducks' health was continually monitored, with a focus on the drinking water quality. Timely feed and water supply were ensured, with detailed records kept. The feeding and vaccination protocols followed standard industry practices. The basal feed met the requirements of the Meat Duck Feeding Standard (NY/T 2122–2012), and its composition and nutritional level were detailed in Table 1.

2.4 Sample collection

On days 21 and 42, two Leizhou black ducks with similar body weights from each group were randomly chosen. After a 12-h fast, blood was drawn from the subclavian vein. Then, 10 mL of blood was allowed to clot at room temperature and centrifuged at 4°C, 3000 rpm for 15 min to separate the serum. This serum was stored at −20°C for later analysis. Following this, the ducks were euthanized, and their thymus, spleen, and bursa were removed and washed with sterile PBS. They were then flash-frozen in liquid nitrogen and stored at −80°C until examination. Each intestinal segment was carefully dissected, and the tissue was prepared for analysis.

2.5 Serum antioxidant index

The serum activities of catalase (CAT), superoxide dismutase (SOD), glutathione peroxidase (GSH-Px), and malondialdehyde (MDA) were quantitatively determined in Lezhou black ducks at 21 and 42 days using commercial enzyme-linked immunosorbent assay

TABLE 1 Dietary composition and nutrient concentrations of basal diets (air-dry basis) in per-centages.

Items	1–21 days	22–42 days
Ingredients		
Corn	61.85	64.60
Soybean meal	28.50	25.30
Wheat bran	3.25	3.14
Fish meal	2.50	2.80
NaCl	0.30	0.30
Limestone	1.30	1.29
$CaHPO_4$	1.28	1.26
DL-Met	0.19	0.21
Premix*	1.00	1.10
Total	100.00	100.00
Nutrition levels		
ME/(MJ/kg)	12.24	12.35
CP	20.70	18.60
Ca	0.98	0.93
AP	0.45	0.40
Lys	1.10	0.86
Met	0.50	0.45

*The dietary premix supplies the following nutritional components per kilogram of starter: Vitamin A, 8,000 International Units (IU); Vitamin D, 2,000 IU; Vitamin E, 20 IU; Vitamin B12, 12 milligrams (mg); Vitamin K3, 2 mg; nicotinic acid, 40 mg; D-pantothenic acid, 15 mg; folic acid, 1 mg; iodine, 0.40 mg; iron, 60 mg; copper, 8 mg; zinc, 60 mg; manganese, 100 mg; and selenium, 0.35 mg.

(ELISA) kits as our previous research report (11) (CAT Cat.BC0200, SOD Cat.BC0170, Solarbio Life Sciences, Beijing, China; GSH-Px Cat. A005-1-1, Nanjing Jiancheng Bioengineering Institute, Nanjing, China; MDA Cat.S0131S, Beyotime, Shanghai, China), in accordance with the manufacturer's protocols.

2.6 Complement and immunoglobulin content determination

The levels of complement component 3 (C3), complement component 4 (C4), and immunoglobulin G (IgG) in serum were quantitatively determined at 21- and 42-days post-immunization using ELISA (Duck C3 Cat.QS48326, C4 Cat.QS48328, IgG Cat.QS48162, Beijing Qisong Biotechnology Co., LTD, Beijing, China), with all procedures conducted in accordance with the manufacturer's protocols.

2.7 Serum cytokine content was determined

The serum concentrations of tumor necrosis factor α (TNF-α), interferon-γ (IFN-γ), interleukin-2 (IL-2), interleukin-4 (IL-4), and interleukin-6 (IL-6) were quantitatively determined using ELISA (Duck TNF-α Cat.QS49067, IFN-γ Cat.QS42217, IL-2 Cat.QS42220, IL-4 Cat.QS42219, IL-6 Cat.QS42215, Beijing Qisong Biotechnology Co., LTD, Beijing, China).

2.8 Analysis of gene expression in immune organs

The HiScript III RT SuperMix for qPCR (Vazyme Biotech Co.,Ltd. Nanjing, China), including gDNA wiper, was used for cDNA synthesis from total RNA. The resulting cDNA served as the template for real-time PCR using the ChamQ SYBR qPCR Master Mix (Vazyme Biotech Co.,Ltd. Nanjing, China) as our previous research report (11). β-actin was employed as the housekeeping gene for relative quantification of TNF-α, IFN-γ, IL-2, IL-4, IL-6, SOD, GSH-Px, and CAT. Primers were designed and synthesized by Shanghai Shenggong based on GenBank sequences; their sequences were listed in Table 2.

The optimized PCR reaction mixture contains 20 μL of reaction solution, composed of 10 μL 2× ChamQ Universal SYBR qPCR Master Mix, 0.4 μL of each forward and reverse primers (10 μM), 1.0 μL cDNA template, and the rest filled with ddH_2O to reach a total volume of 20 μL. The PCR amplification process starts with a denaturation step at 95°C for 30 s, followed by 40 cycles of denaturation at 95°C for 10 s and annealing at 60°C for 30 s. After amplification, a melting curve analysis was performed from 95°C to 60°C over 60 s, with a final hold at 95°C for 15 s to collect data. The relative expression level of mRNA was calculated using the $2^{-\Delta\Delta Ct}$ method.

2.9 Serum barrier factor determination

The serum levels of DAO and D-LA in 42-day-old Leizhou black ducks were quantitatively assayed using a commercial ELISA from Beijing Solarbio Science and Technology Co., Ltd. China, and the results were interpreted in accordance with the manufacturer's instructions.

2.10 Data analysis

SPSS 26.0 was used for one-way ANOVA, followed by Tukey's HSD *post hoc* test. Statistical significance was set at $p < 0.05$, indicating a significant difference, and $p < 0.01$ denoted an extremely significant difference. Results were expressed as mean ± SD.

To explore the relationship between blood antioxidant, immune indexes and immune organ antioxidant immune indexes, intestinal health indexes of Leizhou black ducks under the influence of *Rhodotorula mucilaginosa* ZTHY2. Spearman's rank correlation analysis was performed utilizing the OmicStudio tools accessible at https://www.omicstudio.cn/tool. A p-value below 0.05 was considered indicative of statistical significance, whereas a trend toward significance was defined as $0.05 \leq p < 0.10$.

3 Results

3.1 The impact of *Rhodotorula mucilaginosa* ZTHY2 on the growth performance of Leizhou black ducks

The growth performance of Leizhou black ducks was assessed following the administration of ZTHY2. At day 42 of the trial, a significant increase in body weight was observed in the Leizhou black ducks supplemented with 2×10^8 CFU/kg and 2×10^9 CFU/kg RM,

TABLE 2 Primers utilized in relative real-time polymerase chain reaction analysis.

Genes	Prime sequence(5′ → 3′)	Gene No.
β-actin	F:CTTCACCACCACAGCCGAG R:AATCCAGGGCGACATAGCAC	NM_001310421.1
TNF-α	F:GCCACAGAGAAGAAGCAAACC R:CCAGCAGAGAGTTGTCAGGA	XM_027471963.2
IFN-γ	F:AGAGACCTCGTGGAACTGTC R:ACAGCTCACTCACAGCCTT	NM_001310417.1
IL-2	F:AAACCTGGGAACAAGCATGAA R:AGCTGCACTCCTTTGTGTCATT	NM_001310373.1
IL-4	F:ATGAGACAGACACCGACATGG R:TGTCACGATGTGCAGCAAGT	XM_032196612.1
IL-6	F:CAGGAGGAGATGTGCGAGAAGT R:CGAAGCCAGCCAGGAGACA	XM_027450925.2
SOD	F:AGCGACGACCTGGGCAAAG R:GCACTTGGCTATTCCGATGAC	XM_005019265.5
CAT	F:GTGCGTGACTGACAACCAAGG R:CTGAAATACACATGCGGCTCT	XM_027458335.2
GSH-Px	F:CACCAGGAGAATGCCACCAAC R:TCCCGTTCACCTCGCACTTC	XM_027467953.2

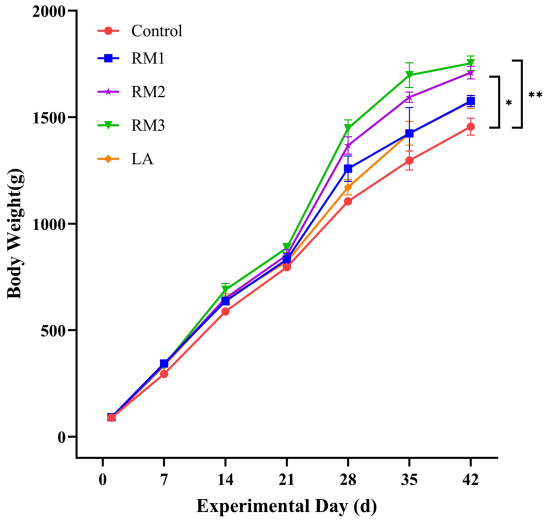

FIGURE 1
Illustrates the effect of *R. mucilaginosa* ZTHY2 on the average body weight of Leizhou black ducks. Control (Control group), RM1 (2×10^7 CFU/kg RM group), RM2 (2×10^8 CFU/kg RM group), RM3 (2×10^9 CFU/kg RM group), LA (2×10^9 CFU/kg LA group).* $p < 0.05$; ** $p < 0.01$.

when compared to the control group ($p < 0.05$ and $p < 0.01$), as depicted in Figure 1.

3.2 The modulatory effect of *Rhodotorula mucilaginosa* ZTHY2 on the antioxidant capacity in Leizhou black ducks

The study meticulously evaluated the impact of ZTHY2 on the antioxidant capacity of 21 and 42-day-old Leizhou black ducks. Figure 2 outlines the effects of *Rhodotorula mucilaginosa* ZTHY2 on the antioxidant functions of these ducks. By the 21st day (Figures 2A–D), the group supplemented with 2×10^7 CFU/kg of RM showed a significant increase in serum Superoxide Dwas mutase (SOD) activity ($p < 0.05$), while the groups treated with 2×10^8 CFU/kg and 2×10^9 CFU/kg of RM demonstrated enhanced serum Catalase (CAT) activity ($p < 0.05$ or $p < 0.01$), respectively. Moreover, the groups treated with 2×10^8 CFU/kg and 2×10^9 CFU/kg of RM significantly elevated both Glutathione Peroxidase (GSH-Px) and SOD activities ($p < 0.01$) compared to the control group. Additionally, at 21 days, the group was supplemented with 2×10^9 CFU/kg of RM showed a significant reduction in serum Malondialdehyde (MDA) content ($p < 0.05$), whereas the group treated with 2×10^9 CFU/kg of LA showed a significant increase in serum GSH-Px activity ($p < 0.05$) compared to the controls.

At the 42-day (Figures 2E–H), treatment with a dosage of 2×10^9 CFU/kg RM markedly enhanced the serum activities of catalase (CAT), superoxide dis-mutase (SOD), and glutathione peroxidase (GSH-Px), while significantly reducing malondialdehyde (MDA) levels ($p < 0.01$) when compared to the control group. Moreover, the groups administered with 2×10^8 CFU/kg RM and 2×10^9 CFU/kg LA exhibited significant elevations in CAT activity and reductions in MDA levels ($p < 0.05$). Additionally, SOD activity was significantly increased ($p < 0.05$) in the group treated with 2×10^8 CFU/kg RM.

3.3 The modulatory impact of *Rhodotorula mucilaginosa* ZTHY2 on the complement system and immunoglobulin levels in Leizhou black ducks

The study evaluated the impact of ZTHY2 on complement and immunoglobulin levels in Leizhou black ducks. Figure 3 shows that supplementing with 2×10^8 CFU/kg of the yeast significantly increased C4 levels compared to the control ($p < 0.05$). At day 21(Figures 3A–C), the 2×10^9 CFU/kg dose elevated C3 and C4 serum levels ($p < 0.05$ or $p < 0.01$), and both doses increased IgG levels at day 21 ($p < 0.01$). Furthermore, at day 42(Figures 3D–F), the 2×10^9 CFU/kg yeast raised C3 and C4 levels compared to the control ($p < 0.05$ or $p < 0.01$), while IgG levels were upregulated in both RM groups at day 42 ($p < 0.05$ or $p < 0.01$).

3.4 Effect of *Rhodotorula mucilaginosa* ZTHY2 on serum immune function of Leizhou black ducks

Figure 4 first illustrated the impact of ZTHY2 on the serum immune function in 21-day-old Leizhou black ducks (Figures 4A–E). ZTHY2 exhibits incremental effects on the serum levels of IL-2, IL-4, IL-6, TNF-α, and IFN-γ. Notably, a dosage of 2×10^8 CFU/kg significantly increases ($p < 0.05$) the serum IFN-γ level in the RM group compared to the control, with highly significant ($p < 0.01$) boosts in IL-4 and TNF-α levels. In the RM group treated with 2×10^9 CFU/kg, both IL-2 and IFN-γ levels were significantly elevated ($p < 0.05$) alongside highly significant ($p < 0.01$) increases in IL-4, IL-6, and TNF-α levels. Additionally, the LA group treated with 2×10^9 CFU/kg shows a significant ($p < 0.05$) rise in serum IL-6 levels compared to the control.

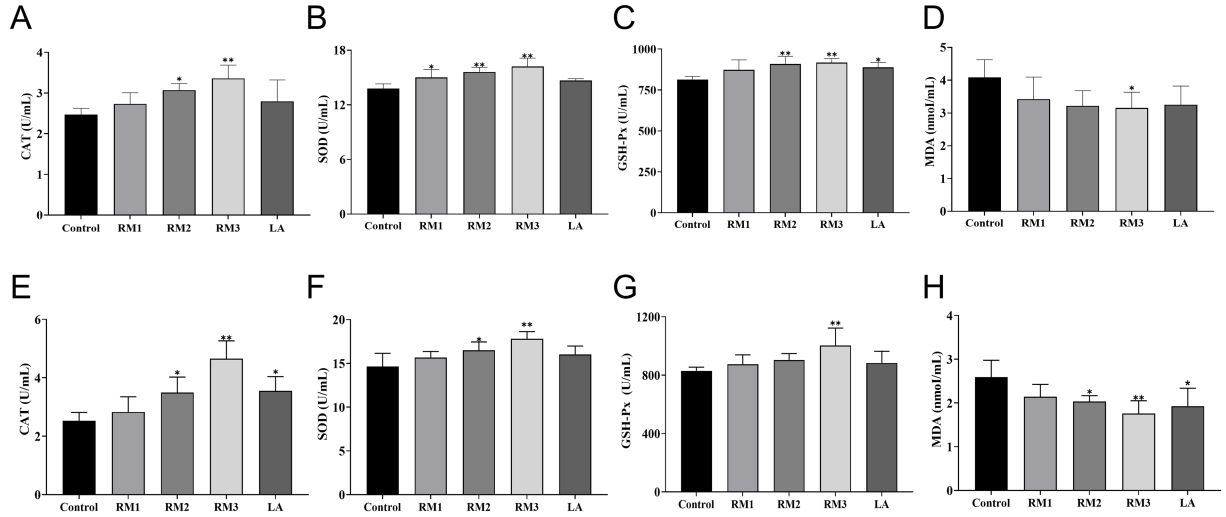

FIGURE 2
Effect of *R. mucilaginosa* ZTHY2 on the antioxidant function of Leizhou black ducks. The CAT, SOD, GSH-Px and MDA in the serum were detected by the commercial kit. **(A–D)** CAT, SOD, GSH-P and MDA in the serum of Leizhou black ducks at 21 days old. **(E–H)** CAT, SOD, GSH-Px and MDA in the serum of Leizhou black ducks at 42 days old. Compared with control group, $*p < 0.05$, $**p < 0.01$.

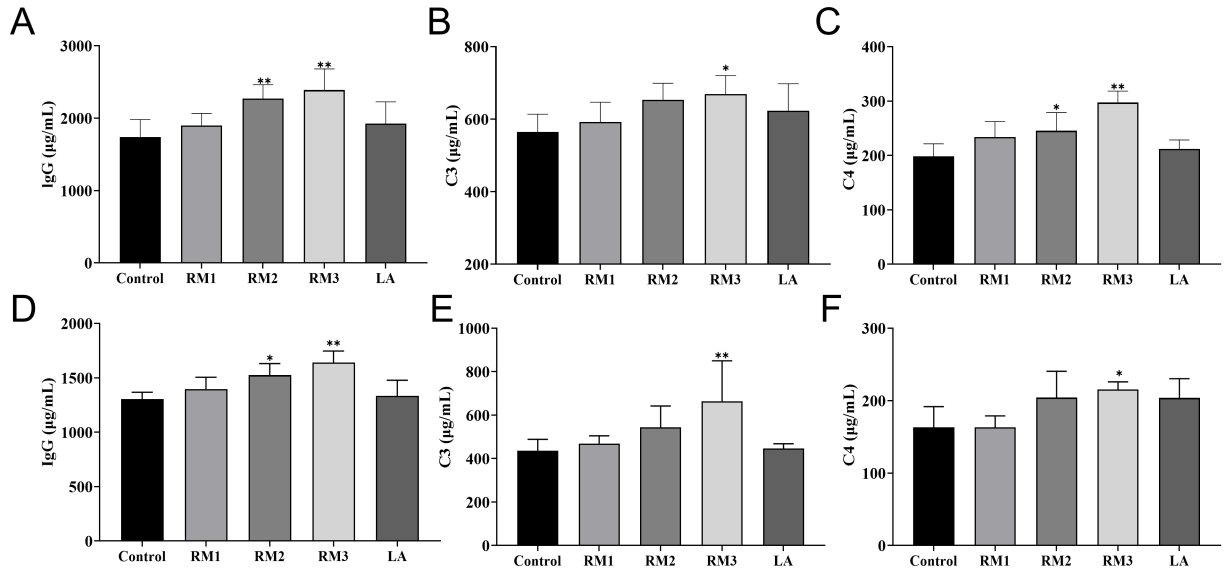

FIGURE 3
Effect of *R.mucilaginosa* ZTHY2 on complement and immunoglobulin levels of Leizhou black ducks. The IgG, C3 and C4 contents in the serum of Leizhou black ducks were detected by ELISA. **(A–C)** The IgG, C3 and C4 contents in the serum of Leizhou black ducks at 21 days old. **(D–F)** The IgG, C3 and C4 contents in the serum of Leizhou black ducks at 42 days old. Compared with control group, $*p < 0.05$, $**p < 0.01$.

The study evaluated the influence of ZTHY2 on the immune system of 42-day-old Leizhou black ducks (Figures 4F–J). Figure 4 second showed that compared to the control, 2×10^7 CFU/kg of the yeast significantly raised serum levels of IL-2, IL-4, IL-6, and TNF-α at day 42 ($p < 0.05$). Moreover, 2×10^8 CFU/kg and 2×10^9 CFU/kg of ZTHY2 markedly increased IL-2, IL-4, IL-6, TNF-α, and IFN-γ ($p < 0.01$), with the 2×10^9 CFU/kg group showing significantly higher IL-4, IL-6, and TNF-α concentrations than the control ($p <$ 0.05 or $p < 0.01$).

3.5 The modulatory effect of *Rhodotorula mucilaginosa* ZTHY2 on the expression levels of cytokines in the immune organs of Leizhou black ducks

The modulatory effects of ZTHY2 on the cytokine expression profiles in the immune organs of 21-day-old Leizhou black ducks were detailed in Figures 5A–C. Compared to the control group, the 2×10^9 CFU/kg RM group exhibited a significant increase in TNF-α mRNA expression ($p < 0.05$) and remarkably higher levels of IFN-γ,

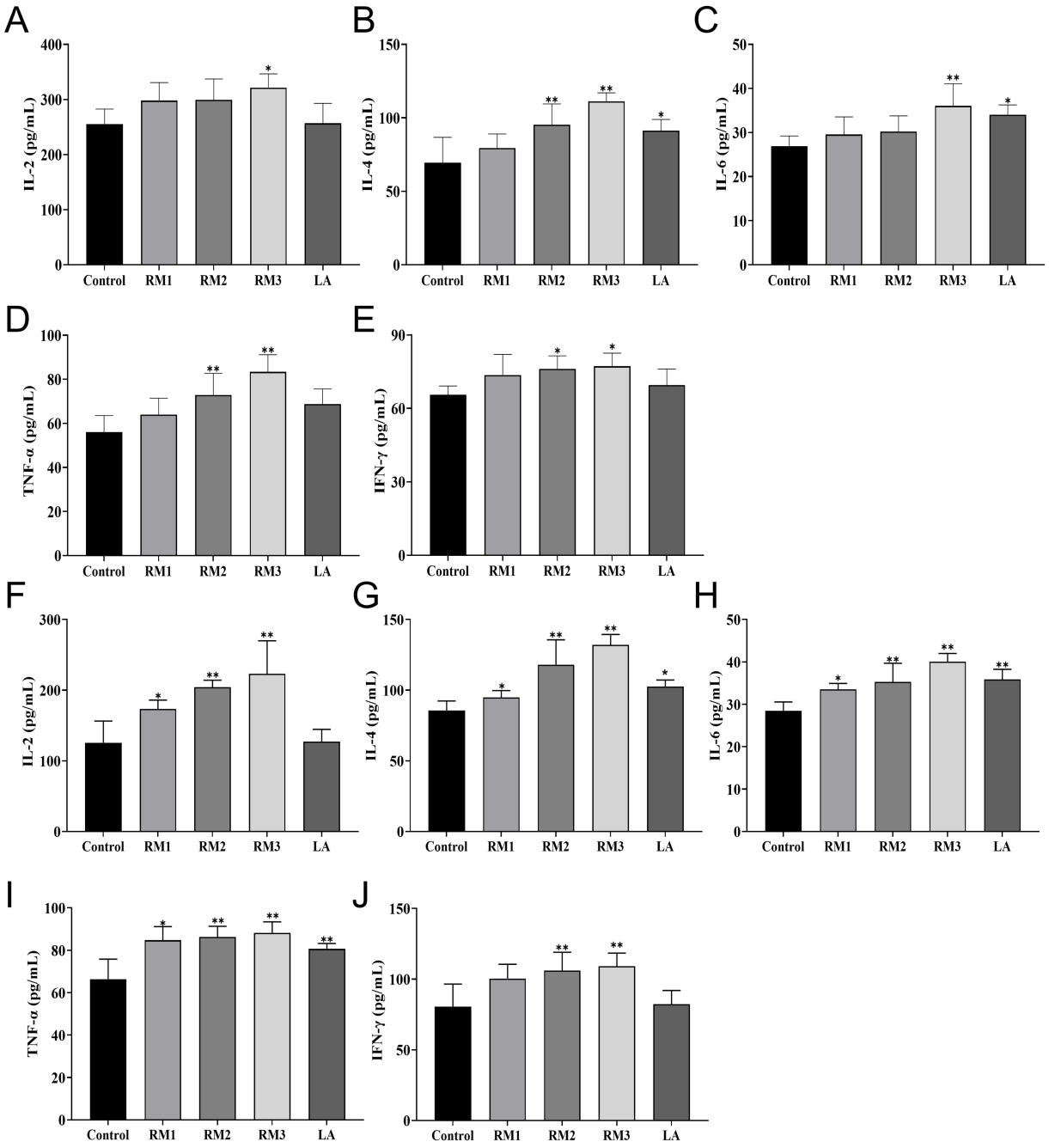

FIGURE 4
Effect of *R.mucilaginosa* ZTHY2 on serum immune function of Leizhou black ducks. The IL-2, IL-4, IL-6, TNF-α and IFN-γ contents in the serum of Leizhou black ducks were detected by ELISA. **(A–E)** The IL-2, IL-4, IL-6, TNF-α and IFN-γ contents in the serum of Leizhou black ducks at 21 days old. **(F–J)** The IL-2, IL-4, IL-6, TNF-α and IFN-γ contents in the serum of Leizhou black ducks at 42 days old. Compared with control group, *$p < 0.05$, **$p < 0.01$.

IL-2, IL-4, and IL-6 mRNA expression ($p < 0.01$) in spleen (Figure 5A). The thymus showed significantly increased expression levels of IL-2 and IL-4 mRNA in the 2 × 10^7 CFU/kg RM group at day 21 ($p < 0.05$ or $p < 0.01$), while the 2×10^8 CFU/kg RM group demonstrated significantly elevated expression levels of IL-2 and IL-6 mRNA at day 21 ($p < 0.05$). In addition, the expressions of IFN-γ, IL-2, and IL-6 mRNA were significantly upregulated in the thymus of the 2 × 10^9CFU/kg RM group ($p < 0.01$ or $p < 0.05$), as well as in the thymus of the 2×10^9 CFU/kg LA group for both IL-2 and IL-6 mRNA ($p < 0.01$; Figure 5B). Furthermore, there was an upregulation observed in bursa with regards to IFN-γ, IL-2, IL-4, IL-6 and TNF-α mRNAs expressions in 2×10^9 CFU/kg RM group at 21 days ($p < 0.05$ or $p < 0.01$; Figure 5C).

The modulatory effects of ZTHY2 on the cytokine expression profiles in the immune organs of 42-day-old Leizhou black ducks were detailed in Figures 5D–F. As shown in Figure 5, compared

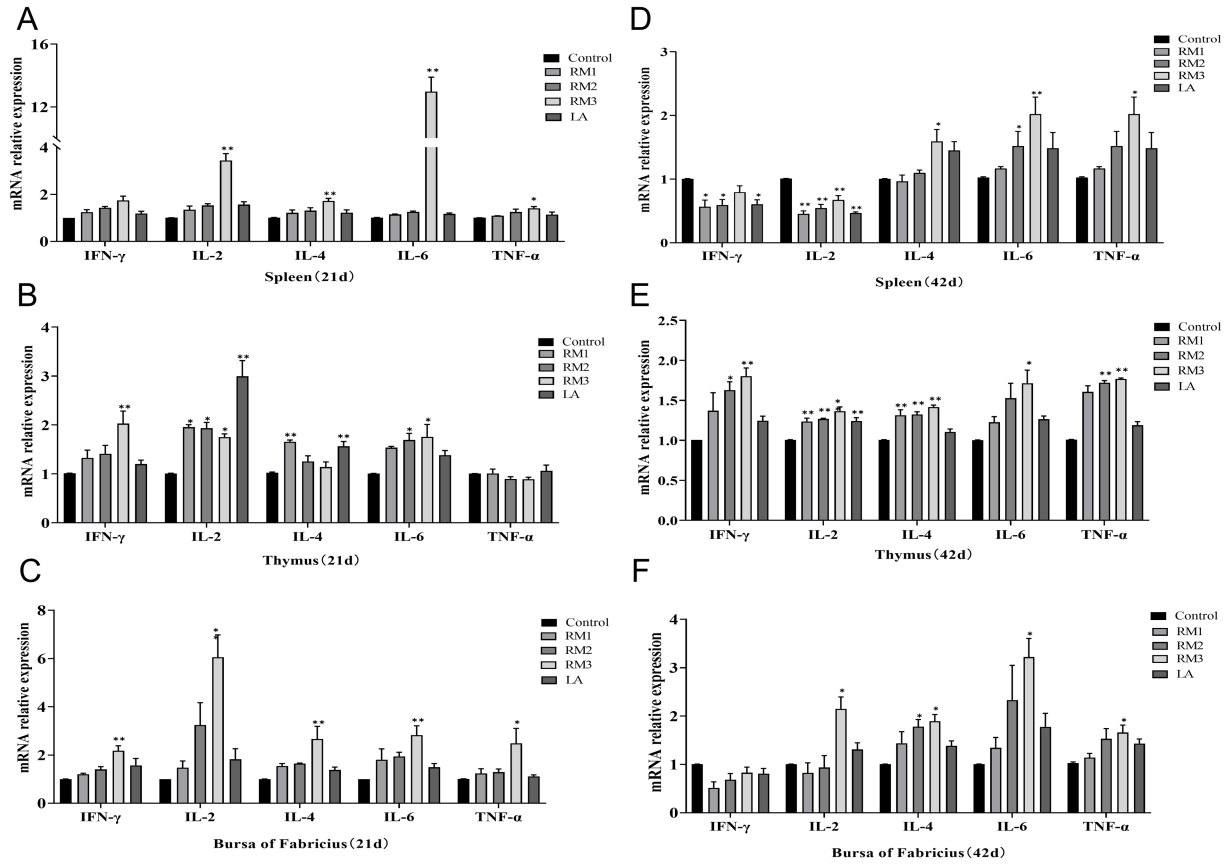

FIGURE 5
Effect of *R. mucilaginosa* ZTHY2 on expression levels of cytokines in the immune organs of Leizhou black ducks. The IL-2, IL-4, IL-6, TNF-α and IFN-γ contents in the spleen, thymus and bursa were detected by ELISA. **(A–C)** The IL-2, IL-4, IL-6, TNF-α and IFN-γ contents in the spleen, thymus and bursa of Leizhou black ducks at 21 days old. **(D–F)** The IL-2, IL-4, IL-6, TNF-α and IFN-γ contents in the spleen, thymus and bursa of Leizhou black ducks at 42 days old. Compared with control group, *$p < 0.05$, **$p < 0.01$.

with the control group, the expression levels of IFN-γ and IL-2 mRNA in spleen of 2×10^8 CFU/kg and 2×10^7 CFU/kg RM groups were down-regulated for 42 days ($p < 0.05$ or $p < 0.01$). The 2 × 10^8 CFU/kg RM group significantly increased the expression of IL-6 mRNA on 42 days ($p < 0.05$), while the 2×10^9 CFU/kg RM group and 2×10^9 CFU/kg LA group significantly decreased the expression of IL-6 mRNA on 42 days ($p < 0.05$; Figure 5D). At the same time, the mRNA expression levels of IL-4, IL-6 and TNF-α were up-regulated in the 2×10^9 CFU/kg RM group ($p < 0.05$ or $p < 0.01$). The expression of IL-2 and IL-4 mRNA in thymus of 2×10^7 CFU/kg RM group was significantly increased on day 42 ($p < 0.01$). The expression levels of IFN-γ, IL-2, IL-4 and TNF-α mRNA in thymus of 2×10^8 CFU/kg RM group were up-regulated for 42 days ($p < 0.05$ or $p < 0.01$). The expression of IFN-γ, IL-2, IL-4, IL-6 and TNF-α mRNA in thymus of 2×10^9 CFU/kg RM group increased for 42 days ($p < 0.05$ or $p < 0.01$; Figure 5E). The 2×10^8 CFU/kg RM group significantly increased the mRNA expression of IL-4 in the bursa for 42 days ($p < 0.05$), and the 2×10^9 CFU/kg RM group significantly increased the mRNA expression of IL-2, IL-4, IL-6, and TNF-α ($p < 0.05$ or $p < 0.01$; Figure 5F).

3.6 The influence of *Rhodotorula mucilaginosa* ZTHY2 on the mRNA expression levels of CAT, SOD, and GSH-Px in the immune organs of Leizhou black ducks

Figure 6 was playing the impact of ZTHY2 on the mRNA expression of antioxidant enzymes CAT, SOD, and GSH-Px in the immune organs of 21-day-old Leizhou black ducks (Figures 6A–C). The 2×10^9 CFU/kg RM group exhibited a significant upregulation of CAT, SOD, and GSH-Px mRNA in the spleen at 21 days (Figure 6A), and also showed increased expression of CAT and SOD in the thymus at the same timepoint ($p < 0.05$ or $p < 0.01$; Figure 6B). Additionally, a trend of increased mRNA expression for CAT, SOD, and GSH-Px was observed in the Bursa after ZTHY2 treatment at 21 days, though these differences were not significant ($p > 0.05$; Figure 6C).

It was illustrated the significant modulatory effects of 2×10^9 CFU/kg of Gum Red Yeast ZTHY2 on the mRNA expression of antioxidant enzymes Catalase (CAT), Super-oxide Dismutase (SOD), and Glutathione Peroxidase (GSH-Px) in the immune organs of 42-day-old Leizhou black ducks in Figures 6D–F. The yeast significantly elevated mRNA levels of these

enzymes in the spleen, thymus, and bursa at day 42, reaching statistical significance at $p < 0.05$ or $p < 0.01$ (Figures 6D–F). Notably, a specific upregulation of CAT and SOD mRNA expression in the bursa was observed in the group supplemented with 2×10^9 CFU/kg of ZTHY2 at day 42 ($p < 0.05$ or $p < 0.01$; Figure 6F).

3.7 The influence of *Rhodotorula mucilaginosa* ZTHY2 on the serum DAO and D-LA concentrations in Leizhou black ducks

D-lactic acid (D-LA) and diamine oxidase (DAO) as sensitive biomarkers reflecting alterations in intestinal mucosal permeability and epithelial barrier function. Figure 7 illustrated the impact of ZTHY2 on Leizhou black duck serum diamine oxidase (DAO) and D-lactic acid (D-LA) levels. Dietary inclusion of ZTHY2 significantly decreased DAO and D-LA concentrations compared to the control ($p < 0.05$ or $p < 0.01$), with the most beneficial effects observed at a dosage of 2×10^9 CFU/kg.

3.8 Correlation analysis of blood antioxidant, immune indexes and immune organ antioxidant immune indexes, intestinal health indexes of Leizhou black ducks under the influence of *Rhodotorula mucilaginosa* ZTHY2

After subjecting Leizhou black ducks to ZTHY2, we conducted a Spearman correlation analysis on their blood antioxidant and immune indices, as well as the antioxidant and immune indices of their immune organs. The results showed positive correlations between blood antioxidant enzymes (CAT, GSH-Px, SOD) and bursal antioxidant enzymes (CAT, SOD, IL-6, TNF-α) in the bursa ($p < 0.05$), while MDA was inversely associated with these enzymes ($p < 0.05$). Beyond C4, various blood immune indices were positively associated with bursal antioxidant enzymes (CAT, SOD, GSH-Px) and cytokines (IL-4, IL-6, TNF-α) ($p < 0.05$), as shown in Figure 8A. In the spleen, blood antioxidant enzymes were positively correlated with spleen enzymes and cytokines (IL-6, TNF-α) ($p < 0.05$), and MDA was negatively correlated with GSH-Px and SOD ($p < 0.05$), as shown in Figure 8B. In the thymus, blood antioxidant indices were positively

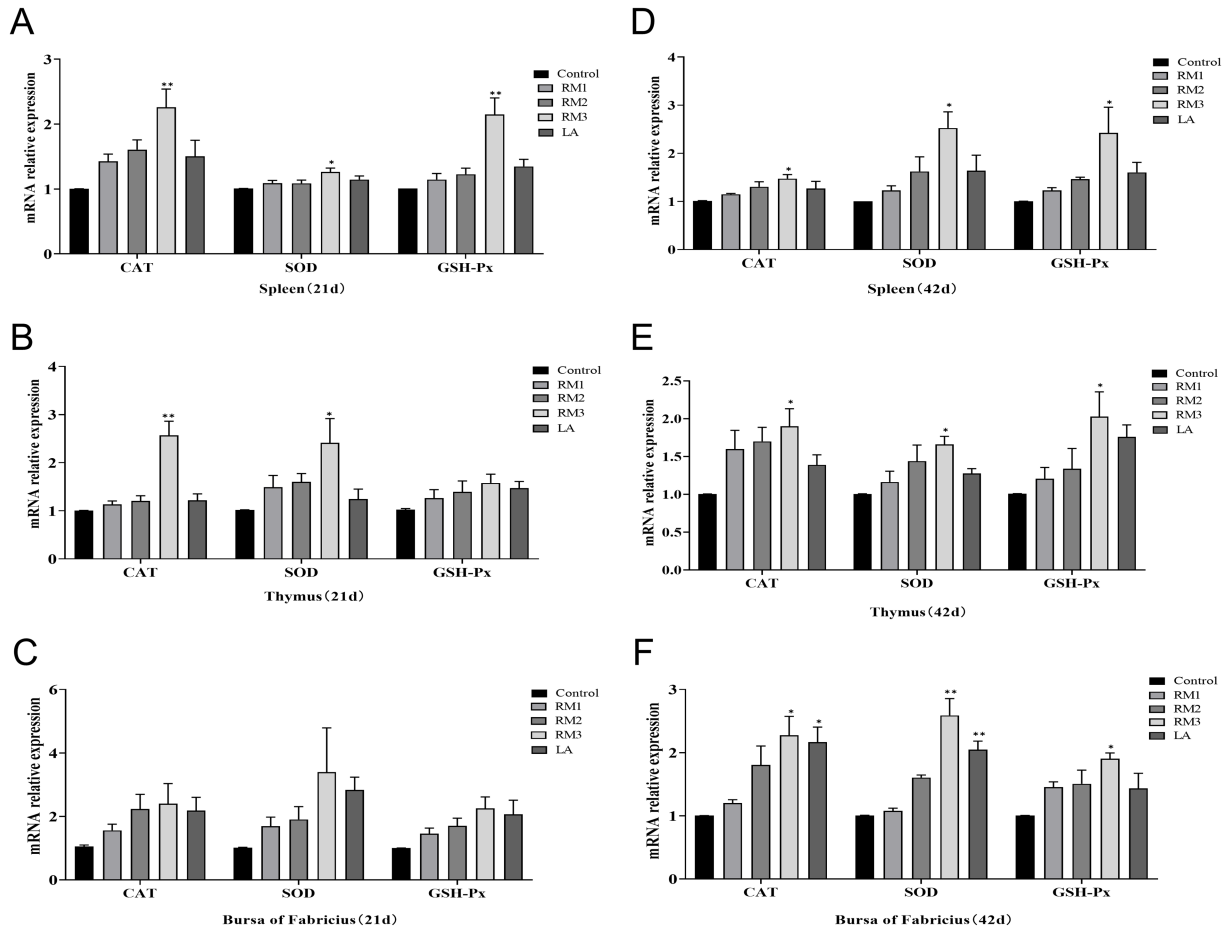

FIGURE 6
Effect of *R. mucilaginosa* ZTHY2 on CAT, SOD, GSH-Px mRNA expression levels in immune organs of Leizhou black ducks. The gene expressions of CAT, SOD and GSH-Px in the spleen, thymus and bursa were detected by qPCR. **(A–C)** The gene expressions of CAT, SOD and GSH-Px in the spleen, thymus and bursa were detected of Leizhou black ducks at 21 days old. **(D–F)** The gene expressions of CAT, SOD and GSH-Px in the spleen, thymus and bursa were detected of Leizhou black ducks at 42 days old. Compared with control group, *$p < 0.05$, **$p < 0.01$.

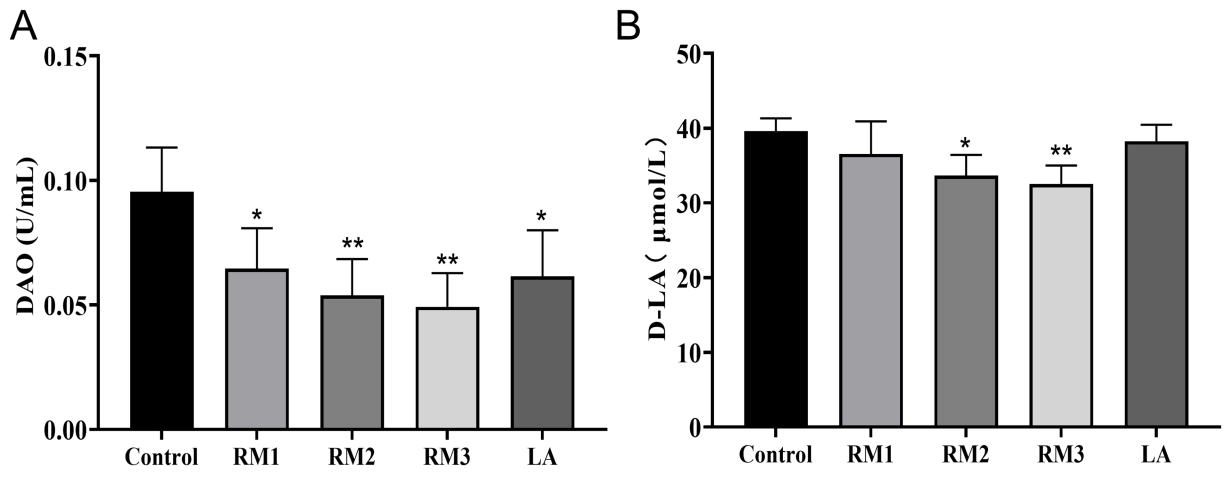

FIGURE 7
The influence of *Rhodotorula mucilaginosa* ZTHY2 on the serum DAO and D-LA concentrations in Leizhou black ducks. The content of DAO and D-LA in serum of Leizhou black ducks were detected by commercial kit. **(A)** The content of DAO in serum of Leizhou black ducks at 42 days old. **(B)** The content of D-LA in serum of Leizhou black ducks at 42 days old. Compared with control group, $*p < 0.05$, $**p < 0.01$.

correlated with thymic indices (GSH-Px, SOD, IL-6, IL-2) ($p < 0.05$, IL-2 significantly at $p < 0.01$), while MDA was negatively correlated with GSH-Px ($p < 0.05$), as illustrated in Figure 8C. A Spearman correlation analysis on the antioxidant and immune indices of the thymus and bursa revealed that all other indices in the bursa were positively correlated with those in the thymus, except for IL-2 and IFN-γ ($p < 0.05$, IL-2 significantly at $p < 0.01$), as depicted in Figure 8D.

Spearman correlation analysis revealed negative associations between Leizhou black duck blood parameters, antioxidant and immune indices, and intestinal health markers after treatment with ZTHY2. DAO and D-LA negatively correlated with GSH-Px, SOD, TNF-α, IL-2, IgG, C3, IFN-γ, and IL-4 in blood ($p < 0.05$; Figure 9A). In the bursa, DAO and D-LA were inversely related to GSH-Px, IL-4, IL-6, and TNF-α ($p < 0.05$; Figure 9B). In the spleen, only DAO was associated with TNF-α and IL-6 negatively ($p < 0.05$; Figure 9C). Within the thymus, DAO and D-LA generally exhibited negative correlations with intestinal health markers, except for GSH-Px ($p < 0.05$), particularly with IL-2 ($p < 0.01$; Figure 9D).

4 Discussion

4.1 The impact of *Rhodotorula mucilaginosa* ZTHY2 on the growth performance of Leizhou black ducks

Rhodosaccharomyces colloides, which is rich in cellular proteins, small molecular peptides, vitamins, and unsaturated fatty acids, is easily absorbed and utilized by animals, directly participating in their metabolic processes (14). One of the key benefits is the natural flavor it brings. It contains nucleotides that release aromatic compounds and amino acids that contribute to a rich umami taste. These elements work together harmoniously to significantly enhance the feed's flavor, thus stimulating the animals' appetite (12, 24). This natural flavor enhancement not only makes the feed more appealing to the animals but also encourages them to eat more actively, which is vital for their overall health and growth efficiency.

In our study, we specifically examined the effects of *Rhodotorula mucilaginosa* ZTHY2 on the growth performance of Leizhou black ducks. During the experiment, we added 2×10^8 CFU/kg and 2×10^9 CFU/kg of ZTHY2 to the feed, respectively. The results showed that, compared to the control group, the Leizhou black ducks supplemented with ZTHY2 experienced a notable increase in body weight. This weight gain can be attributed to the fact that the ducklings' thermoregulatory and digestive systems are not fully developed at the beginning of their growth, making additional nutritional support especially important (25). Based on these findings, we can conclude that adding ZTHY2 to the feed significantly improves the growth performance of Leizhou black ducks. This additive not only provides essential nutrients but also enhances the feed's palatability with its unique flavor, thereby boosting the animals' appetite and growth. These results provide a strong scientific basis for the use of *Rhodotorula mucilaginosa* ZTHY2 in animal nutrition and offer valuable insights for future feeding practices.

4.2 *Rhodotorula mucilaginosa* ZTHY2 strain could enhance the antioxidant capacity of Leizhou black ducks

In healthy animals, a delicate balance was maintained between antioxidant enzymes and reactive oxygen species (ROS). However, during livestock and poultry breeding, the utilization of inappropriate feed additives or the repercussions of environmental stress could unsettle the equilibrium of the oxidation/antioxidant systems, culminating in the excessive aggregation of ROS and the induction of oxidative stress (26). An increased concentration of ROS could lead to the degradation of cellular membrane structure and function, triggering lipid peroxidation, protein oxidation, and DNA damage (27). Antioxidant enzymes, such as superoxide dismutase (SOD), glutathione peroxidase (GSH-Px), and catalase (CAT), were capable

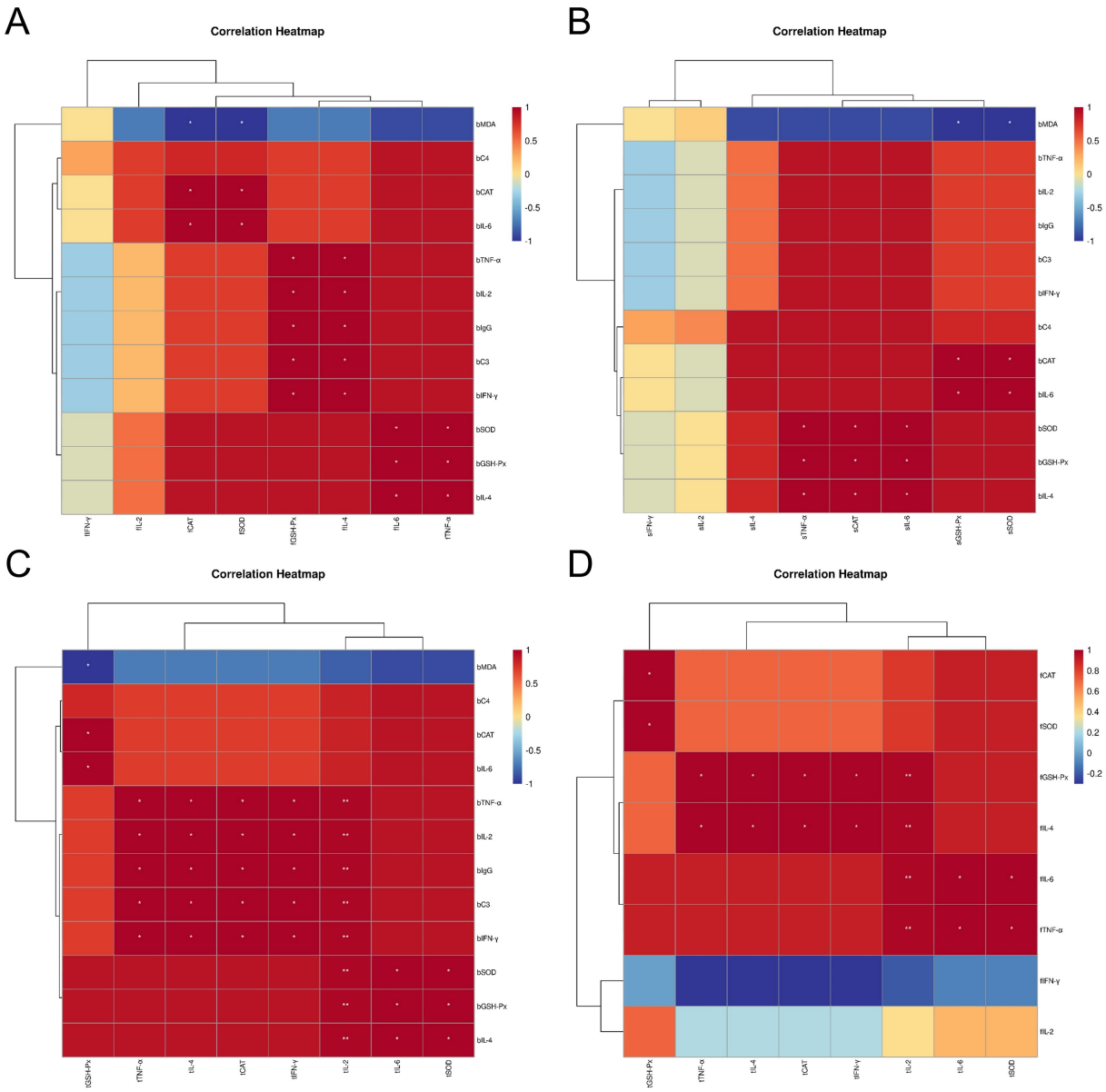

FIGURE 8
Spearman correlation analysis between the blood antioxidant and immune indexes and immune organ antioxidant and immune indexes of Leizhou black ducks under the action of ZTHY2. **(A)** Blood and bursa; **(B)** Blood and spleen; **(C)** Blood and thymus; **(D)** thymus and bursa.

of neutralizing excessive ROS, reducing the production of malondialdehyde (MDA), a byproduct of membrane lipid peroxidation, thereby alleviating oxidative stress and maintaining the homeostasis of the oxidation/antioxidant systems *in vivo* (28).

Throughout growth, *Rhodoyeast* could produce carotenoids like astaxanthin, exopolysaccharides, and other metabolites, which participate in the regulation of various oxidoreductase-related metabolic pathways (13). These natural antioxidants potentiate their antioxidant efficacy by promoting the nuclear translocation of nuclear factor erythroid 2-related factor 2 (Nrf2), modulating the expression of downstream antioxidant enzyme genes, and scavenging oxygen free radicals within cells (14, 15, 29). Nrf2, a member of the basic leucine zipper transcription factor family, plays a key role in regulation of cellular redox homeostasis by inducing various detoxifying and antioxidant enzymes, such as the oxidative stress marker malondialdehyde (MDA), and the antioxidant enzyme superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPX), and glutathione reductase (GR) (30). Hu et al. reported that weaning piglets fed daily with 3×10^{10} CFU of red yeast exhibited a significant enhancement in the activities of serum antioxidant enzymes, including total antioxidant capacity (T-AOC) and GSH-Px, along with a reduction in MDA content (24).The study showed that incorporating ZTHY2 into the diet of Leizhou Black ducks enhanced their serum antioxidant activities, including catalase (CAT), glutathione peroxidase (GSH-Px), and superoxide dismutase (SOD) on days 21 and 42. This was accompanied by a decrease in malondialdehyde (MDA) content in a dose-dependent manner.

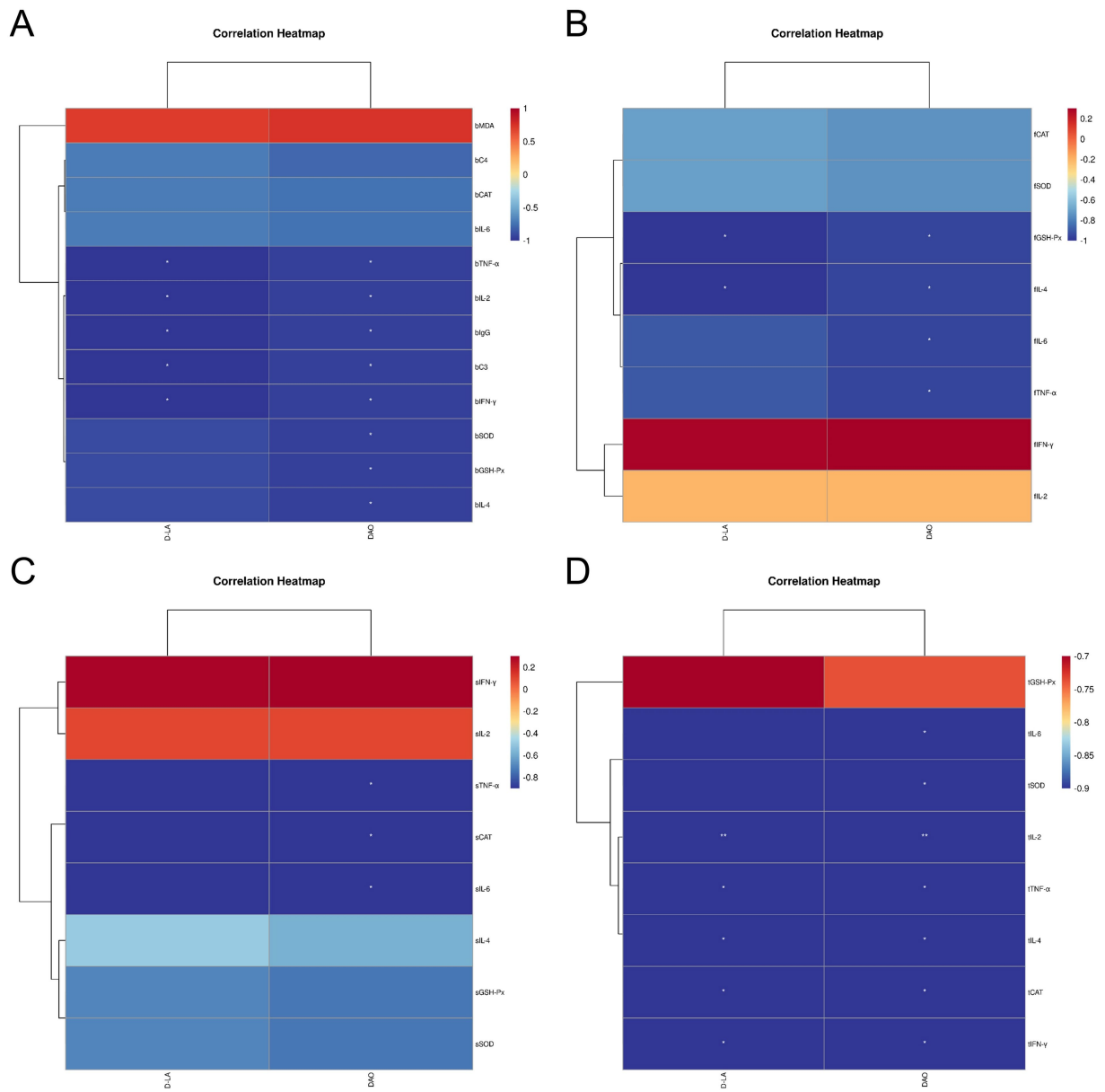

FIGURE 9
Spearman correlation analysis between the blood, immune organ antioxidant, immune indexes and intestinal health indexes (DAO and D-LA, as D.D.) of Leizhou black ducks under the action of ZTHY2. **(A)** Blood and D.D.; **(B)** Bursa and D.D.; **(C)** spleen and D.D.; **(D)** thymus and D.D.

Quantitative polymerase chain reaction (qPCR) confirmed that yeast supplementation upregulated the mRNA expression of antioxidant enzymes in the spleen, thymus, and bursa at both 21 and 42 days, with the strongest effects at a supplemental level of 2×10^9 CFU/kg. These findings indicate that ZTHY2 inhibits lipid peroxide production by improving serum antioxidant activities and increasing the expression of antioxidant-related genes, thereby strengthening antioxidant capacity and maintaining redox balance. Furthermore, increased antioxidant capacity within the body reduces oxidative damage to proteins and lipids in muscle tissue, while improved antioxidant enzyme activity had a positive impact on meat quality (31). This evidence reinforces the idea that ZTHY2 could improve the muscle quality and nutritional value of Leizhou Black ducks.

4.3 *Rhodotorula mucilaginosa* ZTHY2 strain had been shown to enhance the humoral immune response of Leizhou black ducks

IgG, C3 and C4 were the key parameters for assessing humoral immunity, working in concert to bolster the avian immune response and enhance disease resistance. Research indicates that the inclusion of 0.10% yeast nucleotides or yeast β-glucan in the diet could elevate the levels of C3, C4, and IgG in serum, thereby enhancing both cellular and humoral immune functions (32). Additionally, doses of 2×10^8 CFU/kg and 2×10^9 CFU/kg of ZTHY2 elevated the serum IgG concentrations at 42-day intervals. IgG constitutes the predominant immunoglobulin present in avian serum, being produced through the stimulation and differentiation of B cells. It

possesses a specific binding capacity for pathogenic microorganisms, thereby offering a protective barrier against infections and was crucial for the augmentation of the humoral immune response in birds (33).

The complement system serves as an integral linkage between the adaptive and innate immune responses, with complement components C3 and C4, which were also integral parts of immunoglobulins, aiding antibodies and phagocytes in the host's defense mechanisms against pathogens. These components were pivotal in immune regulation (34). C3 was the most copious component within the complement system and was pivotal in both the classical and alternative activation pathways, whereas C4 participates in the early stages of the immune response, bolstering subsequent reactions and exhibiting various non-specific immune functions, such as the prevention of immune complex deposition, enhancing phagocytosis, and toxin neutralization (35, 36).

Our experiment found that supplementing with 2×10^9 CFU/kg of ZTHY2 markedly increased the serum levels of C3 and C4 in 42-day Leizhou black ducks. These results indicate that ZTHY2 could stimulate the production of immunoglobulins and complement in the serum of Leizhou black ducks, thereby enhancing humoral immunity and disease resistance in livestock and poultry. The most effective dose was found to be 2×10^9 CFU/kg ZTHY2 added to the feed.

4.4 *Rhodotorula mucilaginosa* ZTHY2 strain had been shown to enhance the organ immune function of Leizhou black ducks

Our prior research had demonstrated that ZTHY2 could significantly augment the proliferation of T and B lymphocytes in immunosuppressed mice, concomitant with an increase in the serum secretion of IL-2, IL-4, IL-6, TNF-α, IFN-γ, and immunoglobulins, thereby significantly enhancing the immune function in mice (11). The immune organs of avian species predominantly encompass the spleen, thymus, and bursa, which serve as the structural underpinnings of the avian immune response and were critical venues for the differentiation and maturation of immune cells (37). The bursa, an exclusive immune organ in birds, was pivotal in the formative stages of B cells, facilitating their early development, proliferation, and the expression of immunoglobulins. The thymus was the anatomical site where T cells originate, proliferate, differentiate, and achieve maturity, functioning as a pivotal barrier and regulatory entity in both cellular and innate immunity. The spleen was capable of manufacturing a spectrum of immune factors that modulate the body's immune homeostasis, and it executes specific immune functions through the mediation of both T-cell and B-cell-driven humoral and cellular immunity, thereby providing a defense mechanism against microbial incursions and offering direct resistance to extrinsic infections (38–40).

Th2 cell-derived interleukins, including IL-4 and IL-6, significantly contribute to humoral immunity by fostering B cell proliferation and differentiation, aiding in the secretion of immunoglobulins, and exerting multifaceted regulation over immune activity. The intricate interplay and collaboration between Th1 and Th2 cytokines establish a complex cytokine network that mutually maintains the foundations of immune homeostasis (41, 42). Interleukin-2 (IL-2), tumor necrosis factor-alpha (TNF-α), and interferon-gamma (IFN-γ) represent pivotal Th1 cytokines instrumental in macrophage activation and the orchestration of cellular immune responses. IL-2 and IFN-γ particularly emerge as the principal elements within the IL-2-IFN-γ-NKC immune regulatory network, collaboratively activating cellular immunity and promoting tumor cell apoptosis (43). TNF-α, a critical mediator in the inflammatory response, effectively activates neutrophils and lymphocytes while stimulating the secretion of interleukin-6 (IL-6), thereby enhancing the immune system's defense mechanisms against pathogenic infections (44).

By day 42, ZTHY2 modulated mRNA expression of these cytokines in the spleen, decreasing IFN-γ and IL-2 expression and increasing TNF-α and IL-4 expression. Conversely, in the thymus and bursa of Farsi, ZTHY2 induced a variable up-regulation of cytokine expression. These results indicate that ZTHY2 regulates cytokine expression within immune organs, maintaining the balance of Th1/Th2 cell populations. This promotes antagonistic and synergistic interactions, enhancing the body's overall immune function. The mechanism may involve the enrichment of ZTHY2 with metabolically active components such as mannooligosaccharides, β-glucan, and nucleotides. These stimulate the complement cascade, activate and proliferate B lymphocytes, and facilitate antibody production, including immunoglobulins (45). Conversely, the augmentation of T-cell proliferation and macrophage activation instigates these immune cells to recruit and secrete a diverse array of cytokines, thereby exerting regulatory functions within the immune response (46). Other yeast studies have also found that they could activate the TLR4-NF-κB signaling pathway (16) or TLR4-NF-κB signaling pathway and enhance macrophage function (17). Liver is an important metabolic organ and immune organ. Mycotoxins in various feeds and feed materials can induce liver inflammation and affect body homeostasis and health (47). Transcriptional co-activator with PDZ-binding motif (TAZ), one of core modules of the Hippo pathway, involves inflammatory cell infiltration in the liver, and might ameliorate the inflammatory response through the Nrf2-reactive oxygen species (ROS)-nuclear factor κB (NF-κB) pathway (30). The above pathways and their key regulatory genes can be used as potential research directions, in the future to explore the regulation of oxidation-inflammation balance by *Rhodotorula mucilaginosa* ZTHY2 through the "gut-liver" axis.

In summary, ZTHY2 enhances immune function by promoting the growth and maturation of T and B cells, orchestrating both cell-mediated and humoral responses, and regulating the release of immune regulators along with the upregulation of immune-related genes, thus maintaining immune balance and improving immune efficacy.

4.5 *Rhodotorula mucilaginosa* ZTHY2 had the potential to enhance the intestinal permeability in Leizhou black ducks

D-lactic acid (D-LA), an organic acid derived from the metabolism of intestinal microbiota, and diamine oxidase (DAO), an intracellular enzyme expressed in epithelial cells of the intestinal tract, function as sensitive biomarkers reflecting alterations in intestinal mucosal permeability and epithelial barrier function (48). Under

normal physiological conditions, serum levels of D-LA and DAO were typically low. However, disruptions in the equilibrium of gut microbiota or intestinal mucosal injury, which result in increased permeability, frequently lead to elevated serum concentrations of DAO and D-LA. In the current study, the administration of ZTHY2 was found to significantly decrease serum levels of DAO and D-LA in Leizhou black ducks, thereby exerting a protective effect on the intestinal mucosa and preserving its integrity.

5 Conclusion

Rhodotorula mucilaginosa ZTHY2 could enhance the body's antioxidant capacity, reduce lipid oxidation damage, resist the occurrence of stress reactions, thereby maintaining immune homeostasis and improving immune function. It also had potential to enhance intestinal barrier function and improve digestive absorption in the intestines. The most efficacious concentration of ZTHY2 had been determined to be 2×10^9 CFU/kg. This study aligns with the forward-thinking trend of "feed without resistance," encompassing significant implications for the healthful and sustainable advancement of the aquaculture industry.

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 studies were approved by all experimental protocols were approved by the Animal Ethics Committee of Guangdong Ocean University, China (IACUC No. GDOU-LAE-2020-007). The studies were conducted in accordance with the local legislation and institutional requirements. Written informed consent was obtained from the owners for the participation of their animals in this study.

Author contributions

JW: Writing – original draft, Writing – review & editing. YH: Writing – original draft. NZ: Conceptualization, Investigation, Methodology, Writing – original draft. WY: Conceptualization, Data curation, Methodology, Writing – original draft. ZC: Writing – original draft, Writing – review & editing.

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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.2025.1494892/full#supplementary-material

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A low-protein soybean-free diet improves carcass traits and meat quality and modulates the colonic microbiota in Daweizi pigs

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Introduction: Soybean meal is an excellent protein source and is widely used in pig feed. However, the Americas account for more than 80% of global soybean production, so European and Asia swine production largely depends on soybean imports. The use of safe and functional unconventional feed sources can effectively alleviate worldwide protein shortage problems.

Methods: Here, we formulated a low-protein soybean-free diet (LPNS) for growing and fattening pigs using rice, potatoes, tea, and other unconventional feed sources. Thirty-six healthy Daweizi pigs (average body weight 23.60 ± 1.34 kg) were raised under the same conditions and randomly assigned to two dietary treatments: (1) Con group, corn-soybean base meal; (2) LPNS group. When the average weight of pigs in the group reached 85 kg, two pigs per pen were randomly selected and euthanized for collection of the colonic digesta and carcass traits and for meat quality determination.

Results: Compared with the corn-soybean based diet, the LPNS diet decreased the average daily gain (ADG) and feed conversion ratio (FCR) of Daweizi pigs but had a lower cost per kilogram of gain. In addition, the LPNS diet significantly increased leanness and decreased the fat-skin rate and bone rate of Daweizi pigs. The cooking loss of meat decreased, and unsaturated fatty acids such as C22:6 and *n*−3 PUFA significantly increased in the LPNS group. Moreover, the purine content in the meat substantially decreased with the LPNS diet. The 16S rDNA analysis revealed that the LPNS diet greatly modified the composition of the colonic microbiota community, with a decrease in the Firmicutes/Bacteroidetes ratio and an increase in the abundance of *Lactobacillus* spp.

Discussion: The use of functional herbs along with a low-protein diet helped to regulate fat and purine metabolism in fatty-type pigs. The LPNS diet formulated with unconventional-feed sources not only helps reduce the feed cost in swine production but also improves the carcass traits and meat quality of pigs, which is more suitable for small-scale pig farming.

KEYWORDS

fatty-type pig, low protein diet, meat quality, gut microbiota, carcass traits

1 Introduction

Soybean meal is an excellent protein source and is widely used in pig feed. However, the Americas account for more than 80% of global soybean production, so European and Asia swine production largely depends on soybean imports (1). The use of alternative feedstuffs as substitutes for soybeans has been investigated worldwide to alleviate protein shortage problems and reduce feed costs in swine production (2–4).

The Daweizi pig is a fatty-type pig breed in China and is characterized by tender meat and tolerance to rough feeding (5). However, compared with lean-type pigs, such as Duroc × Landrace × Large White pigs, Daweizi pigs have a lower growth rate and feed efficiency, a higher fat percentage, and a lower lean mass rate (6). Moreover, like most fat-type Chinese domestic pigs, the nutritional requirements and feeding practices of Daweizi pigs differ from those of lean-type pigs. The current nutritional standards for lean-type pigs refer mainly to NRC 2012, but the recommended dietary protein levels are not applicable for fatty-type pigs. For most fatty-type pigs, appropriately reducing dietary protein level and using non-conventional protein sources can not only reduce the cost of feed but also help to regulate the health of pigs (7, 8). For example, dietary partially replaced with cassava residue can regulate the lipid metabolism and antioxidant capacity of Huanjiang Mini-pigs (a Chinese fatty-type pig breed) (9). However, few studies have used soybean-free meals for fatty-type pig production.

The food-feed competition is a global challenge. Use of food-not feed resources, such as biofuel co-produces, food waste, and other safe-edible by-products, not only decrease the feed costs but also avoid cost associated with disposal (10). Corn is a cereal crop that is most commonly cultivated worldwide. It is usually used as a sustenance source for humans and livestock, as well as to manufacture ethanol and citric acid (11). Corn residue after extraction of citric acid by fermentation (RCC meal) is non-toxic and cost-effective. RCC meal contains more than 25% crude protein and is rich in amino acids (Supplementary Table S1). It's usually used as a protein feed resource in China (12). In addition, nearly 30% of the world's rice production is attributed to China (13). Rice bran, a nutrient-rich byproduct of rice production, contains 17.5% crude protein, antioxidants, vitamins, and minerals and is widely used as animal feed (14). Furthermore, the province of Hunan, where Daweizi pigs originated, is abundant in citrus and tea. Black tea consumption has numerous health benefits, such as cholesterol reduction, cardiovascular protection, and antioxidation (15). Dried citrus peel (tangerine peel) is a traditional Chinese medicine with antioxidant and anti-inflammatory effects (16). In this study, we developed a low-protein, soybean-free diet (LPNS diet) that included RCC meal, rice bran, black tea powder, tangerine peel powder, and other unconventional feedstuffs. The effects of the LPNS diet on the growth performance, carcass traits, meat quality, and intestinal microbiota of Daweizi pigs were investigated.

2 Materials and methods

2.1 Preparation of the low-protein soybean-free diet

Rice, rice bran, RCC meal, sweet potatoes, black tea powder, and tangerine-peel powder were initially mixed and steamed for 1 h. The ingredients were subsequently cooled and dried to a moisture content of approximately 10%, then pulverized and blended. Finally, the mixture was thoroughly blended with soybean oil and premix and stored in a dry and cool place to be used within 3 days. The nutritional levels and ingredient components are shown in Table 1.

2.2 Animals and treatments

The animal experiment was reviewed and approved by the Animal Care and Use Committee of the Institute of Subtropical Agriculture, Chinese Academy of Sciences (IACUC # 202302).

Thirty-six healthy Daweizi pigs (average body weight 23.60 ± 1.34 kg) were raised under the same conditions at Hunan Tianfu Agriculture & Animal Husbandry Ecology Co., Ltd., Changsha, China. The pigs were randomly assigned to two dietary treatments, with four replicates per group and four pigs per replicate. Each pen consisted of a concrete wallow (4.0 m × 2.0 m) and free access to feed and water. The dietary treatment groups were as follows: (1) Con group, which was fed with a conventional diet based on corn and soybean meal; and (2) LPNS group, which was fed a with the low-protein, soybean-free diet. The nutritional level of the conventional diet (see Table 1) met the National Research Council (2012) nutrient recommendation (17). The crude protein and some amino acids contents in the LPNS diet (20–50 kg and 50–85 kg) are lower than those in the Pig Nutrient Requirements of China (GB/T 39235-2020) (18). When the average weight of pigs in the group reached 50 kg and 85 kg (the finishing weight), the number of feeding days was recorded, and feed intake, body weight gain and feed efficiency were calculated with a pen as the experimental unit. After that, two pigs per pen were randomly selected with a 12 h fast. Then, the selected pigs were electrical stunning with 250 V before being slaughtered. The colonic digesta and longissimus thoracis (LT) muscle were collected for further analysis.

2.3 Assessment of carcass traits

The carcass traits of the Daweizi pigs were determined according to previous study (19). Briefly, the carcass sides were processed into primal cuts, and the half-carcass weight was recorded (the head, hair, hooves, tail, and internal organs were not included). The mean backfat thickness was measured with a ruler at three sites in the right dorsal midline of the right carcass: the shoulder, the last rib, and the lumbar-sacral junction. The carcass oblique length and the carcass straight length were also measured. The outline of the loin eye area (LEA) of the longissimus thoracis (LT) muscle was traced with an acetate film between the last 3rd and 4th ribs, perpendicular to the dorsal midline, and the area of LEA was subsequently calculated via planimetry. The skin with the subcutaneous fat, lean, and bone of the left carcass was separated from the right carcass and weighed.

2.4 Assessment of meat quality

The LT muscle at the junction of the waist and sacrum was collected and separated into several portions. Half was stored in a refrigerator at 2–4°C for meat quality determination, and the other half was stored at −80°C for further analysis. The pH value and color ($L^*a^*b^*$) of the LT muscle at 24 h after sampling were determined by

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TABLE 1 Composition and analyzed nutrients levels of the experimental diet (as-fed basis, %).

Con diet		LPNS diet		
Ingredient %		Ingredient %	20 kg–50 kg	50 kg–85 kg
Corn	58.5	Rice bran	35	30
Soybean meal	17	Rice	25	34
Wheat bran	2	RCC meal	25	20
Limestone	1	Sweet potatoes	10	6
Rice	11.5	Corn oil	1	1
Brown rice	5	Tangerine-peel powder	—	1
Corn germ meal	2.5	Black tea powder	—	4
Premix	2.5[a]	Premix	4[b]	4[b]
Total	100	Total	100	100
Analyzed nutrient content		Analyzed nutrient content		
Gross energy MJ/kg	17.46	Gross energy MJ/kg	18.98	18.70
Crude protein %	16.23	Crude protein %	10.55	9.577
Ether extract %	6.60	Ether extract %	11.67	10.85
Crude fiber %	7.28	Crude fiber %	8.93	9.27
Lysine %	1.31	Lysine %	0.81	0.48
Methionine %	0.15	Methionine %	0.11	0.11
Arginine %	0.93	Arginine %	0.54	0.55
Histidine %	0.43	Histidine %	0.24	0.22
Leucine %	1.42	Leucine %	0.70	0.69
Isoleucine %	0.68	Isoleucine %	0.31	0.36
Phenylalanine %	0.97	Phenylalanine %	0.57	0.54
Threonine %	0.70	Threonine %	0.44	0.66
Valine %	0.86	Valine %	0.56	0.54
Total calcium, mg/kg	7677.42	Total calcium, mg/kg	7642.45	6890.37
Total phosphorus, mg/kg	3190.30	Total phosphorus, mg/kg	5087.39	4181.97
Copper mg/kg	24.48	Copper mg/kg	29.11	20.39
Iron mg/kg	376.43	Iron mg/kg	237.98	217.08
Zinc mg/kg	112.95	Zinc mg/kg	135.43	156.19
Magnesium mg/kg	2166.49	Magnesium mg/kg	3371.88	2725.27
Manganese mg/kg	104.59	Manganese mg/kg	158.40	162.91
Selenium, mg/kg	0.70	Selenium, mg/kg	0.53	6.60

[a]The premix provided the following per kg of diets: VA 10700 IU, VD_3 2,300 IU, VE 45 mg, VK3 2.5 mg, VB_1 2.5 mg, VB_2 7.5 mg, niacinamide 29 mg, D-pantothenic acid 14.5 mg, folic acid 1.0 mg, VB_6 2.0 mg, VB_{12} 0.02 mg, biotin 0.15 mg, Fe ($FeSO_4{\cdot}H_2O$) 100 mg, Cu ($CuSO_4{\cdot}5H_2O$) 20 mg, Mn ($MnSO_4{\cdot}H_2O$) 45 mg, Zn ($ZnSO_4{\cdot}H_2O$) 80 mg, I (CaI_2O_6) 0.4 mg, Se (Na_2SeO_3) 0.35 mg, Co 0.15 mg, rice bran meal 1.088%, betaine 0.012%, choline chloride 0.08%, antioxidant 0.02%, sodium chloride 0.32%, sodium bicarbonate 0.025%, potassium chloride 0.025%, $CaCO_3$ 1.20%, calcium bicarbonate 0.25%, antimicrobial peptide 0.04%, glucin 0.01%, fungicide 0.05%, methionine 0.01%, threonine 0.05%, lysine 0.65%, glucose oxidase 0.02%, multienzyme complexes 0.025%.
[b]The premix provided the following per kg of diets: VA 10700 IU, VD_3 2,300 IU, VE 45 mg, VK_3 2.5 mg, VB_1 2.5 mg, VB_2 7.5 mg, niacinamide 29 mg, D-pantothenic acid 14.5 mg, folic acid 1.0 mg, VB_6 2.0 mg, VB_{12} 0.02 mg, biotin 0.15 mg, Fe ($FeSO_4{\cdot}H_2O$) 100 mg, Cu ($CuSO_4{\cdot}5H_2O$) 20 mg, Mn ($MnSO_4{\cdot}H_2O$) 45 mg, Zn ($ZnSO_4{\cdot}H_2O$) 80 mg, I (CaI_2O_6) 0.4 mg, Se (Na_2SeO_3) 0.35 mg, Co 0.15 mg, rice bran meal 1.73%, betaine 0.01%, choline chloride 0.08%, antioxidant 0.02%, sodium chloride 0.35%, sodium bicarbonate 0.03%, potassium chloride 0.03%, $CaCO_3$ 1.0%, calcium bicarbonate 0.2%, glucin 0.01%, fungicide 0.05%, methionine 0.005%, threonine 0.04%, lysine 0.295%, multienzyme complexes 0.025%.

using a hand-held pH meter and a colorimeter. Meat color traits (a^*, redness, and b^*, yellowness, L^*, lightness) were measured with a CR-410 hand-held chromameter (Kinica Minolta Sensing Inc., Osaka, Japan). The marbling score was determined by a five-member trained sensory panel using visual standard cards. The shear force was determined via a Warner–Bratzler shear force device (TA.XT Plus, Stable Micro Systems, Godalming, United Kingdom). The cooking loss and drip loss of meat were measured as previously described (20).

2.5 Determination of fatty acid composition

The extraction of fatty acids from LT muscle was conducted according to a previous study (20). The determination of the muscular fatty acid composition was performed on a GC/MS system consisting of a 7890B GC/5977A mass selective detector (Agilent Technologies, Inc. United States). The saturated fatty acid ratio (SFA), monounsaturated fatty acid ratio (MUFA), polyunsaturated fatty acids

ratio (PUFA), $\sum n-3$, $\sum n-6$, and $\sum n-6/\sum n-3$ were calculated according to a previous study (21).

2.6 Determination of the purine concentration

The concentrations of xanthine, adenine, guanine, hypoxanthine, and the purine metabolites creatinine, adenosine, and guanosine in the LT muscle were determined (22). Briefly, the samples were minced thoroughly, and 0.200 g ± 0.005 g was weighted. Three milliliters of 10% (v/v) perchloric acid was added to each meat sample and mixed. The mixture was subsequently incubated at 99°C for 60 min. After cooling, the pH of the mixture was adjusted to 7.0 ± 0.1 with 1 mol/L KOH, and the volume was adjusted to 10 mL. After shaking well, the supernatant was obtained by centrifugation (6,000 r/min, 10 min) and filtered through a 0.22 μm filter for HPLC analysis.

2.7 The colonic microbiota composition

In accordance with the instruction manual, microbial DNA was isolated from the colonic digesta via a genomic DNA extraction kit (TIANGEN, Beijing, China). The isolated DNA was separated via 1% agarose gel electrophoresis, and its concentration was quantified via a Nanodrop 2000. The 16S rDNA was amplified via PCR using primers for the V3–V4 region (341F: 5′-CCTAYGGGRBGCASCAG-3′; 806R: 5′-GGACTACHVGGGGTWTCTAAT-3′). After being purified, the PCR products were sequenced on the Illumina HiSeq platform (Novogene Bioinformatics Technology Co., Ltd., Beijing, China). The α-diversity, β-diversity, and principal coordinate analyses (PCoAs) of the colonic microbiota were analyzed (23).

2.8 Statistical analysis

The pen was used as an experimental unit for body weight, ADG, and FCR analysis. Individual pigs constituted the experimental unit for the analysis of carcass traits, meat quality, fatty acid and purine concentrations, and the colonic microbiota. Two-tailed independent-sample *t*-tests (SPSS 25.0 software) were performed to analyze differences across groups. Differences were considered significant at $p < 0.05$ (*), $p < 0.01$ (**) and $p < 0.001$ (***).

3 Results

3.1 Growth performance

The growth performance of the pigs is presented in Table 2. Compared with the Con group, the LPNS group had a significantly greater FCR value at the 20–50 kg stage and a significantly lower ADG at the 50–85 kg stage ($p < 0.05$). Moreover, the ADG of the LPNS group was significantly lower than that of the Con group at all stages ($p < 0.05$), and the time to slaughter was prolonged.

TABLE 2 The effect of LPNS on the growth performance of Daweizi pigs.

	Con	LPNS	*p*
Initial body weight, kg	24.66 ± 2.35	22.54 ± 1.38	0.886
Final body weight, kg	85.86 ± 4.75	85.16 ± 2.3	0.999
20 kg–50 kg			
ADG/kg	0.45 ± 0.03	0.43 ± 0.02	0.599
ADFI/kg	1.09 ± 0.15	1.38 ± 0.06	0.123
FCR	2.43 ± 0.28	3.21 ± 0.03*	0.032
50 kg–85 kg			
ADG/kg	0.45 ± 0.03*	0.32 ± 0.02	0.011
ADFI/kg	1.95 ± 0.11	1.73 ± 0.07	0.143
F/G	4.37 ± 0.38	5.54 ± 0.41	0.081
20–85 kg			
Feeding period, day	136	173	
ADG/kg	0.45 ± 0.02*	0.36 ± 0.01	0.036
ADFI/kg	1.83 ± 0.14	1.68 ± 0.07	0.375
FCR	4.05 ± 0.16	4.64 ± 0.19	0.055

*$p < 0.05$.

3.2 Carcass traits and meat quality

The result for carcass traits and meat quality are shown in Tables 3, 4. Compared with the Con diet, the LPNS diet significantly increased leanness and decreased the skin fat rate and bone rate of the Daweizi pigs ($p < 0.05$). In addition, the LPNS group had a lower b^* value and cooking loss than the Con group ($p < 0.05$).

3.3 Fatty acid profile

The fatty acid profile of LT muscle is presented in Table 5. Compared with the Con diet, the LPNS diet significantly increased the concentrations of C18:2*n*6, C20:3*n*3 and C22:6 in the LT muscle ($p < 0.05$). The concentrations of C18:3*n*3, C20:4*n*6 and C20:5*n*3 were elevated ($0.05 < p < 0.10$). The concentration of PUFA in pork was greater in the LPNS group than in the Con group, as were the concentrations of total *n*−3 and total *n*−6 ($p < 0.05$).

3.4 Purine profile

The purine profile of the LT muscle is presented in Table 6. Compared with the Con diet, the LPNS diet significantly reduced the contents of xanthine, hypoxanthine and guanine in the meat ($p < 0.05$). The total purine content in the LPNS group was also significantly lower ($p < 0.05$), with a value approximately half that of the Con group. The contents of purine metabolites, such as creatinine and guanosine, were significantly lower in the LPNS group than in the Con group ($p < 0.05$).

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TABLE 3 The effect of LPNS on the carcass trait of Daweizi pigs.

	Con	LPNS	*p*
Slaughter rate, %	70.46 ± 0.50	69.67 ± 0.51	0.286
Lean meat rate, %	36.56 ± 0.58	41.51 ± 1.28*	0.003
Fat + skin rate, %	54.50 ± 0.61**	49.11 ± 1.04	0.001
Bone rate, %	9.75 ± 0.30*	8.11 ± 0.69	0.047
Loin muscle area, mm^2	12.38 ± 0.65	13.78 ± 1.03	0.269
Skin thickness, mm	4.43 ± 0.19	4.42 ± 0.39	0.982
Backfat thickness, mm	52.14 ± 2.91	45.39 ± 2.68	0.110
Carcass straight length, cm	84.13 ± 0.79	84.58 ± 1.01	0.731
Carcass oblique length, cm	74.00 ± 0.78	72.60 ± 0.87	0.251

*$p < 0.05$; ** $0.001 \leq p < 0.01$.

TABLE 4 The effect of LPNS on the meat quality of Daweizi pigs.

	Con	LPNS	*p*
pH	6.22 ± 0.09	6.78 ± 0.24	0.062
L	47.08 ± 1.01	46.86 ± 0.92	0.875
a	14.75 ± 0.37	15.06 ± 0.21	0.480
b	4.82 ± 0.37*	3.69 ± 0.33	0.038
Drip losses, %	2.75 ± 0.30	3.14 ± 0.49	0.511
Cooking losses, %	25.25 ± 1.00*	16.59 ± 1.78	0.001
Crude protein (dry matter), %	81.51 ± 1.87	84.08 ± 1.46	0.299
Crude fat (dry matter), %	16.01 ± 2.07	13.04 ± 1.27	0.244

*$p < 0.05$; ** $0.001 \leq p < 0.01$.

3.5 Colonic microbiota

An average of 811 OTUs were detected from the colonic microbiota. The number of observed species and the Chao 1, Shannon and Simpson indices did not differ significantly between the groups (Table 7). The top 10 phyla accounted for approximately 90% of the total phyla; the dominant phylum was *Firmicutes*, followed by *Bacteroidetes*, which accounted for more than 80% of the total phyla (Figure 1A). The top 10 genera are presented in Figure 1B. The most abundant genus in the Con group was *Streptococcus*, whereas the most abundant genus in the LPNS group was *Lactobacillus*. The principal component analysis (PCA) plots revealed a difference in clustering between the two groups (Figure 1C).

The differences in the relative abundance of the microbiota at the phylum and genus levels were analyzed and are shown in Figures 2, 3. Compared with the Con diet, the LPNS diet significantly decreased the *Firmicutes* and *Euryarchaeota* levels and the Fir/Bac ratio at the phylum level ($p < 0.05$). The *Bacteroidota* level in the LPNS group was greater than that in the Con group. At the genus level, the relative abundances of *Lactobacillus*, *Rikenellaceae_RC9_gut_group* and *Eubacterium_ventriosum_group* were significantly greater in the LPNS group than in the Con group ($p < 0.05$). The relative abundances of *Streptococcus*, *UCG0002*, *Methanobrevibacter*, *Clostridi-um_sensu_stricto_1*, and *Terrisporobacter* were significantly lower than those in the Con group ($p < 0.05$).

TABLE 5 The effect of LPNS on the fatty acid profile of longissimus dorsi muscle of Daweizi pigs (% of total fatty acids).

	Con	LPNS	*p*
C14:0	0.74 ± 0.12	0.84 ± 0.22	0.699
C15:0	0.011 ± 0.002	0.015 ± 0.002	0.171
C15:1	0.006 ± 0.001	0.008 ± 0.001	0.386
C16:0	14.14 ± 2.04	15.98 ± 3.94	0.673
C16:1	1.71 ± 0.19	1.59 ± 0.36	0.775
C17:0	0.06 ± 0.01	0.10 ± 0.02	0.092
C17:1	0.05 ± 0.01	0.07 ± 0.01	0.240
C18:0	7.75 ± 1.38	8.72 ± 2.11	0.702
C18:1*n*9	21.48 ± 3.04	24.97 ± 6.78	0.632
C18:2*n*6	3.56 ± 0.61	6.00 ± 0.79	0.028
C18:3*n*6	0.023 ± 0.004	0.028 ± 0.003	0.385
C18:3*n*3	0.07 ± 0.02	0.16 ± 0.04	0.057
C20:0	0.14 ± 0.02	0.17 ± 0.04	0.481
C20:1	0.47 ± 0.09	0.54 ± 0.15	0.677
C20:2	0.17 ± 0.04	0.24 ± 0.05	0.260
C21:0	0.016 ± 0.002	0.0207 ± 0.002	0.149
C20:3*n*6	0.103 ± 0.016	0.137 ± 0.014	0.132
C20:4*n*6	0.75 ± 0.11	1.04 ± 0.10	0.068
C20:3*n*3	0.036 ± 0.006	0.056 ± 0.006*	0.035
C20:5*n*3	0.027 ± 0.004	0.038 ± 0.004	0.071
C22:0	0.029 ± 0.003	0.037 ± 0.003	0.079
C22:1*n*9	0.020 ± 0.002	0.0245 ± 0.003	0.216
C23:0	0.014 ± 0.002	0.019 ± 0.002	0.151
C24:0	0.034 ± 0.004	0.045 ± 0.005*	0.105
C22:6	0.030 ± 0.004	0.045 ± 0.005	0.022
SFA	22.94 ± 3.56	25.94 ± 6.31	0.675
MUFA	23.74 ± 3.28	27.20 ± 7.29	0.658
PUFA	4.77 ± 0.79	7.74 ± 0.90*	0.026
$\sum n-3$	0.14 ± 0.02	0.25 ± 0.04*	0.024
$\sum n-6$	4.43 ± 0.72	7.20 ± 0.81*	0.024
$\sum n-6/\sum n-3$	32.44 ± 1.00	29.80 ± 1.52	0.160

SFA = C14:0 + C15:0 + C16:0 + C17:0 + C18:0 + C20:0 + C21:0 + C22:0 + C23:0 + C24:0. MUFA = C15:1 + C16:1 + C17:1 + C18:1*n*9 + C20:1 + C22:1*n*9 + C24:1. PUFA = C18:2*n*6 + C18:3*n*6 + C18:3*n*3 + C20:2 + C20:3*n*6 + C20:4*n*6 + C20:3*n*3 + C20:5*n*3 + C22:6. $\sum n-3$ = C18:3*n*3 + C20:3*n*3 + C20:5*n*3. $\sum n-6$ = C18:2*n*6 + C18:3*n*6 + C20:3*n*6 + C20:4*n*6. *$p < 0.05$.

4 Discussion

The advantages and disadvantages of reducing dietary protein with free amino acid supplementation have drawn great attention in recent years. Growth performance is an important indicator for

TABLE 6 The effect of LPNS on the purine profile of longissimus dorsi muscle of Daweizi pigs (μg/g).

	Con	LPNS	*p*
Zanthine	0.105 ± 0.022*	0.052 ± 0.006	0.038
Adenine	0.028 ± 0.009	0.020 ± 0.003	0.433
Guanine	0.262 ± 0.143*	0.098 ± 0.028	0.016
Hypoxanthine	146.17 ± 16.06***	62.08 ± 9.55	<0.001
Creatinine	191.23 ± 9.60***	128.13 ± 6.10	<0.001
Adenosine	0.079 ± 0.031	0.161 ± 0.029	0.073
Guanosine	4.16 ± 0.41**	2.47 ± 0.25	0.004
Total[a]	146.56 ± 16.11**	62.25 ± 9.58	0.001

[a]Total = Zanthine + Adenine + Guanine + Hypoxanthine.
*$p < 0.05$; ** $0.001 \leq p < 0.01$; ***$p < 0.001$.

TABLE 7 The effect of LPNS diet on the alpha diversity of microbiota in the colon.

	Con	LPNS	*p*
Observed OTUs	780.9 ± 25.9	841.1 ± 24.4	0.113
Chao 1	782.0 ± 25.9	842.4 ± 24.4	0.111
Shannon	7.14 ± 0.23	7.62 ± 0.13	0.095
Simpson	0.96 ± 0.01	0.98 ± 0.00	0.142

evaluating low-protein diets. During the growing and finishing stages, a dietary crude protein reduction of 3% or less of the NRC-recommended amount does not affect growth performance (19, 24). However, if the reduction is more than 3%, e.g., 6% or more, the growth performance of pigs is significantly lower than that of pigs fed a high-protein diet (25–27). Unlike Long White pigs or Large White pigs, Daweizi pigs have rough feeding resistance and are better adapted to very-low-protein diets, as they lived on leaves, roots, and berries in the wild before being domesticated (5). Therefore, we designed a very-low-protein diets in the present study. The CP content of the LPNS diet was approximately 10%, which was 3% lower than the Pig Nutrient Requirements of China and 6% lower than the NRC 2012. The CP content of the Con diet was approximately 16%, which was 3% greater than the Pig Nutrient Requirements of China. After being fed the LPNS diet, the ADG of the pigs in the growing stage did not differ from that of the Con group, but the ADG of the pigs in the fattening stage decreased. Studies have shown that the concentration of branched-chain amino acids in a low-protein diet is a limiting factor affecting the growth performance of fattening pigs (28); therefore, we hypothesized that increasing the content of branched-chain amino acids in the LPNS diet may help improve the growth performance of Daweizi pigs.

The use of a low-protein diet has caused great concern, in part, because of its effect on fat deposition. Many studies have shown that feeding with an LP diet increases the amount of dietary energy available for fat deposition (29, 30). Supplementation with functional amino acids, plants, probiotics, etc., may help reduce excess fat deposits in the body. Tea polyphenols, polysaccharides, and other functional substances can accelerate the rate of body metabolism and promote fat decomposition (31, 32). Tangerine peel powder, a traditional Chinese medicine, has been proven to lower blood cholesterol (33). In the present study, the LPNS diet did not lead to fatter carcasses but rather to leaner carcasses, suggesting that supplementation of black tea and tangerine peel in the low-protein diet may help to decrease fat deposition caused by the low-protein diet. This may also be related to the different breeds of pigs, as fat pigs were used in this study, whereas all the pigs reported to have been used in other studies were lean pigs. Since this study used fatty-type pigs, while all other reports used lean-type pigs, the pig breeds may also have contributed to the differential results.

In addition to growth performance, meat quality is a key indicator of farming efficiency (34). Tea powder is reported to significantly improve the meat color and increase the water retention (increased moisture content) of meat (35–37). In this study, the LPNS diet, which contains 2% black tea powder, significantly improved the meat quality by reducing the yellowness value and cooking losses. Meat is an important source of unsaturated fatty acids for humans. The fatty acid composition of pork can be influenced by feed components. For example, pigs cannot synthesize linoleic acid, and its content in tissues is highly correlated with dietary intake (38). The LPNS diet contains a large proportion of rice and sweet potatoes, both of which are rich in linoleic acid, so the linoleic acid content in meat is significantly elevated (39). On the other hand, linoleic acid is a metabolic precursor of *n*−3 PUFAs, including C20:5 and C22:6 (40). In this study, we found that the contents of C20:5, C22:6, and *n*−3 PUFAs in meat were significantly elevated in the LPNS group. PUFA *n*−3 intake is very important for reducing the risk of cardiovascular disease in humans. However, PUFA *n*−3 intake in adults is very low, and increasing the level of PUFA *n*−3 in meat is essential to help consumers meet the minimum nutritional requirements (40). High-protein diets are usually high in purines, and soybeans are a purine-rich food. Low-protein diets can reduce purine intake (41). After consumption of the LPNS diet, the meat of the Daweizi pigs had a very low purine content, without affecting the fat and protein levels, making it a high-quality meat. Nowadays there are a lot of people suffering from hyperuricosuria and they need to reduce the purine intake. So the pork from the LPNS group may meet the dietary needs of patients with hyperuricosuria.

The type and amount of feed ingredients can affect the composition of gut microorganisms and thus the metabolism of pigs (42). In the present study, the intestinal Fir/Bac ratio was significantly reduced after LPNS treatment, and this change was associated with a low body fat percentage. Moreover, the LPNS group showed a significant reduction in the abundance of intestinal *Euryarchaeota*, one of the few archaea known to colonize humans and animals, which contain many methanogens. Methanogens are hydrogenotrophic groups involved in interspecies hydrogen transfer. H2 utilization and transfer between bacteria and methanogens can increase energy absorption in the gut, which is associated with obesity and is clinically harmful to the host (43). In addition, many *Lactobacillus* species have been shown to degrade purines (44, 45). The ability of LPNS to reduce purines in meat may be related not only to the low-protein diet but also to the increased number of *Lactobacillus* in the gut. The relative abundances of both the *Rikenellaceae RC9 gut group* and the *Eubacterium ventriosum group* were significantly elevated in the colon, and both alleviated intestinal inflammation and increased fiber utilization in the diet (46, 47). LPNS diets significantly reduced intestinal

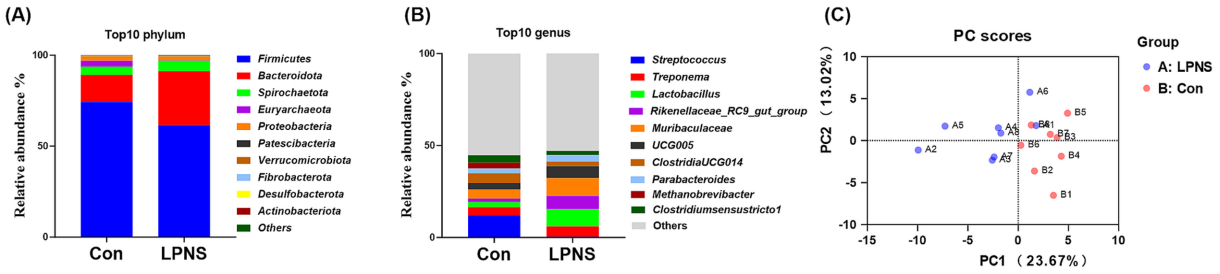

FIGURE 1
Effects of LPNS diet on the microbiota diversity in the colon of Daweizi pigs.

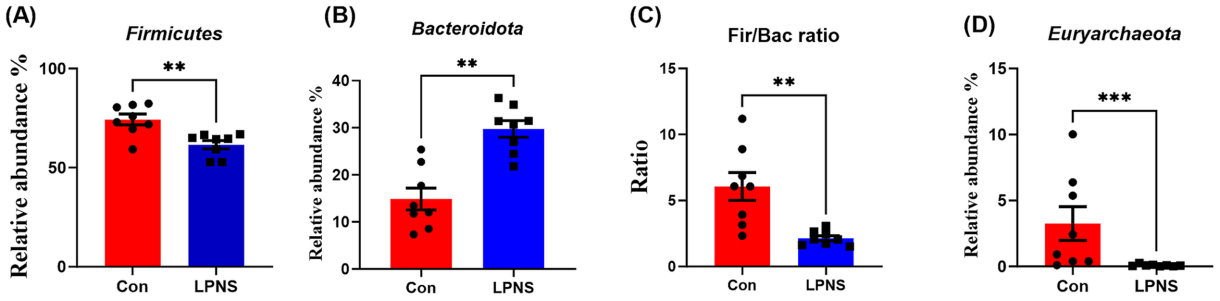

FIGURE 2
Taxonomy difference of intestinal microbiota at the phylum level between the two groups.

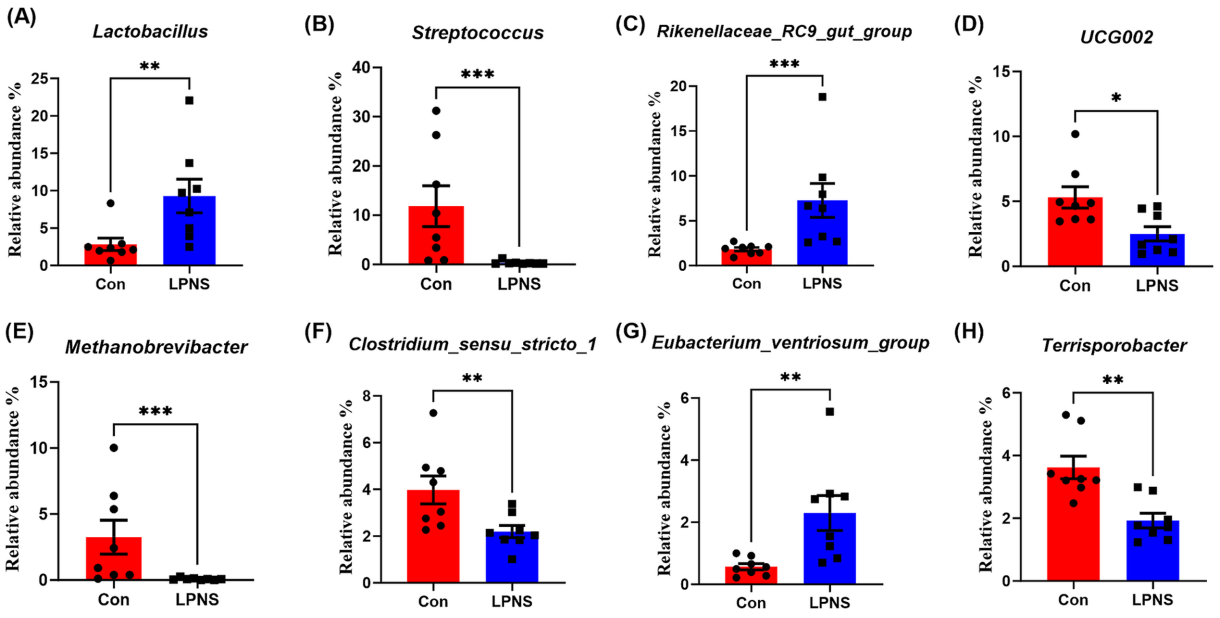

FIGURE 3
Taxonomy difference of intestinal microbiota at the genus level between the two groups.

Clostridium_sensu_stricto_1 and *Streptococcus*, which are thought to be positively correlated with the expression of intestinal proinflammatory factors (48, 49). These results suggest that LPNS diets are more helpful in maintaining intestinal microecological homeostasis and promoting intestinal health.

5 Conclusion

We designed a low-protein soybean-free (LPNS) diet. Compared with the corn-soybean meal-type diet, the LPNS diet significantly improved the carcass traits and meat quality of Daweizi

pigs, although it affected the feed-to-weight ratio. The use of functional herbs along with a low-protein diet helped to regulate fat and purine metabolism in fatty-type pigs. The use of other alternative feed sources that could be grown locally for fatty-type local pig breeds, which are cost-effective and suitable for small-scale pig farming, is suggested.

Data availability statement

The data presented in the study are deposited in the NCBI repository, accession number: BioProject PRJNA1171598.

Ethics statement

The animal studies were approved by the Animal Care and Use Committee of the Institute of Subtropical Agriculture, Chinese Academy of Sciences. The studies were conducted in accordance with the local legislation and institutional requirements. Written informed consent was obtained from the owners for the participation of their animals in this study.

Author contributions

HF: Formal analysis, Methodology, Writing – original draft. TY: Investigation, Methodology, Writing – original draft. HN: Methodology, Supervision, Writing – review & editing. JLi: Investigation, Methodology, Writing – review & editing. FL: Methodology, Writing – review & editing. JLiu: Writing – review & editing, Software. YY: Funding acquisition, Supervision, Writing – review & editing.

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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.2024.1516198/full#supplementary-material

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Effect of best bet methane abatement feed on feed intake, digestibility, live weight change, and methane emission in local Menz breed sheep in Ethiopia

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This study continued the *in vitro* screening of locally available ruminant feedstuffs for optimum nutrient composition and low methane (CH_4) production in Ethiopia. The best bet feeds from the *in vitro* study, hereafter called the test feeds, include dried leaves of *Acacia nilotica*, *Ziziphus spina-christi*, and brewery spent grains (BSG). The study involves four treatments: Control, Acacia, BSG, and Ziziphus; each treatment provided an equivalent crude protein and estimated enteric CH_4 emissions using Modeling and a Laser CH_4 detector (LMD). The experiment was designed as a randomized complete block, using initial weight as the blocking factor for 21 yearling castrated Menz sheep. The study spanned 90 days, and digestibility trials were carried out following a month of the feeding trial. The control group exhibited a significantly ($p < 0.001$) lower dry matter intake (DMI) compared to the test feed group, which had a higher intake, particularly in the Ziziphus group. However, the Ziziphus group demonstrated significantly ($p < 0.01$) lower CP digestibility than the other groups. The test diet also led to a significantly ($p < 0.001$) higher weight gain. Notably, the Ziziphus group demonstrated superior performance in weight change (BWC), final body weight (FBW), and average daily gain (ADG). Similar results were observed for CH_4 production (g/day), CH_4 yield (g/kg DMI), and CH_4 intensity (g CH_4/kg ADG) using both CH_4 measuring methods. The CH_4 emission intensity was significantly ($p < 0.04$) lower in the test feed groups than in the control group. The control group emitted 808.7 and 825.3 g of CH_4, while the Ziziphus group emitted 220 and 265.3 g of CH_4 per kg of ADG using the Modeling and LMD methods, respectively. This study indicates that LMD could yield biologically plausible data for sheep. Although the small sample size in the Ziziphus group was a limitation of this study, leaf meals from *Ziziphus spina-christi and Acacia nilotica*, which are rich in condensed tannins (CTs), have resulted in considerable weight gain and enhanced feed efficiency, thereby making these leaf meals a viable and sustainable feed option for ruminants in Ethiopia.

KEYWORDS

condensed tannins, laser methane detector, local sheep, methane emission, modeling, protein efficiency ratio, *Ziziphus spina-christi*

1 Introduction

In Ethiopian farm households, financial income from sheep and goat production constitutes 40%, which also equates to 19% of the subsistence food value from all livestock production and 25% of the country's domestic meat consumption (1, 2). Small ruminants also contribute about 2% of the national gross domestic product (GDP) (3). Ethiopia claims an estimated 38 million sheep, 99.6% of which are indigenous breeds. The country also supports 14 traditional sheep-breeding communities (4, 5).

The scenario of sheep production in Ethiopia relies on low input systems such as poor quality and quantity of feed resources, lack of appropriate feeding system, poor production and reproduction traits, and low productive and reproductive performance. This low-productivity production system uses more energy to produce each unit of animal product than those with high-productivity. The low-input system is responsible for the bulk of CH_4 emissions (6). Based on CSA (4) report, grazing is the primary type of feeding (57.8%), followed by crop residue (29.8%). Hay and by-products comprise about (6.7%) and (1.5%) of the total feeds, with the remaining (4.2%) other feed types, such as improved forage.

Ruminants that consume low-quality feed are known to produce more CH_4 per unit of product compared to those on higher-quality diets (7). As a result, animal nutritionists are urged to investigate alternative feed resources that can be integrated with existing dietary components to lessen CH_4 emissions while maintaining productivity (8). Enhanced feeding could significantly improve ruminants' digestive efficiency and lower CH_4 emissions by as much as 50% per unit of feed intake (9). In Ethiopia, some of the top feed sources with low CH_4 yields include *Acacia nilotica (L.)* (6.6 g/kg DM), *Ziziphus spina-christi* (7.8 g/kg DM), and BSG (8.1 g/kg DM) (10).

This study sets out to assess locally available feed that improves the feeding value of the existing feed resource, increases or maintains animal productivity, and reduces CH_4 intensity (i.e., g CH_4/kg product) in the local Menz sheep breed in Ethiopia.

The selection criteria for test feeds in this trial were based on the *in vitro* output, focusing on low CH_4 yield and optimal nutritional content from feed sources available locally in Ethiopia (10). Therefore, the study's objective was to evaluate the effects of best-bet feeds on enteric CH_4 emissions, weight gain, digestibility, and methane emission in local Menz sheep breeds. This research supports the country's goal of adopting climate-smart agriculture within the livestock sector (11).

2 Materials and methods

2.1 Study area

The experiment was conducted at Debre Berhan Agricultural Research Center (DBARC). The experiment site is located in the central Highlands of Ethiopia about 120 km northeast of Addis Ababa, at an altitude of 2,800 m above sea level. The geographical location of DBARC is from 09°35′ 45″ to 09° 36′ 45″ north latitude and 39° 29′ 40″ to 39°31′ 30″ east longitude.

2.2 Experimental animals and management

Twenty-one yearling Menz sheep with a mean initial live body weight of 22.7 ± 1.7 kg (mean ± SD) were purchased from a nearby local livestock market. All sheep purchased were Burdizzo castrated and vaccinated for pasteurellosis, sheep pox, and anthrax during a 15-day quarantine period. In addition, both internal and external parasites were treated with ivermectin.

2.3 Experimental feed preparation and feeding

The three test feeds in this experiment include BSG, dried leaves of *Acacia nilotica*, and *Ziziphus spina-Christi.*

Acacia nilotica and *Ziziphus spina-christi* leaves were harvested from Debre Berhan University research site and farmers' trees in Showarobit. The branches were pruned and placed on a canvas to sun dry. Afterward, the leaves were removed by gently striking the branches with sticks. The BSG was obtained from Dashen Brewery factory and sun-dried on a canvas floor. The bulk of the dried foliage and BSG were stored in jute bags for subsequent feeding experiments. The other feed ingredients used for the experiment were Wheat bran (WB), Niger seed cake (NG), and salt lick. Control diet constituted only WB and NG. Supplements were divided into two halves and provided at 8:30 h and 14:00 h. Additionally, grass hay, dominated by *Andropogon amethystinus Steud hay*, was chopped manually and fed *ad libtum* along with salt lick as a basal diet to all animals. Water was available freely, daily feed offered, and refusals were recorded.

2.4 Experimental treatments and design

The treatment feeds were calibrated such that the test feeds contained equivalent amounts of crude protein to the control diet. The feeding experiment was conducted using a randomized complete block design for 90 days. Six animals were assigned to each treatment, except for the Ziziphus group, which had only three animals due to a shortage of *Ziziphus spina-christi* at the time of the experiment. A possible limitation of the study is that only three sheep were used for the Ziziphus group, and one animal from the BSG group had to be dropped due to a sudden unexplained drop in intake.

2.5 Data collection

2.5.1 Feed intake measurement

The diet offered, and orts were recorded daily for each animal to measure DMI. Representative samples of feed offered per batch and refused per animal were collected every 3 days to determine dry matter.

2.5.2 Live weight measurement

Initial body weight was measured by taking the mean of two consecutive weights after overnight fasting before the beginning of the actual feeding trial and every 15 days thereafter using hanging digital balance with 10 g graduation.

2.5.3 Apparent digestibility trial

At 31 days of the feeding trial, animals were fitted with fecal collection bags for digestibility study for 10 consecutive days, where the first 3 days were used as adaptation and the remaining 7 days as sampling. All the daily fecal outputs were collected, weighed, and recorded for each animal, and a sub-sample of approximately 100 g was taken from each animal after thorough mixing and stored at -20°C. The frozen daily fecal output was thawed, pooled for the sampling days per animal and treatment, and dried at 65°C for 72 h. Dried samples were ground to pass through a 1 mm sieve and stored in a plastic bag for chemical analysis.

2.6 Enteric CH_4 measurements

In the final week of the experiment, a portable laser CH_4 detector LMD (Crowcon Detection Instruments Ltd., Tokyo, Japan), was utilized for three consecutive days before and after the morning and evening feedings. The concentration of CH_4 was measured by directing a green laser beam toward the nostrils of the sheep for 3 continuous minutes to estimate the CH_4 concentration at 1 m from the resting animal in their feeding pen (Figure 1). The LMD was linked to a tablet with the GasViewer app through Bluetooth for data export and storage. The LMD's output is a time series of CH_4 emission values from a single animal, encompassing both eructation and respiration, indicative of the respiratory cycle. The nonlinear generalized reduced gradient (nonlinear GRG) method in Excel 2019 (Microsoft Corporation, Redmond, WA, USA) was employed for calculating eructation and respiration, as documented by Kobayashi et al. (12).

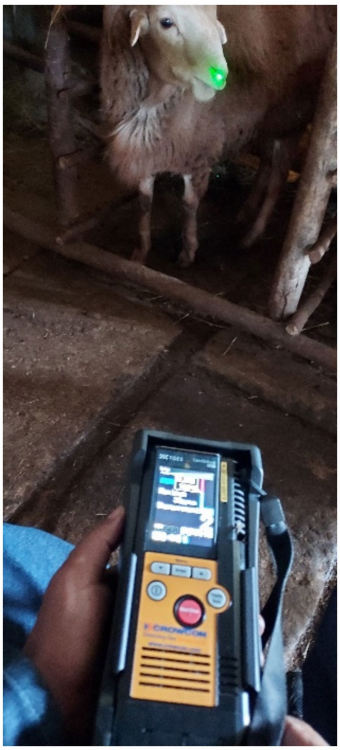

FIGURE 1
Demonstrating LMD measurement during the experiment.

The CH_4 levels, recorded in parts per million (ppm), were then converted to grams per day using a formula adapted from Lanzoni et al. (13), specifically tailored for sheep.

$$CH4\left(\frac{g}{\min}\right) = CH4\ average * V * R * \alpha * \beta * 10^{-6}$$

$$CH4\left(\frac{g}{day}\right) = CH4\left(\frac{g}{\min}\right) * 1440\left(\frac{\min}{day}\right)$$

Where, V represents the tidal volume (which is 12 mL/kg body weight); R denotes the respiratory rate (resting respiratory rate of sheep ranges from 16 to 34 breaths per minute according to Reece et al. (14), and an average of 25/min is used); α is the conversion factor for (CH_4 0.000667 g/mL); and, β denotes factor representing the dilution correction, (which accounts for the discrepancy between breath and total methane production in sheep and typically assumed to range from 5 to 8, with an average value of 6.5 being adopted). The value 1,440 represents the number of minutes per day. The CH_4 average utilized in this study represents the mean of peaks and troughs, aiding in capturing each respiratory cycle (15). In addition, the current dilution factor assumed the difference in tidal volume, CH_4 production, and respiratory rate between sheep and large ruminants.

In addition, enteric CH_4 emissions were estimated using a model developed from an intercontinental database (16). Using the following formula:

$$CH4\left(\frac{g}{day}\right) = -0.669 + 9.19 * DMI + 0.0495 * OMD + 0.169 * BW$$

Where DMI denotes dry matter intake, OMD denotes organic matter digestibility, and BW denotes body weight.

2.7 Chemical analysis

The chemical composition of the feed ingredients is presented in (Table 1) and the proportion of the treatment feed ingredients (Table 2). A detailed procedure, including *in vitro* CH_4 yield and condensed tannin content (CTs), of the test feed can be found in Bekele et al. (10).

2.8 Calculations for derived values

Daily feed intake (g/d) of both supplement and basal feeds was determined by the difference between the amount of feed offered and its constituents (OM, CP, NDF) and refusal on a dry matter basis.

The average daily live weight gain (ADG, g/d) was calculated by subtracting initial weight from final weight and dividing by the days of feeding.

Apparent total tract digestibility coefficients of feed DM and its nutrients were calculated from the ingested and excreted amounts in the feces of each component.

TABLE 1 Chemical composition of the experimental feed ingredient used for *in vivo* feeding study.

Feed ingrident	DM, g/kg	Chemical composition (g/kg DM)			Total CTs in mg/g
		NDF	CP	OM	
Grass hay (Andropogon amethystinus Steud)	944	652	46	916	–
Acacia nilotica (L.)	946	142	204	940	15.3
Ziziphus spina-christi	927	254	158	918	340.8
Brewery's spent grain	934	519	253	957	–
Wheat bran	923	380	166	942	–
Niger seed cake	940	191	442	924	–

NDF: NDF assayed with a heat-stable amylase and expressed inclusive of residual ash; CP: crude protein; DM: dry matter; OM: organic matter; CTs: condensed tannins including soluble and cell-bound. Source: Bekele et al. (10).

TABLE 2 Proportion of the treatment feed ingredients on a DM basis for the *in vivo* trial.

Treatments	Wheat bran (g)	Noug seed cake (g)	Test feed (g)	Grass hay(g)
Control	200	200	0	*Ad libitum*
Acacia	100	100	304	*Ad libitum*
BSG	100	100	243.2	*Ad libitum*
Ziziphus	100	100	380	*Ad libitum*

Additionally, calculations were performed to derive data for protein efficiency ratio (PER) and CH_4 emission intensity from the literature that did not provide direct values.

2.9 Data analysis

The analysis of variance (ANOVA) for data on feed intake, body weight change (BWC), and digestibility was conducted using R version 4.3.0 (17). Initial body weight (IBW) served as a covariate in the statistical analysis of ADG, BWC, and final body weight (FBW). This was performed using the 'lm' function with the following model:

$$Y_{ij} = \mu + T_i + B_j + e_{ij}$$

Where

Y_{ij} is an observed variable for the i^{th} treatment, j^{th} block. μ is the overall mean, T_i is i^{th} treatment, B_j is j^{th} block, and e_{ij} is the residual error for the i^{th} treatment and j^{th} block. The results were considered statistically significant when $p < 0.05$. Duncan's Multiple Range Test method was employed for *post hoc* analysis. Simple linear regression analysis was also performed using the "ggscatter" function from the "ggpubr" package.

3 Results

3.1 Feed intake and apparent digestibility

The Ziziphus group demonstrated significantly ($p < 0.001$) higher DM and OM intake than others, while the BSG group showed the highest NDF intake. Additionally, the Ziziphus group exhibited the lowest CP digestibility (Table 3).

3.2 Body weight change

The test diet groups resulted in significantly more ADG than the control group (Table 4). Among the treatments, the Ziziphus group showed significantly ($p < 0.001$) the highest ADG, FE, and PER. Additionally, sheep fed with dried leaves (Ziziphus and Acacia groups) had significantly higher ADG than those solely on agro-industrial by-products (BSG and Control groups), as shown by the weight trend in Figure 2.

3.3 Enteric CH_4 emission

Table 5 presents the estimated enteric CH_4 emissions derived from modeling and LMD methods. The Ziziphus group recorded the highest CH_4 production yet the lowest CH_4 intensity among all treatment groups. Conversely, the control group had the lowest CH_4 production but the highest CH_4 intensity according to both methods. The results from the LMD and modeling were consistent across treatments, showing a strong correlation between CH_4 production and intensity, as evidenced by high (R^2_{adj} = 0.68–0.99), as depicted in Figure 3. This study found that feed efficiency (FE) significantly influenced CH_4 intensity, and the effect of FE on CH_4 intensity was greater than that of CT intake. The optimal R^2_{adj} value for FE was 0.70 (Figure 4), whereas the R^2_{adj} for total CT intake was only 0.011 (Figure 5).

Figure 5 illustrates the impact of CTs on FE, ADG, and CH_4 intensity. An incremental increase in total CT intake correlates with a 0.031% increase in FE and a 0.41 g/d rise in ADG. Moreover, CH_4 intensity is reduced by 3.2 grams per day.

4 Discussion

In this *in vivo* study, we employed modeling and LMD methods to estimate enteric CH_4 emissions. Furthermore, the trial results indicated that CT intake affects ADG, FE, and CH_4 emissions. In addition, FE had a notable effect on weight gain and CH_4 intensity.

4.1 Feed intake and apparent digestibility

The experimental diet, designed to be iso-nitrogenous, led to a notably higher consumption of *Ziziphus spina-christi dried leaves* in the Ziziphus group. Consequently, this group showed a

TABLE 3 Dry matter, nutrient intake (g) and digestibility coefficient of Menz sheep fed natural pasture hay as a basal diet and supplemented with control and test feed.

Intake (g)	Treatments				SEM	*p*-value
	Control	Acacia	BSG	Ziziphus		
DM	1085[d]	1185[b]	1124[c]	1254[a]	3.1	0.001
NDF	569[c]	558[d]	633[a]	607[b]	1.7	0.001
CP	154	157	155	155	0.46	0.14
OM	997[c]	1093[b]	762[d]	1150[a]	2.4	0.001
Digestibility						
DM	0.68	0.70	0.66	0.64	0.01	0.5
NDF	0.60	0.63	0.62	0.57	0.02	0.75
CP	0.81[a]	0.77[a]	0.79[a]	0.67[b]	0.01	0.003
OM	0.70	0.72	0.68	0.67	0.01	0.5

NDF, NDF assayed with a heat-stable amylase and expressed inclusive of residual ash; CP, crude protein; OM, organic matter: [a,b,c,d]Mean values within a row with different superscript letters differ significantly ($p < 0.05$). Supplement: Niger seed cake, wheat bran, brewery-spent grain, and test feed.

TABLE 4 Body weight change, feed efficiency and protein efficiency ratio of Menz sheep fed natural pasture hay as basal diet and supplemented with control and test feed.

Variables	Treatments				SEM	*p*-value
	Control	Acacia	BSG	Ziziphus		
IBW(kg)	22.4	22.9	22.8	22.4	0.20	0.7
FBW(kg)	24.8[c]	27.6[b]	27.0[b]	30.3[a]	0.33	0.001
BWC(kg)	2.4[c]	4.7[b]	4.1b[c]	7.9[a]	0.29	0.001
ADG(g)	27.0[c]	52.4[b]	45.1[bc]	87.7[a]	3.15	0.001
FE (%)	2.5[c]	4.5[b]	4.0[bc]	7.0[a]	0.28	0.003
FCR	52[a]	23.4[b]	27.4[ab]	14.3[b]	4.14	0.04
PER	0.17[c]	0.33[b]	0.29[bc]	0.57[a]	0.020	0.001

IBW, initial body weight; FBW, final body weight; BWC, body weight change after feeding supplement (FBW-IBW); ADG, average daily gain; FE, feed efficiency calculated as average daily gain (ADG)/DMI; PER, protein efficiency ratio calculated as ADG/CP intake: [a,b,c]Mean values within a row with different superscript letters differ significantly ($p < 0.05$).

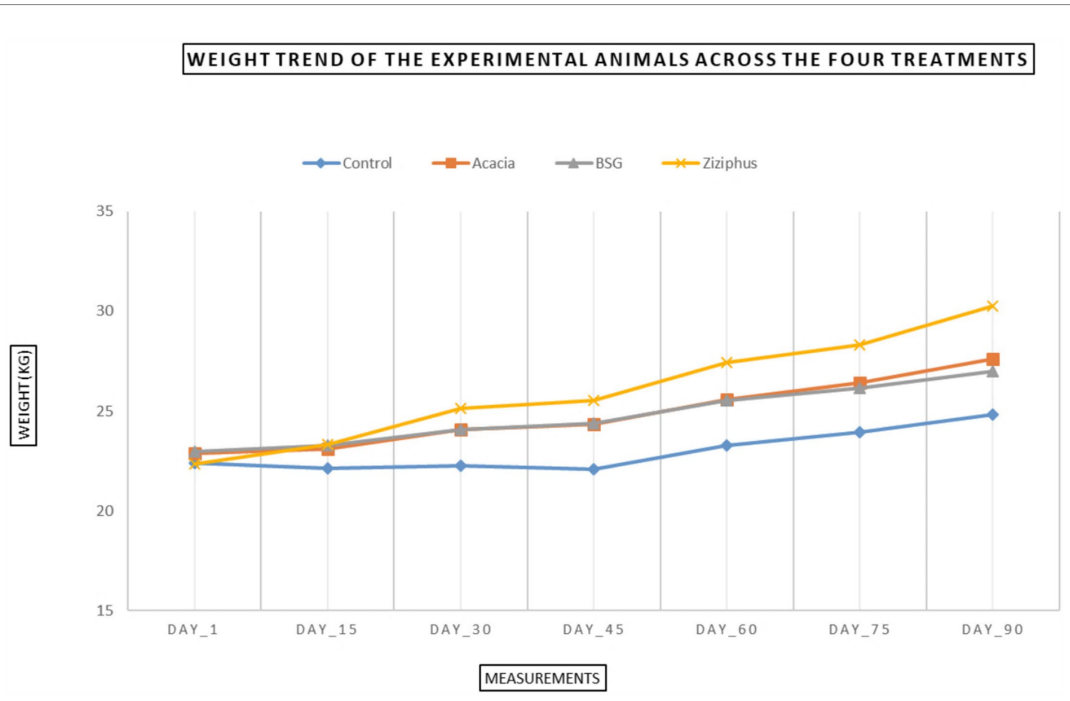

FIGURE 2
Trend in weight change over 90 days of the experimental period for Menz sheep.

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TABLE 5 Enteric CH_4 production, yield, and intensity of Menz sheep fed natural pasture hay as basal diet and supplemented with control and test feed using modeling and laser CH_4 detector methods.

Enteric CH_4	Treatments				SEM	*p*-value
	Control	Acacia	BSG	Ziziphus		
CH_4 production (g/day)						
Modeling	17^{d}	18.4^{b}	17.6^{c}	19.3^{a}	0.06	0.001
LMD	17.8^{c}	20.0^{b}	19.8^{b}	23.3^{a}	0.29	0.001
Average	17.4	19.2	18.7	21.3		
SD	0.49	1.06	1.56	2.83		
CV (%)	3	6	8	13		
CH4 yield (g/kg DMI)						
Modeling	15.6	15.6	15.6	15.4	0.04	0.25
LMD	16.3^{b}	16.8^{b}	17.6^{ab}	18.5^{a}	0.23	0.04
Average	16.0	16.2	16.6	17		
SD	0.49	0.85	1.4	2.2		
CV (%)	3	5	9	13		
CH4 intensity (g CH4/kg ADG)						
Modeling	808.7^{a}	363.5^{b}	428.4^{ab}	220^{b}	64.41	0.04
LMD	825.3^{a}	391^{b}	478^{ab}	265.3^{b}	61.51	0.04
Average	817	377.3	453.1	242.7		
SD	11.7	19.4	34.9	32		
CV (%)	1	5	8	13		

CH_4, methane; DMI, dry matter intake; ADG, average daily gain; ^{a,b,c,d}Mean values within a row with different superscript letters differ significantly ($p < 0.05$).

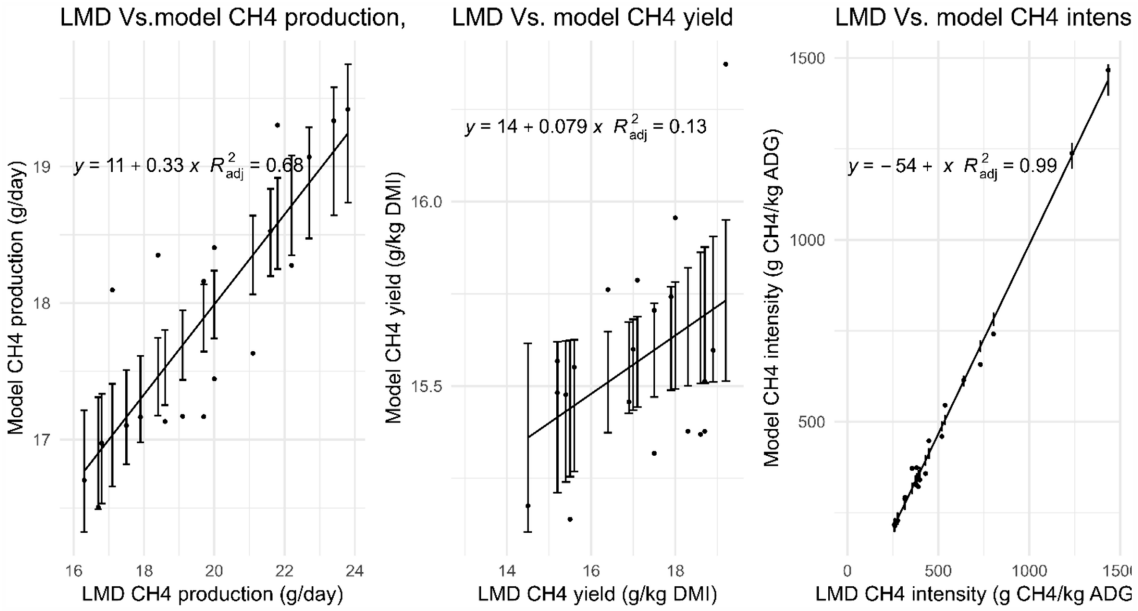

FIGURE 3
Relationship between enteric CH_4 emissions measured by LMD Vs. Modeling from local Menz sheep breed in Ethiopia.

significantly ($p < 0.001$) greater supplement and total DMI. This is attributed to the bulkiness of *Ziziphus spina-christi* compared to the other treatment groups.

The research indicated that there were no significant differences ($p > 0.05$) in the intake of crude protein (CP) among the treatment groups, and the average daily consumption of CP met the satisfactory levels as defined by Kearl (18) and NRC (19). Nevertheless, OM, FE, and PER were significantly ($p < 0.001$) higher in the Ziziphus group compared to other treatments. The protein efficiency ratio in this study ranged from 0.18 to 0.57, which is lower than the ratios found in afar

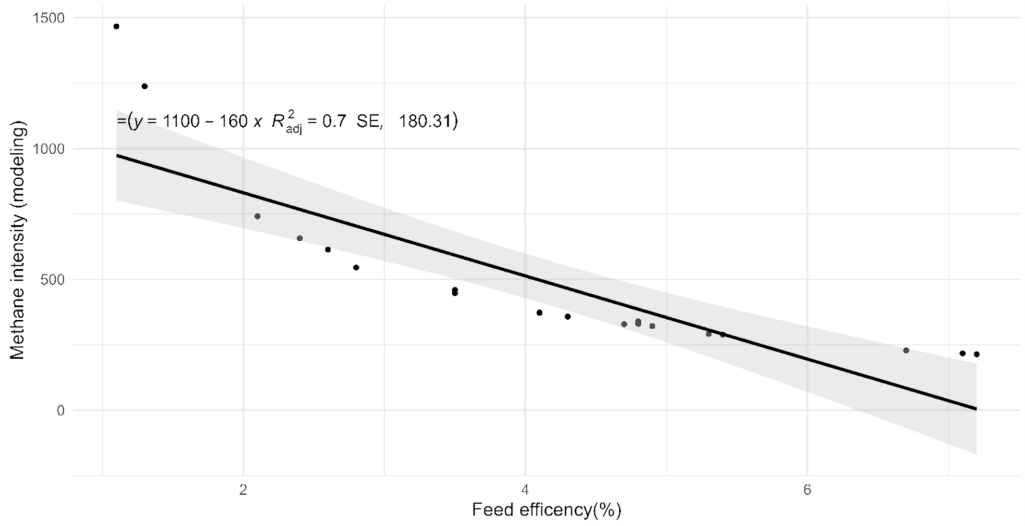

FIGURE 4
Feed efficiency effect on CH_4 intensity by the Menz sheep.

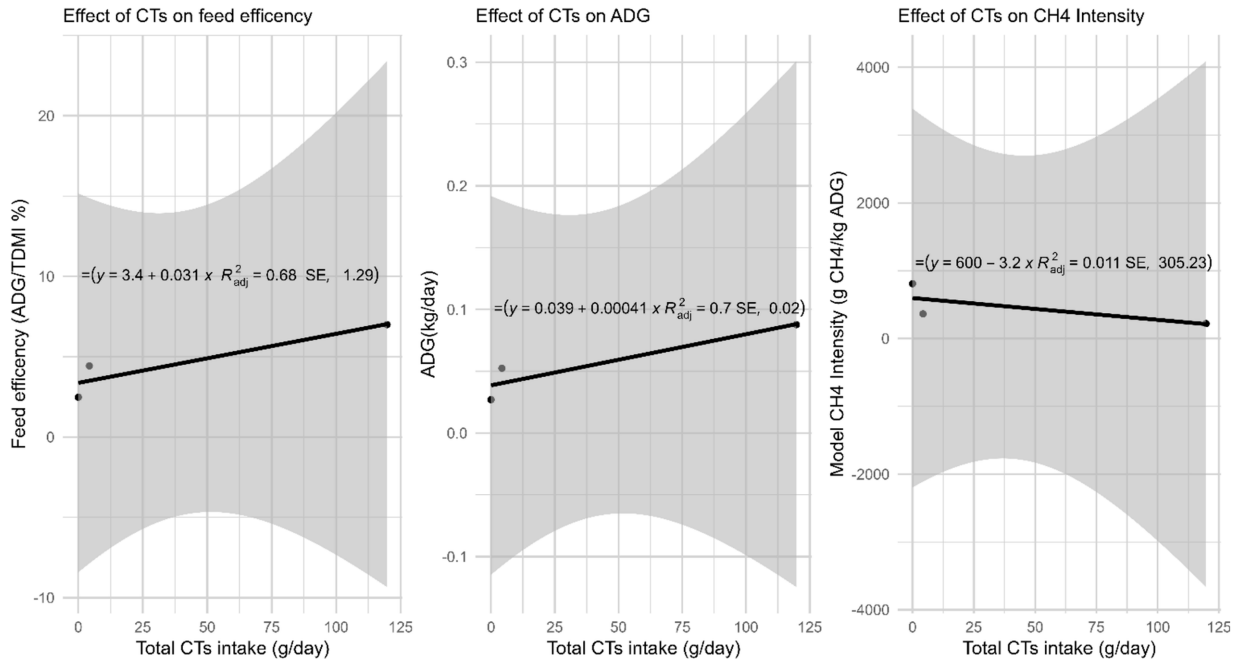

FIGURE 5
The effect of CTs intake on feed efficiency, average daily gain and CH_4 intensity of Menz Sheep (Average of FE, ADG and CH_4 intensity from modelling and total CTs values were used from Control, Acacia and Ziziphus group to construct the scatter plot).

sheep fed varying levels of *Brassica carinata* cake (0.65–0.83) (20) and Menze sheep supplemented with tagasaste leaves (0.73 to 0.91). However, the Ziziphus group showed a comparable PER value to goats supplemented with *Ziziphus spina-christi* leaves in Ethiopia (21).

The higher PER in the Ziziphus group may be attributed to the higher levels of bypass protein available. Valizadeh et al. (22) observed that lambs fed with low, medium, and high levels of bypass protein exhibited increased ADG and PER proportional to the bypass protein levels. Additionally, the notable PER in the Ziziphus group could stem from the increased DMI, and the beneficial effects of tannins on nitrogen utilization efficiency. Orzuna-Orzuna et al. (23) reported that in ruminants, dietary supplementation of CTs enhances the efficiency of ingested feed by increasing the duodenal flow of microbial protein and amino acids without losses in the rumen (24, 25). Protein Efficiency Ratio measures the nutritive value of protein sources. The higher the PER value of a protein, the more beneficial it is to the animal. It is also the easiest method of assessing the quality of proteins (26).

The group fed with Ziziphus exhibited significantly ($p < 0.01$) lower CP digestibility when compared to other groups. This difference is likely attributed to the high levels of CT present in the Ziziphus group. Kumar (62) noted that condensed tannins significantly affect digestibility, yet the influence of CT on rumen digestion differs depending on their concentration, type, and activity. Additionally, except for BSG, agro-industrial by-products typically have low fiber content and high digestibility, as noted by Mengistu et al. (27).

4.2 Body weight change

Supplements sourced from indigenous plants significantly ($p < 0.001$) enhanced BWC and ADG compared to the Control and BSG groups. This effect may be attributed to the beneficial role of secondary plant metabolites such as CTs (28). Min et al. (29) noted that moderate levels of tannins in forage legumes offer multiple benefits for ruminants, such as improved growth rates. Reducing rumen forage protein degradation due to reversible binding to these proteins and reducing the populations of proteolytic rumen bacteria increases essential amino acid absorption from the small intestine.

In the current *in vivo* study, the ADG ranged from 27 g/day for the control group to 87 g/day for the Ziziphus group. This resulted in a corresponding WC of 2.4 kg to 7.9 kg, respectively, over a period of 90 days during the feeding trial. The Ziziphus group showed superior results compared to similar research conducted elsewhere in Ethiopia. For example, Hailecherkos et al. (30) found that supplementing tree lucerne (*Chamaecytisus palmensis*) dried leaves or concentrate mixture to Washera sheep resulted in an ADG of 51.1 to 82.2 g/day. Worku et al. (31) reported that supplementing Kafa sheep with rice bran, Sesbania (*Sesbania sesban*) leaf, and their mixtures resulted in an ADG of 42.4 to 86.1 g/day. Kokeb et al. (32) found that supplementing Dorper-Menz crossbred sheep fed with local brewery by-product (*Atella*) and concentrate mixture led to an ADG of 42.2 to 73.2 g/day. Bonsi et al. (33) studied the effect of protein supplement sources (cottonseed cake, sundried leaves of *Leucaena leucocephala*, and sundried leaves of *Sesbania sesban*) on Menz sheep and found an ADG of 32.6 to 62.9 g/day. Our findings showed lower results compared to Ali et al. (34), who reported an ADG of 46.7 to 190 g/day for Bati goat breeds in Ethiopia when supplemented with sun-dried *Ziziphus spina-christi*. Mangara (35) also observed superior weight gain in goats fed with *Ziziphus spina-christi* than those fed with *Combretum adenogonium*. The enhanced performance of the Ziziphus group may be attributed to its high CT content (10). Basyony et al. (36) suggested that *Ziziphus spina-christi* leaves could be used as a natural growth promoter in rabbit diets. Its active ingredients have been shown to have antibacterial and antifungal properties (37).

4.3 Enteric CH_4 emission from sheep

Several methods have been developed to measure CH_4 emissions from ruminants. All methods have different scopes of applications, advantages, and disadvantages, and none of them is perfect in all aspects. Respiration chambers yield the most accurate measures of total enteric CH_4 emissions from ruminants. Modeling and LMD are non-invasive, non-contact, user-friendly, and cost-effective methods compared to the respiration chamber, rendering them suitable for resource-constrained countries such as Ethiopia (38–40).

4.3.1 Methane production

Comparable results were observed from the LMD values and reports of other studies using respiration chambers. For instance, Pelchen and Peters (41) found 22.2 g/day from 1,137 sheep observations under various feeding conditions. Pinares-Patiño et al. (40) reported 22.7 g/day in sheep-fed grass and 18.6 g/day in those fed pellets. The CH_4 production from modeling in our study was lower than that reported by Belanche et al. (16), which was 19.9 g/kg of sheep from an intercontinental database. However, it was higher than Amaral et al. (42), who reported 10.9–15.5 g/day for sheep grazing on pearl millet swards, measured using the sulfur hexafluoride (SF6) tracer technique. Chagunda et al. (43) reported that ruminating cows produced higher CH_4 production using LMD than estimated by empirical modeling, which aligns with our findings. In a separate study, Chagunda et al. (44) found that LMD measurements showed higher means and variation than those taken from metabolic chambers in sheep and cattle. However, the trend of the measurements from the LMD and the metabolic chamber was similar. Therefore, LMD could be a plausible technique for CH_4 emission studies in Sheep if a large number of animal data is taken.

This study observed that higher total intake relates to increased CH_4 production in both CH_4 estimation methods. According to Gebbels et al. (45), the main factor leading to increased net CH_4 emissions in sheep meat and wool enterprises is the rise in total feed intake. In line with this, Patra et al. (46) identified feed intake as the most significant predictor of CH_4 production in sheep. Belanche et al. (16) also highlighted the importance of DMI for predicting enteric CH_4 emissions in sheep where DMI alone explains 80–91% of the variation in CH_4 production in sheep (47). Consistent with our findings on identifying treatment differences using LMD methods, Kang et al. (48) also detected variations in CH_4 emissions from cattle based on forage intake levels utilizing the LMD method. They recommended its application for assessing the effects of dietary treatments on CH_4 concentrations in cattle.

4.3.2 Methane yield

CH_4 yield reflects the methanogenic potential of the digestive process and correlates with CH_4 production (49). In the current study, the LMD CH_4 yield ranged from 16.3 to 18.5 g CH_4/kg DMI, the highest observed in the Ziziphus group. The higher CH_4 yield in the Ziziphus group may stem from variations in the feed's chemical composition and degradability (50, 51). Starsmore et al. (52) stated that DMI influences CH_4 emissions. Higher DMI provides more material for fermentation in the rumen, which is positively linked to CH_4 emissions.

Washaya et al. (53) fed Xhosa lop-eared goats in South Africa a forage having secondary metabolites such as tannins, phenolic, and saponins and measured the CH_4 using LMD and found CH_4 yield of 12.6 to 13.1 g/kg DMI. Waghorn et al. (54) conducted indoor trials with sheep in metabolism crates and observed CH_4 yields ranging from 11.5 g CH_4/kg DMI with lotus to 25.7 g CH_4/kg DMI with pasture. The current *in vivo* study contradicted our previous *in vitro*

findings, showing a low CH_4 yield from the indigenous plant feed source (10). Any *in vitro* methodology and batch culture are handy when evaluating treatments, feeds, or additives. They provide a quick response to elucidate a treatment's potential impact on fermentation. However, they cannot necessarily be directly applied to make assumptions of responses *in vivo* (55). The most accurate way to evaluate the nutritional value of any feedstuff is to feed it to the appropriate class of animal using feeding trials, which is the standard measure of digestibility (56).

4.3.3 Methane emission intensity

Methane intensity strongly depends on milk or meat production output (49). Savian et al. (57) and Silva et al. (58) observed that CH_4 emissions intensity from grazing sheep varied from 159 to 285 g CH_4/kg ADG, and from young bulls consuming soybean lipids, it ranged between 105 to 169 g CH_4/kg ADG, respectively, as measured by the SF6 tracer technique. El-Zaiat et al. (59) also observed a range of 93 to 131 g CH_4/kg ADG in lambs supplemented with encapsulated nitrate and cashew nutshell liquid, using open-circuit respiration chambers for measurement.

The higher CH_4 emission intensity observed in our study could be attributed to the lower fattening efficiency of the Menze breed. Indigenous sheep breeds in Ethiopia, particularly the Menz breed, are often regarded as low-producers, with the Menz breed being notably slow-growing (60). Kurihara et al. (61) highlighted the variation in CH_4 emission intensity associated with live weight gain, diet quality, and fattening efficiency.

5 Conclusion

In this research, the test feed outperformed the control group in terms of body weight gain and CH_4 emission intensity in sheep. The Ziziphus group notably showed a significantly greater increase in final body weight. The average enteric CH_4 emission, as measured by the two methods, displayed concordance in all CH_4 variables, such as CH_4 production and CH_4 intensity, as evidenced by strong R^2. A laser CH_4 detector could potentially estimate CH_4 in sheep where there is no access to other measurement equipment. It is also a friendly and economical method for estimating CH_4 in a country like Ethiopia. Methane emission intensity is the ideal variable related to the fattening efficiency of the sheep. Furthermore, possibly due to secondary metabolites such as CTs, the Acacia and Ziziphus groups exhibited optimal body weight gain in sheep compared to the control diet, suggesting their suitability for sustainable ruminant production in tropical regions. This study identified that the indigenous Menz breed sheep have low fattening efficiency, highlighting the need for breed improvement. In our results, feed efficiency promotes better weight gain, which leads to lower CH_4 per unit of average daily gain. In conclusion, supporting research and extension services to promote the utilization of leaf meals in the diet of ruminant livestock is a sustainable feeding option in Ethiopia.

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 approved by Brook Lemma Addis Ababa University, Tesfay Tessema Addis Ababa University, Addis Simachew Addis Ababa University. The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

WB: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Software, Writing – original draft. AZ: Conceptualization, Supervision, Writing – review & editing. AS: Supervision, Writing – review & editing. NK: Data curation, Writing – review & editing.

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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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The effect of *Broussonetia papyrifera* silage on intestinal health indicators and fecal bacterial composition in Kazakh sheep

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Hybrid *Broussonetia papyrifera* shows great promise for use in antibiotic-free feed, potentially contributing to the green and sustainable development of the animal husbandry industry. In this study, we investigated the impact of *Broussonetia papyrifera* silage on the intestinal health of Kazakh sheep. Forty healthy male Kazakh sheep, aged 5 months and weighing an average of 28.28 ± 1.14 kg, were randomly assigned to either a control or an experimental group, each comprising four replicates, with five sheep per replicate. The control group was fed a basal diet, while the experimental group received a diet supplemented with 20% *Broussonetia papyrifera* silage (dry matter basis). The 70-day experiment included a 10-day adaptation phase followed by a 60-day feeding trial. The results showed that there was no significant difference in growth performance or apparent nutrient digestibility between the experimental and control groups ($p > 0.05$). However, the experimental group exhibited significantly greater total antioxidant capacity, alongside higher contents of superoxide dismutase, catalase, glutathione peroxidase, immunoglobulins A, M, and G, and interleukins-2, −6, and −8 in the intestinal mucosa; in contrast, malondialdehyde and interleukin-4 contents were significantly reduced ($p < 0.01$). Furthermore, the dietary inclusion of *Broussonetia papyrifera* silage resulted in a reduction in the relative abundance of the bacterial genera *Turicibacter* and *Romboutsia* ($p < 0.05$). In conclusion, the feeding of *Broussonetia papyrifera* silage to Kazakh sheep significantly enhanced immune function, increased antioxidant capacity, and reduced the relative abundance of potentially pathogenic bacteria in the sheep without negatively impacting their growth or nutrient digestion, thus supporting the overall health of the animals.

KEYWORDS

***Broussonetia papyrifera*, antibiotic-free feed, immunity, antioxidation, fecal bacteria**

1 Introduction

Livestock husbandry represents a critical source of meat for humans. The use of antibiotics in livestock feed has been banned in many countries to mitigate the risk of animal-derived antibiotic resistance, thereby safeguarding human health (1). Accordingly, research interest has increasingly focused on the development of alternative, antibiotic-free feed additives that can enhance nonspecific immunity in animals (2, 3). Additionally, the advancement of "green, efficient, and safe" feeds, free not only from antibiotics but also from hormones and exogenous chemical agents, represents a significant trend for the future development of the feed industry.

Broussonetia papyrifera, a cultivar recently developed by the Institute of Botany at the Chinese Academy of Sciences, is rich in diverse bioactive compounds such as flavonoids, polysaccharides, and terpenoids (4). It was recently shown that *B. papyrifera* ensilage treatment enhances its assimilation and absorption by animals (5). The silage derived from *B. papyrifera* holds significant potential for applications related to animal health and is being explored as a potential alternative to antibiotics (6–11). *B. papyrifera* silage has been shown to benefit animal health by improving growth performance, immune function, and antioxidant capacity, as well as through its modulatory effects on ruminal bacterial communities (12–18). While numerous studies have investigated the effects of *B. papyrifera* silage on ruminal bacteria, few have addressed its impact on fecal bacteria in animals. Bacteria in feces reflect the broader microbial ecosystem of the digestive tract of ruminants and offer insights into the effects of diet, digestive efficiency, microbial health, and immune status (19). *B. papyrifera* silage has shown different effects on the health of different animals such as sheep, goats, rabbits, cows, donkeys, and piglets (12–16). In summary, incorporating *Broussonetia papyrifera* silage into livestock diets can reduce reliance on antibiotics and exogenous chemicals, thereby promoting more sustainable green development by enhancing feed efficiency and minimizing environmental pollution in agricultural cycles.

Kazakh sheep, originating in China, are highly valued for their ability to endure harsh environments, a quality that is particularly beneficial for nomadic herders in arid and semi-arid regions. To the best of our knowledge, research on the effects of *B. papyrifera* silage on the health of Kazakh sheep is limited. Therefore, in this study, we explored the effects of *B. papyrifera* silage on growth performance, intestinal health, and fecal bacterial composition in these animals, aiming to provide a scientific foundation for its application in green, antibiotic-free ruminant breeding.

2 Materials and methods

The animal research protocol used in this study was approved by the biological ethics committee of Shihezi University (Shihezi, China) in March 2023, under approval number A2023-129.

2.1 Preparation of silage from *Broussonetia papyrifera*

The experimental site was located at the *B. papyrifera* demonstration base of the Seventh Agricultural Science Institute in Xinjiang Province (N 44°20′, E 83°51′, elevation 450 m). *B. papyrifera* was harvested on April 20, 2023, at a height of 120 cm, leaving a stubble of approximately 10 cm, and the entire plant was cut into 2–3 cm-long pieces. The plants were then inoculated with 1×10^5 CFU/g *Lactiplantibacillus plantarum* (isolated from *B. papyrifera* silage) and thoroughly mixed (4). The *B. papyrifera* silage was then wrapped and sealed, with each package weighing approximately 80 kg. The silage underwent fermentation for 60 days at a storage temperature of 24°C. Nutrient analysis was performed on the dry matter of *Broussonetia papyrifera* silage after fermentation, and the results are presented in Table 1.

TABLE 1 Nutritional levels of *Broussonetia papyrifera* silage (dry matter basis).[1]

Item	Content	SEM
pH	4.58	0.004
Dry matter	35.11	0.102
Crude protein	17.88	0.101
Neutral detergent fiber	42.78	0.303
Acid detergent fiber	30.16	0.635
Water-soluble carbohydrate	8.32	0.106
Crude ash	10.42	0.069

[1]$n = 4$; SEM, standard error of the mean.

2.2 Animal experimentation and experimental design

A total of 40 male Kazakh sheep (castrated rams), approximately 5 months old and weighing an average of 28.28 ± 1.14 kg, were selected for the study. The sheep were randomly divided into a control group (CK) and an experimental group (GS), each with four replicates (five sheep per replicate). The control group was fed a basal diet, while the experimental group received a total mixed ration containing *B. papyrifera* silage (20% dry matter). The formulation of the experimental diet followed the Chinese Sheep Feeding Standard (NY/T 816–2021) and was designed to meet the nutritional requirements for the growth phase of sheep. Detailed information on the composition and nutrient levels of the experimental diet is provided in Table 2. The crude-to-refined ingredient ratio was 4:6. The feeding period lasted for 10 days, followed by a 60-day trial period. The experiment was conducted in Jinghe County, Xinjiang Province (N 82°30′49.20″, E 44°31′19.58″, elevation 384 m). Each sheep was individually housed in pens and had ad libitum access to water throughout the study. The sheep were fed twice daily at 08:00 and 18:00 h, with feed leftovers restricted to approximately 5%.

2.3 Sample collection

On the final day of the experimental period, five sheep from each group with body weights closest to the average weight of the group were randomly selected for fecal sample collection. Samples were obtained from the sheep 4 h after the morning feeding. Rectal fecal samples (approximately 0.2 g each) were stored in a liquid nitrogen tank for microbial community analysis. Subsequently, 3 sheep from each group (6 sheep in total) were randomly selected from those that provided fecal samples for slaughter. After slaughter, the abdominal cavity was quickly opened, and the middle sections of the duodenum, jejunum, and ileum were extracted and stored at −80°C for subsequent analysis (20).

2.4 Measurement of indicators

2.4.1 Feed nutrient levels

Dry matter content was determined according to the AOAC standard procedure. Calcium (Ca) content was determined using

TABLE 2 Composition and nutrient levels of the experimental diets (dry matter basis).

Item	Control group	Experimental group
Ingredients		
Corn (%)	35.00	35.00
Wheat straw (%)	15.00	15.00
Soybean meal (%)	6.00	6.00
Cottonseed meal (%)	3.00	3.00
Alfalfa hay (%)	15.00	12.00
Total corn silage (%)	22.00	5.00
Broussonetia papyrifera silage (%)	-	20.00
NaCl (%)	0.30	0.30
Baking soda (%)	0.70	0.70
Premix[1]	3.00	3.00
Total	100.00	100.00
Nutrient levels		
Metabolic energy (MJ/kg)	11.15	11.19
Crude protein (%)	14.12	13.92
Neutral detergent fiber (%)	35.55	35.81
Acid detergent fiber (%)	26.30	26.36
Calcium (%)	0.64	0.63
Phosphorus (%)	0.52	0.53

[1]Each kg of premix contained the following: vitamin A, 3,000 IU; vitamin D, 7,500 IU; vitamin E, 19 IU; Cu, 18.5 mg; Zn, 118.5 mg; Fe, 81 mg; Mn, 118.5 mg; Se, 0.75 mg; and Co, 1.75 mg. Metabolic energy was a calculated value, while the others were measured values.

AOAC official method 968.08, phosphorus (P) content was determined using AOAC official method 965.17, acid detergent fiber (ADF) content was determined using AOAC official method 973.18, the nitrogen content of the feed was measured using the Kjeldahl method, and the neutral detergent fiber (NDF) content was determined according to Van Soest (21).

2.4.2 Growth

On days 1 and 60 of the experiment, the body weight of the sheep was measured in the morning after an overnight fast.

2.4.3 Apparent nutrient digestibility

On day 53 of the experiment, five sheep were randomly selected from each group, and fecal samples were continuously collected from these sheep for 7 days using the total feces collection method. The collected feces were thoroughly mixed, and 10% of the sample was treated with 10% sulfuric acid for nitrogen preservation. The samples were then dried at 65°C and stored for subsequent determination of fecal nutrient contents. During this period, feed and leftover feed samples were also collected, and their nutrient content was determined using the method outlined in Section 2.4.1. The apparent digestibility of nutrients in the experimental animals was calculated based on the acid-insoluble ash (AIA) content (21).

2.4.4 Determination of antioxidant, immunoglobulin, and cytokine contents

Duodenal, jejunal, and ileal mucosal tissues were individually weighed to 0.1 g. Subsequently, 0.9 mL of prechilled 0.9% physiological saline was added to each sample, and the mixture was homogenized using a homogenizer to obtain a 10% tissue homogenate. A 0.5-mL aliquot of the resulting homogenate was then centrifuged at 2000 rpm for 10 min at 4°C and the resulting supernatant was stored at −20°C for subsequent analysis (22). Intestinal tissue indices in the supernatants were analyzed using commercial kits (Shanghai Meilian Biological Technology Co., Ltd., Shanghai, China) according to the manufacturer's instructions. The antioxidant indices measured were total antioxidant capacity (T-AOC) and superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GSH-Px), and malondialdehyde (MDA) contents. The immunoglobulins assessed were immunoglobulin (IgA), IgG, and IgM. The cytokines evaluated included tumor necrosis factor-alpha (TNF-α), interleukin-2 (IL-2), IL-4, IL-6, and IL-8.

2.4.5 Fecal bacteria

The collected (cryogenically frozen) fecal samples were placed in 200 mL of sterilized triangular flasks, mixed with 50 mL of PBS (pH 7.2), shaken at 200 rpm for 30 min, ultrasonicated at 50 W for 2 min, and subsequently shaken at 150 rpm for another 30 min. The supernatant was then transferred to 50-mL sterilized centrifuge tubes that had been sterilized under high pressure and centrifuged at 1,500 rpm for 1 min. After transferring to new 50-mL sterilized high-speed centrifuge tubes, the samples were centrifuged at 12,000 rpm for 10 min, and the isolated bacteria were collected. DNA was extracted from the fecal samples using a DNA extraction kit (QIAamp PowerFecal DNA Kit, Qiagen, Hilden, Germany) and assessed for concentration and purity using 1% agarose gel electrophoresis.

TABLE 3 The effect of *Broussonetia papyrifera* silage on the growth performance of Kazakh sheep.

Item	Group		SEM	*P*-value
	CK	GS		
Initial weight (kg)	28.26	28.28	0.036	0.859
Final weight (kg)	35.53	35.72	0.015	0.746
Apparent digestibility of dry matter (%)	64.38	63.74	2.521	0.820
Apparent digestibility of crude protein (%)	69.15	68.75	2.156	0.753
Apparent digestibility of crude lipid (%)	83.32	84.31	1.173	0.720
Apparent digestibility of neutral detergent fiber (%)	56.17	53.47	2.906	0.627
Apparent digestibility of acid detergent fiber (%)	45.38	44.99	2.543	0.789

SEM, standard error of the mean. Differences were considered significant at $p < 0.05$.

The V4–V5 region of the bacterial 16S rRNA gene was amplified using the primers 515F (5′-GTGYCAGCMGCCGCGGTAA-3′) and 926R (5′-CCGYCAATTYMTTTRAGT-3′). PCR amplification was performed in 20-μL volumes using the following parameters: 95°C for 3 min, followed by 30 cycles of 94°C for 20 s, 55°C for 20 s, and 72°C for 30 s, with a final extension at 72°C for 10 min. Amplicons were verified by agarose gel electrophoresis, purified, and sent to Meij Biotech for library construction. Sequencing was performed on the Illumina MiSeq platform using paired-end reads (300 bp). Raw reads underwent quality filtering with fastp (v0.19.6) and merging with FLASH (v1.2.11). Denoising was performed using the DADA2 plugin in QIIME2 (default parameters). Denoised sequences (ASVs) were rarefied to 20,000 sequences per sample, yielding an average Good's coverage of 99.09%. Taxonomic classification was conducted using the Naive Bayes classifier in QIIME2 with the SILVA 16S rRNA database (version 138). Subsequent analyses were performed on the Majorbio Cloud Platform.[1] The original data relating to fecal bacteria obtained in this study can be accessed at https://www.ncbi.nlm.nih.gov/sra/PRJNA1167466, with SRA accession number PRJNA1167466.

2.5 Statistical analysis

Data were preprocessed using Excel 2018 and analyzed with SPSS 20.0. Prior to applying independent samples t-tests or one-way ANOVA, data normality was assessed using the Shapiro–Wilk test and homogeneity of variances with Levene's test. Non-parametric tests were applied if assumptions were not met. Post-hoc comparisons were performed using Tukey's test ($p < 0.05$). The classification and abundance of bacteria in the fecal samples were analyzed using the Majorbio Cloud Platform. All of data analysis in the biological cloud platform (see Footnote 1), specific as follows: Alpha is obtained by using the mothur software[2] diversity sobs, chao, shannon index, and USES the Wilxocon rank-sum test for Alpha diversity analysis of differences between groups; The similarity of microbial community structure among samples was tested by PCoA analysis (principal coordinate analysis) based on bray-curtis distance algorithm, and the PERMANOVA non-parametric test was used to analyze whether the difference in microbial community structure between sample groups was significant. Species were selected for correlation network analysis based on spearman correlation $|r| > 0.6$ and $p < 0.05$.

3 Results

3.1 Growth performance and the apparent digestibility of nutrients

As shown in Table 3, no significant difference in growth performance or apparent nutrient digestibility was detected between the GS and CK groups ($p > 0.05$).

3.2 Antioxidant capacity

As shown in Table 4, T-AOC and the levels of SOD, CAT, and GSH-Px in duodenal, jejunal, and ileal tissues in the GS group were significantly elevated compared to those in the CK group ($p < 0.01$), whereas the MDA content was significantly reduced ($p < 0.01$).

3.3 Immunoglobulin concentrations

As shown in Table 5, the contents of IgA, IgM, and IgG in the duodenal, jejunal, and ileal tissues were all significantly higher in the GS group than in the CK group ($p < 0.01$).

3.4 Cytokine contents

As listed in Table 6, the levels of TNF-α, IL-2, IL-6, and IL-8 in the duodenal, jejunal, and ileal tissues of the GS group were significantly higher than those in the CK group ($p < 0.01$), with only IL-4 contents being significantly lower in the GS group than in the control group ($p < 0.01$).

3.5 Fecal bacteria

As shown in Figure 1, the Sobs, Chao, and Shannon indices were all higher in the GS group than in the CK group; however, the differences were not significant ($p > 0.05$).

1 https://cloud.majorbio.com

2 http://www.mothur.org/wiki/Calculators

TABLE 4 The effect of *Broussonetia papyrifera* silage on the antioxidant capacity of intestinal tissues in Kazakh sheep.

Item	Group		SEM	*P*-value
	CK	GS		
Duodenal mucosa				
T-AOC (pg/mL)	53.82	149.87	19.702	< 0.01
SOD (pg/mL)	70.31	165.57	19.771	< 0.01
CAT (pg/mL)	318.58	715.37	81.032	< 0.01
GSH-Px (pg/L)	217.34	472.67	53.622	< 0.01
MDA (nmol/L)	6.47	2.94	0.741	< 0.01
Jejunal mucosa				
T-AOC (pg/mL)	81.03	156.97	15.897	< 0.01
SOD (pg/mL)	83.49	163.11	16.225	< 0.01
CAT (pg/mL)	337.50	734.30	82.546	< 0.01
GSH-Px (pg/L)	233.61	468.87	48.794	< 0.01
MDA (nmol/L)	5.72	2.88	0.602	< 0.01
Ileal mucosa				
T-AOC (pg/mL)	67.17	153.94	17.827	< 0.01
SOD (pg/mL)	71.69	168.59	19.998	< 0.01
CAT (pg/mL)	393.65	715.37	67.836	< 0.01
GSH-Px (pg/L)	214.12	479.27	54.418	< 0.01
MDA (nmol/L)	6.22	2.94	0.687	< 0.01

SEM, standard error of the mean. Differences were considered significant at $p < 0.05$. T-AOC, total antioxidant capacity; SOD, superoxide dismutase; CAT, catalase; GSH-Px, glutathione peroxidase; MDA, malondialdehyde.

TABLE 5 The effect of *Broussonetia papyrifera* silage on the immunoglobulin content in the intestinal tissues of Kazakh sheep.

Item	Group		SEM	*P*-value
	CK	GS		
Duodenum mucosa				
IgA (μg/mL)	119.85	258.20	29.482	< 0.01
IgM (μg/mL)	3.77	9.49	1.262	< 0.01
IgG (μg/mL)	320.26	802.22	99.066	< 0.01
Jejunal mucosa				
IgA (μg/mL)	111.09	236.18	25.854	< 0.01
IgM (μg/mL)	3.67	9.64	1.303	< 0.01
IgG (μg/mL)	347.73	782.98	89.545	< 0.01
Ileal mucosa				
IgA (μg/mL)	113.00	227.74	23.704	< 0.01
IgM (μg/mL)	2.97	10.11	1.498	< 0.01
IgG (μg/mL)	342.41	881.89	114.105	< 0.01

SEM, standard error of the mean. Differences were considered significant at $p < 0.05$. IgA, immunoglobulin A; IgG, immunoglobulin G; IgM, immunoglobulin M.

Principal coordinate analysis was used for the evaluation of beta diversity among fecal microbes (Figure 2). The results showed that microbial composition differed significantly between the treatment groups ($R = 0.4480$, $p < 0.05$). The R-value indicates the correlation between sample groups, with higher R-values indicating greater dissimilarity between groups.

As shown in Figure 3A, *Firmicutes* was the dominant phylum in fecal bacterial samples collected from both the control (FCK) and *Broussonetia papyrifera* silage treatment (FGS) groups, with relative abundances of 86.34–93.92% and 90.21–94.47%, respectively, followed by *Bacteroidota*, with relative abundances of 2.58–9.28% and 3.10–5.68%, respectively.

TABLE 6 The effect of *Broussonetia papyrifera* silage on the cytokine contents of intestinal tissue in Kazakh sheep.

Item	Group		SEM	*P*-value
	CK	GS		
Duodenal mucosa				
TNF-α (ng/L)	282.81	773.59	99.463	< 0.01
IL-2 (pg/mL)	377.09	867.25	100.765	< 0.01
IL-4 (pg/mL)	94.22	45.07	10.564	< 0.01
IL-6 (pg/mL)	66.35	171.96	22.147	< 0.01
IL-8 (pg/mL)	14.43	38.69	5.028	< 0.01
Jejunal mucosa				
TNF-α (ng/L)	278.77	753.70	96.795	< 0.01
IL-2 (pg/mL)	407.98	935.40	109.476	< 0.01
IL-4 (pg/mL)	95.58	52.43	9.124	< 0.01
IL-6 (pg/mL)	58.69	169.47	22.925	< 0.01
IL-8 (pg/mL)	15.05	41.25	5.407	< 0.01
Ileal mucosa				
TNF-α (ng/L)	275.12	724.87	93.628	< 0.01
IL-2 (pg/mL)	354.27	966.30	128.594	< 0.01
IL-4 (pg/mL)	92.79	50.71	8.802	< 0.01
IL-6 (pg/mL)	67.71	168.88	21.475	< 0.01
IL-8 (pg/mL)	14.59	37.93	4.767	< 0.01

SEM, standard error of the mean. Differences were considered significant at $p < 0.05$. TNF-α, tumor necrosis factor-alpha; IL, interleukin.

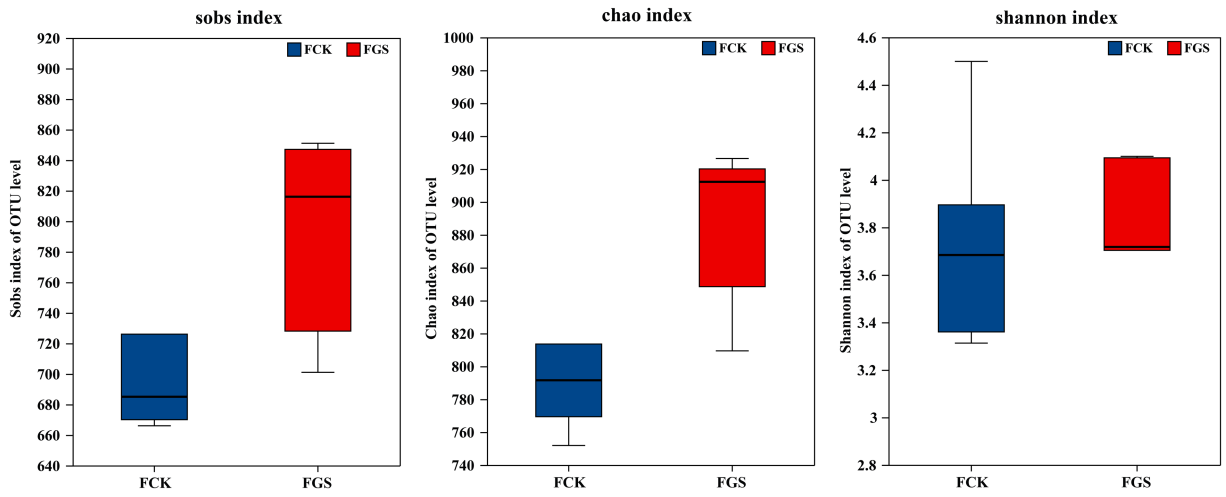

FIGURE 1
Box plots of alpha diversity analysis. FCK, fecal bacterial samples collected from the control group; FGS, fecal bacterial samples collected from the *Broussonetia papyrifera* silage treatment group; OTU, operational taxonomic unit.

At the genus level, *Solibacillus* was the dominant bacterial genus in both the FCK and FGS groups (relative abundances: 15.39–26.05 and 15.78% ~ 41.50%, respectively), followed by *Clostridium_sensu_stricto_1* (relative abundances: 5.17% ~ 21.17 and 7.00% ~ 15.28%, respectively), *Turicibacter* (relative abundances: 8.00% ~ 17.24 and 3.07% ~ 7.10%, respectively), and *Romboutsia* (relative abundances: 9.38% ~ 12.28 and 4.32% ~ 7.09%, respectively) (Figure 3B).

Compared with the FCK group, the abundance of *Acinetobacter*, norank_f__Oscillospiraceae, and *Rhabdanaerobium* was significantly higher in the FGS group ($p < 0.05$), whereas that of *Turicibacter*, *Romboutsia*, CPla-4_termite_group, *Treponema*, *Blautia*, *Mogibacterium*, and Lachnospiraceae_NK3A20_group were significantly lower ($p < 0.05$) (Figure 4).

As shown in Figure 5, fecal bacteria such as *Turicibacter* and *Romboutsia* exhibited marked positive correlations with TNF-α, IL-2,

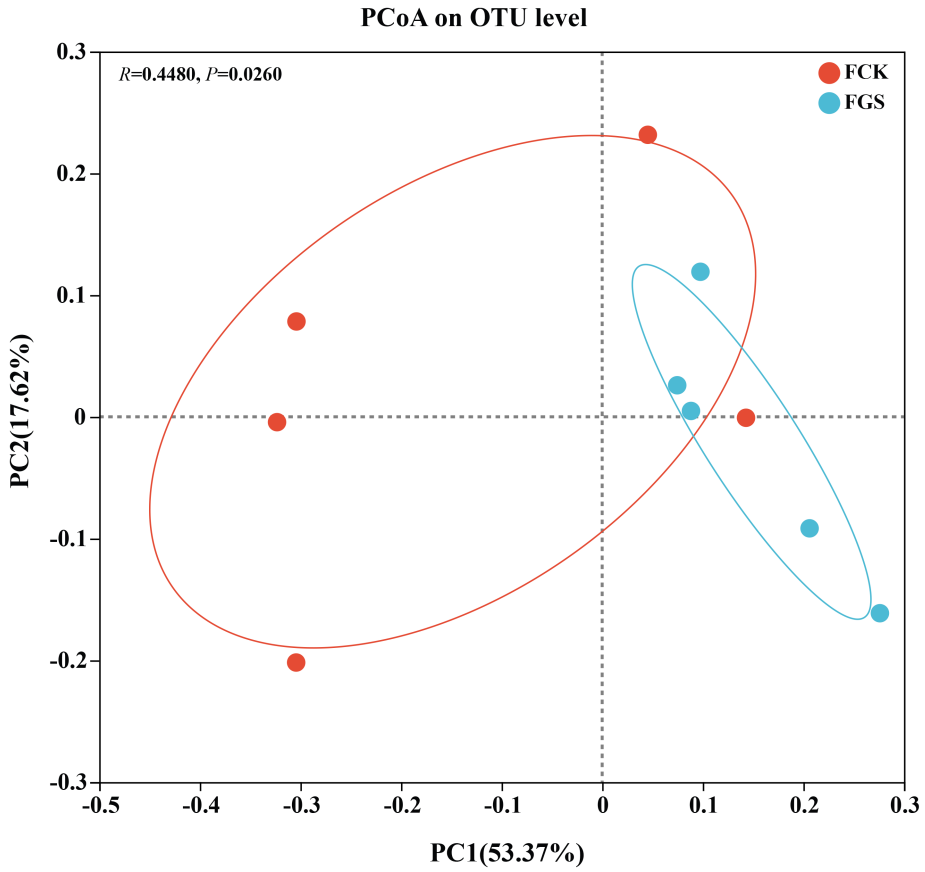

FIGURE 2
Principal coordinate analysis (PCoA). The horizontal and vertical coordinates represent the two selected principal coordinate components. The percentage represents the contribution of each principal coordinate component to the difference in sample composition. The closer the *R*-value is to 1, the greater the between-group difference relative to the within-group difference; conversely, the smaller the *R*-value is, the less significant the between- and within-group differences.

IL-6, IL-8, IgA, IgM, IgG, T-AOC, SOD, CAT, and GSH-Px ($p < 0.05$), while manifesting significant negative correlations with IL-4 and MDA ($p < 0.05$).

4 Discussion

4.1 Growth performance and apparent digestibility of nutrients

In this study, *B. papyrifera* silage did not affect growth performance or apparent nutrient digestibility in Kazakh sheep. Apparent nutrient digestibility serves as an indicator of both feed nutritional value and the digestive and absorptive capacity of animals. Notably, some studies have suggested that feeding *B. papyrifera* silage can enhance the growth performance of ruminants, potentially due to the resulting variations in dietary nutrient composition (12, 23). However, other research has indicated that feeding *B. papyrifera* silage significantly decreases apparent nutrient digestibility, likely due to the presence of antinutritional factors in *B. papyrifera* that inhibit nutrient absorption (20, 24). Our results differ from these previous reports. We found no significant difference in growth performance between the two groups, which may be attributed to the uniform nutritional level in the diets used in this study. Similarly, no notable differences in apparent nutrient digestibility were observed between the dietary groups, possibly due to the specific lactic acid bacteria preparation used, which may have mitigated the effects of the antinutritional factors associated with *B. papyrifera* silage (4).

4.2 Immunoglobulins, antioxidants, and cytokines

Relatively few studies to date have examined the impact of the dietary addition of *B. papyrifera* silage on the intestinal mucosal immune barrier in sheep. The intestine is the largest immune organ in the body and also harbors the highest density of immune cells. In animals, it plays a critical role in mucosal immunity and serves as the first line of defense against infection (25). The levels of immunoglobulins and cytokines can reflect the functionality of the intestinal immune barrier (26). IgA is the primary antibody type in the intestinal mucosa, with IgG and IgM concentrations being present at only relatively low levels. IgA, secreted by intestinal mucosal epithelial cells, plays a protective role during inflammatory responses in the intestinal mucosa (27). Cytokines are key regulators of inflammation and the immune responses triggered by infection or

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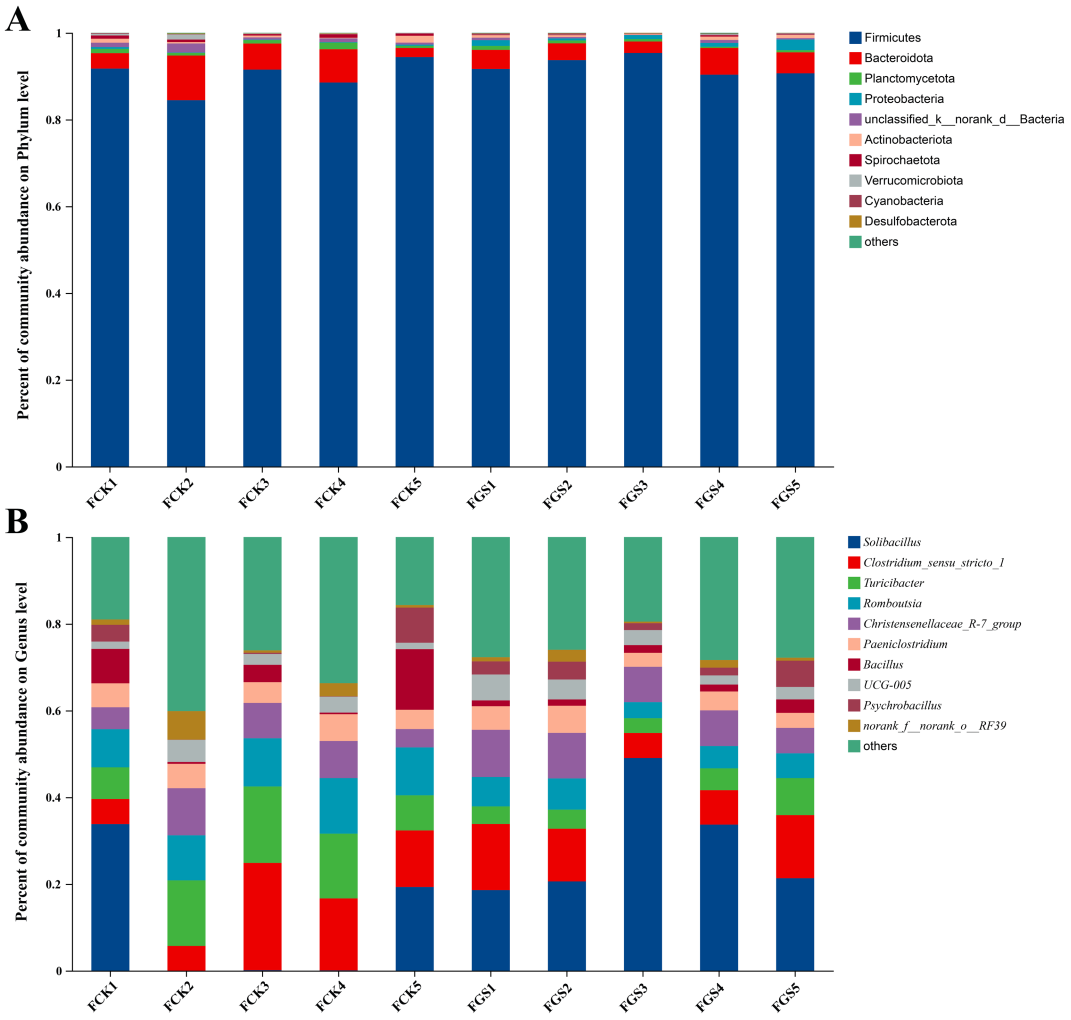

FIGURE 3
Fecal bacterial communities at the phylum **(A)** and genus **(B)** levels. FCK, fecal bacterial samples collected from the control group; FGS, fecal bacterial samples collected from the *Broussonetia papyrifera* silage treatment group.

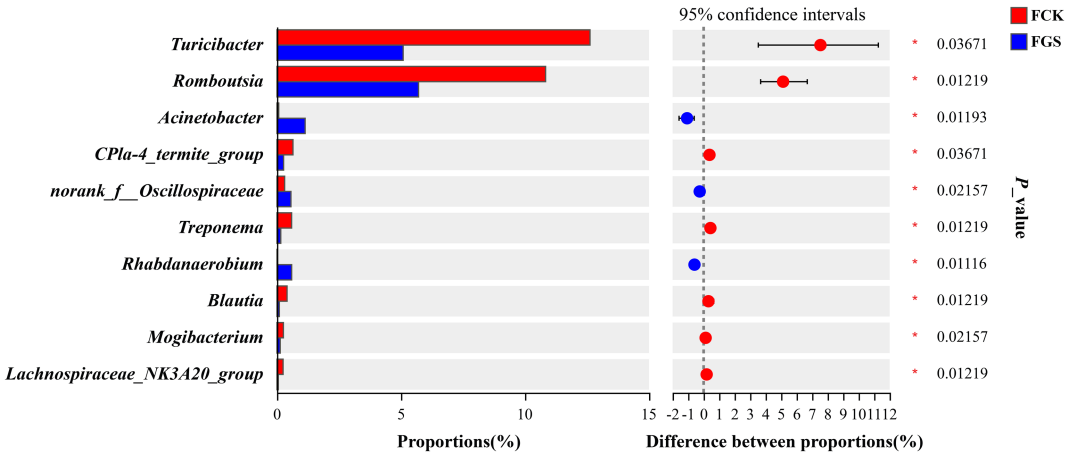

FIGURE 4
Bar chart for the multi-genera difference analysis. The *y*-axis represents different genera and the colored boxes represent the FCK (red) and FGS (blue) groups. The *x*-axis represents the average relative abundance of a particular genus in the different groups. FCK, fecal bacterial samples collected from the control group; FGS, fecal bacterial samples collected from the *Broussonetia papyrifera* silage treatment group.

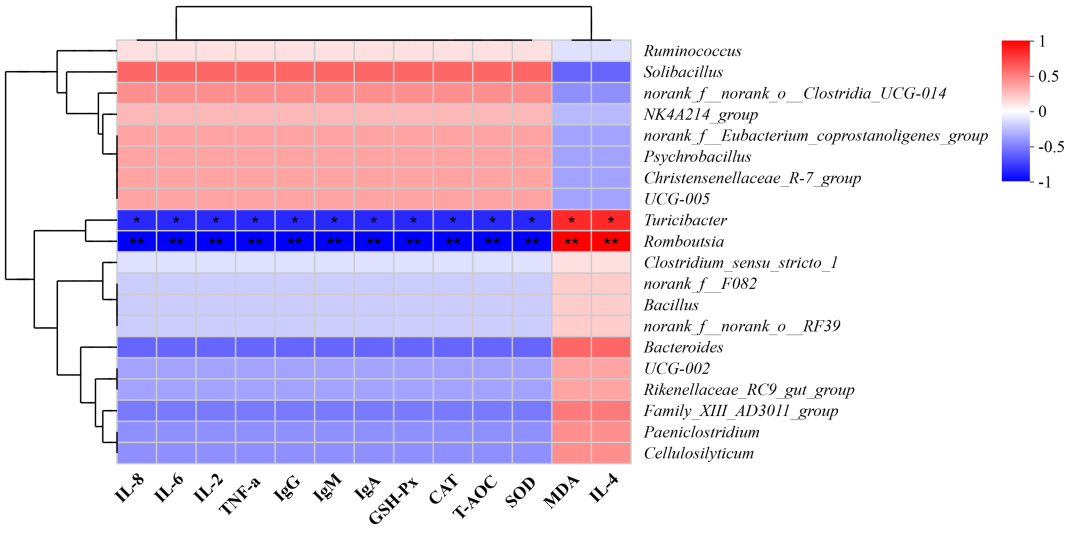

FIGURE 5
A correlation heatmap of the association of fecal bacteria with antioxidant indexes, immunoglobulin concentrations, and cytokine levels in mucosal tissue of the duodenum, jejunum, and ileum. Antioxidant indexes: T-AOC, total antioxidant capacity; SOD, superoxide dismutase; CAT, catalase; GSH-Px, glutathione peroxidase; MDA, malondialdehyde. Immunoglobulins: IgA, immunoglobulin A; IgG, immunoglobulin G; IgM, immunoglobulin M. Cytokines: TNF-α, tumor necrosis factor-alpha; IL, interleukin. The x- and y-axes represent environmental factors and species, respectively. Correlation coefficients (R-values) and p-values were calculated. The R-values are displayed in different colors on the chart; the color range for the R-values is shown on the right of the map. *$p \leq 0.05$, **$p \leq 0.01$.

injury (28). T-AOC and SOD, CAT, and GSH-Px contents are essential indicators of the antioxidant capacity of animals, while MDA levels reflect the extent of lipid peroxidation and cellular damage (29–31). The regulatory mechanisms governing cytokine production are complex. The variations in cytokine concentrations observed in this work may be attributed to the multiple antinutritional factors present in *B. papyrifera* silage, which induce intestinal stress in sheep (22). Additionally, some constituents of *B. papyrifera* silage may influence cytokine activity, a possibility that warrants further investigation. The increase in immunoglobulin concentrations (IgA, IgG, and IgM) in the experimental group may not necessarily indicate pathogen exposure but rather reflects the modulation of the intestinal immune system by bioactive compounds present in *B. papyrifera* silage. The dietary incorporation of *B. papyrifera* silage, which contains flavonoids, polyphenolic compounds, and alkaloids, can enhance intestinal health by improving intestinal morphology (16, 32, 33). Although a rise in antioxidant enzymes could suggest mild stress due to bioactive compounds in *B. papyrifera* silage, the overall reduction in MDA levels indicates that these compounds help mitigate oxidative damage, thereby supporting the beneficial antioxidant effects rather than harmful stress. Our results corroborate previous research findings, namely, that the addition of *B. papyrifera* silage in the diet enhances both immunity and antioxidant capacity in animals. Our findings further suggest that such dietary inclusion may bolster the intestinal immune function of sheep and promote overall intestinal health.

4.3 Fecal bacteria

Variations in the intestinal bacterial community can reflect the health status and production performance of the host. These communities interact through various signaling pathways, influencing their host's nutrient metabolism and immune function (34, 35). Several studies have established that the gastrointestinal microbiota of ruminants is predominantly composed of *Firmicutes* and *Bacteroidetes*, in line with the results of this study (36–39). *Firmicutes* primarily facilitate cellulose decomposition, while *Bacteroidetes* enhance carbohydrate utilization by animals (40, 41). In the present study, we noted an increasing trend in the relative abundance of *Firmicutes* in sheep of the GS group, indicative of an improvement in their fiber-decomposing capacity. Conversely, there was a decreasing trend in the abundance of *Bacteroidetes*, suggesting that structural changes within the intestinal bacterial community of the sheep may be linked to antinutritional factors present in *B. papyrifera* silage.

In this work, the relative abundances of *Turicibacter* and *Romboutsia* demonstrated a significant negative correlation with the health of their hosts. *Turicibacter* has been positively associated with inflammation and identified as a target microbe for colitis (42). Additionally, research has shown that mice engaged in treadmill running exhibit a lower fecal abundance of *Turicibacter*, whereas sedentary or forced-exercise mice display the opposite pattern (43). Literature relating to *Romboutsia* remains limited; however, its abundance is significantly elevated in the colonic chyme of rats with irritable bowel syndrome (44). The abundance of *lactobacilli* was increased, whereas that of *Romboutsia* was decreased, in the feces of hens fed diets supplemented with astragalus polysaccharides, indicative of a potential competitive exclusion relationship between these two genera (45). The *B. papyrifera* silage used in this study contained substantial quantities of *lactobacilli*, which may account for the observed decrease in the relative abundance of *Romboutsia*. Notably, the feeding of hybrid mulberry silage led to a significant reduction in the relative abundance of both *Turicibacter* and

Romboutsia, suggesting that such a dietary intervention can mitigate the prevalence of potentially pathogenic bacterial genera in the host.

5 Conclusion

The incorporation of *B. papyrifera* silage in the diet did not impact growth performance or apparent nutrient digestibility in Kazakh sheep. Notably, *B. papyrifera* silage enhanced the immune response and antioxidant capacity of the animals, while concurrently reducing the relative abundance of potentially pathogenic bacteria (*Turicibacter* and *Romboutsia*) in fecal matter. Given the comprehensive bans on antibiotics being introduced within the livestock sector worldwide, *B. papyrifera* silage represents a viable green functional feed for ruminants.

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/, PRJNA1167466.

Ethics statement

The animal study was approved by Biology Ethics Committee of Shihezi University. The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

XZ: Conceptualization, Data curation, Investigation, Methodology, Resources, Software, Writing – original draft, Writing – review & editing. YS: Software, Writing – original draft. SG: Writing – original draft. JY: Conceptualization, Investigation, Writing – original draft. RH: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Resources, Software, Visualization, Writing – original draft, Writing – review & editing. FZ: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Software, Writing – original draft, Writing – review & editing.

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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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Dietary supplementation with Chinese herbal mixture extracts enhances growth performance, immunity, antioxidant capacity, and intestinal microbiota function in calves

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This study examined the effects of dietary supplementation with Chinese herbal mixture extracts (CHE) on growth performance, Immunity, antioxidant capacity, and gut microbiota composition in dairy calves. CHE is a compound extracts powder composed of *Honeysuckle, Astragalus, Officinal magnolia bark,* and *Tangerine peel*. Forty calves were randomly assigned to four groups: basal diet (CON), basal diet + 0.1% CHE (LCHE), basal diet + 0.2% CHE (MCHE), and basal diet + 0.4% CHE (HCHE). The experiment was conducted for 56 days with daily observations, bi-weekly weighing, blood sampling, and fecal collection toward the end. The addition of the CHE group significantly increased the average daily weight gain (ADG) and decreased the feed/gain ratio (F/G) compared to the CON group ($p < 0.05$). The apparent digestibility of crude fat, neutral detergent fiber, and acid detergent fiber was higher in HCHE and MCHE groups ($p < 0.05$). Serum GH and IGF-1 levels increased in MCHE and HCHE groups ($p < 0.05$). The blood biochemical analysis revealed that the levels of CA and GLU in the MCHE group were higher than those in the CON group, while remaining within the normal physiological range. Both the IgG and IFN-γ levels and the serum antioxidant levels were significantly increased in the CHE supplementation group compared with the control group ($p < 0.05$). High-throughput 16S rRNA sequencing revealed changes in gut microbiota, with increased *unclassified Muribaculaceae* and *UCG-005* species in MCHE and HCHE groups ($p < 0.05$). In conclusion, CHE supplementation enhanced digestion, growth performance, immunity, and gut microbiota balance in calves without toxic side effects.Considering both the economic benefits and the effects of the additive, a clinical dosage of 0.2% CHE additive may be recommended.

KEYWORDS

Chinese herbal mixture extracts, growth performance, immunity, antioxidant capacity, intestinal microbiota, calves

1 Introduction

The digestive and metabolic systems of newborn calves are not yet fully developed, resulting in significantly inadequate digestive and metabolic performance and a limited ability to digest solid food. Therefore, calves at this stage are mainly fed whole milk or milk replacer (1), which significantly increases feeding costs. Calves at this stage are very sensitive to environmental changes and pathogenic bacteria, making them susceptible to diseases such as diarrhea and pneumonia, which can severely hinder normal growth and development may even lead to death, posing numerous challenges for farm management (2). Currently, In-feed antibiotics have been widely applied to nursery diets for controlling post-weaning diarrhea and promoting animal health and growth (3). However, in large-scale intensive livestock systems, the inappropriate use of antibiotics can disrupt the gut microbiota, impair immunity, promote antibiotic resistance, and lead to residues that pose risks to human health and the environment (4, 5).

Compared with chemical medicines, traditional Chinese herbs have certain advantages in the prevention and treatment of animal diseases, such as lower toxicity, no resistance, and multiple effects including immune modulation, anti-oxidation, and health maintenance, which makes them important agents for the preventive and treatment of calf diseases (6, 7). Herbal additives based on traditional Chinese herbal theory and produced from various herbs through special processes are healthy and environmentally friendly feed additives. After being fully absorbed by the animal body, their nutrients can regulate physiological functions, promote nutrient absorption, improve health status, and enhance the quality of animal products (8). Moreover, herbal additives are rich in organic compounds such as alkaloids, polysaccharides, and saponins, which can effectively improve metabolic functions (9, 10). Chinese herbal supplementation during the perinatal period in dairy cows has been shown to enhance reproductive performance, elevate antioxidant capacity, and strengthen neonatal calf immunity (11). Dietary inclusion of compound herbal formulations in weaned calves significantly improves growth parameters and modulates immune responses (12). Similarly, dietary supplementation with herbal extracts in Holstein calves has been found to enhance immune function and antioxidant activity, thereby effectively promoting overall health (13). Furthermore, the incorporation of blended herbal mixtures into calf rations reduces physiological oxidative stress and increases antioxidant levels (14).

Honeysuckle, Astragalus, Officinal magnolia bark, and *Tangerine peel* are medicinal and edible Chinese herbal medicines widely used in China since ancient times. These Chinese herbal medicines not only contain carbohydrates, proteins, crude fats, crude fibers, vitamins, minerals and other nutrients but also organic acids, flavonoids, volatile oils, triterpenoid saponins and other active ingredients, which can be used as feed additives in livestock and poultry production (15). *Honeysuckle* is an important medicine for clearing heat and detoxifying agent that has anti-inflammatory, antibacterial, antiviral, anti-tumor hypoglycemic, antioxidant, immunity-boosting and other beneficial effects (16). Studies have shown that *Honeysuckle* extracts, particularly those with varying chlorogenic acid content, can enhance the production performance of Holstein cows, improve rumen fermentation conditions, and boost antioxidant capacity and immune function (17). *Astragalus,* known for its qi-tonifying and immune-regulating properties, exhibits a wide range of biological activities, including cardiovascular and cerebrovascular protection, anti-tumor effects, anti-inflammatory responses, anti-aging properties, and antioxidant activity (18). Dietary supplementation with *Astragalus*-based preparations in broiler feed improves feed efficiency and enhances antioxidant capacity (19). *Officinal magnolia bark*, traditionally used for drying dampness and resolving phlegm, has been found to regulate gastrointestinal hormones, modulate material metabolism, protect the intestinal barrier, and influence gut microbiota composition, thereby contributing to disease prevention and treatment (20). *Tangerine peel*, recognized for its qi-regulating and spleen-strengthening effects, has been demonstrated to improve digestive and absorptive functions, enhance antioxidant activity, and exert antibacterial and bacteriostatic effects. Dietary supplementation with Tangerine peel in broiler feed improves growth performance, immune function, and antioxidant status (21). Based on these findings, we selected *Honeysuckle*, *Astragalus*, *Officinal magnolia bark*, and *Tangerine peel* to formulate a Chinese herbal mixture according to the principles of traditional Chinese medicine, and processed into the extracts. But the effects of this Chinese herbal mixture extracts as feeding additives in calves was unknown. Therefore, this study aimed to determine the efficacy and optimal dosage of the extracts in calves by analyzing the change on the growth performance, immune function, and antioxidant capacity at three doses. The research findings can provide scientific evidence to support the application of traditional Chinese herbal extract as feed additives for calves.

2 Materials and methods

2.1 The CHE preparation

The Chinese herbal mixture extracts (CHE) was composed of *Honeysuckle, Astragalus, Officinal magnolia bark, Tangerine peel,* and their ratio was 1:1:1:1. After cutting, drying, and mixing, the chinese herbal mixture we extracted and concentrated with 10 volumes of distilled water by boiling, the Chinese herbal mixture extracts with a concentration of more than 1.05 g · mL $^{-1}$ was produced. Subsequently, the extracts were spray-dried to obtain the powder, which was characterized as a yellow-brown powder with a subtle aromatic scent. The extract powder exhibited solubility in both water and milk and demonstrated good palatability.

2.2 Animal treatment and experimental design

The experiment was conducted in a large dairy farm in Xi'an, Shaanxi Province. Forty healthy Holstein calves, about 10 days old and with similar body weight (41.91 ± 4.37 kg), were randomly divided into four groups: including three groups with different dosages of CHE (LCHE: basal diet + 0.1% CHE; MCHE: basal diet + 0.2% CHE; HCHE: basal diet + 0.4% CHE) and one control

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TABLE 1 Nutrient levels of forages.

Items	Milk substitute powder (%)	Feed (%)
Water content	≤5.2	≤14.0
CP	≥22	≥20.0
Ash	≤10.0	≤10.0
CF	≤0.3	≤12.0
NaCl	0.6–1.4	0.05–2.0
AP	≥0.6	≥0.3
CA	0.6–1.2	0.4–1.7
Lysine	≥1.7	≥0.3
EE	≥16.05	—
Lactose	≥35.0	—

(CON: basal diet) group, with 10 calves in each group. The CHE was mixed with milk at different doses, while the CON group was fed only milk and starter feed. The pre-test period was 3 days and the formal test period was 56 days. The calves were fed whole milk and starter feed in accordance with the farm's production practices. The whole milk was prepared by mixing milk replacer powder with warm water, and the starter feed consisted mainly of corn (50.0%), soybean meal (37.5%), cottonseed meal (10.0%), limestone (1%), sodium chloride (0.5%), vitamins and trace elements (1%). The nutritional components of the milk replacer and the starter feed are shown in Table 1. The required milk replacer powder and starter feed were provided by the dairy farm. The pelleted premix was supplemented based on actual intake, without restriction, and whole milk was fed daily at 06:30, 13:30 and 20:00 h. Each calf was housed individually to prevent cross-infection. All feeding and management conditions were kept consistent across all groups during the experimental period.

2.3 Sample collection

On days 0, 14, 28, 42 and 56 of the experiment, 10 ml of blood was collected from the jugular vein of each calf using a vacuum collection vessel without any additives. The collected whole blood samples were stored at 4°C for 12 h, then centrifuged at 4,000 rpm for 10 min, and the serum was collected and stored at −20°C for subsequent analysis.

Three days before the end of the experiment, 200 g of feces were randomly collected daily from each calf. Thirty percent of the fecal sample was mixed with 10 % sulfuric acid (0.2 mL/g) for nitrogen fixation and stored at −20°C for crude protein determination. Seventy percent of the fecal samples were stored in sealed bags at −20°C and labeled for determination of the apparent digestibility of nutrients in the feces. At the end of the experiment, 1 to 2 g of feces was collected in a 1.5 mL Eppendorf tube using an anal swab and rapidly stored at −20°C for subsequent fecal microbiota analysis.

2.4 Determination of growth performance indicators

Before feeding, the weight of the amount remaining from the previous meal and the amount added were recorded and used to calculate the average daily feed intake (ADFI). The ADFI of calves refers to the sum of daily dry matter content in the milk and the starter feed.

$$\text{ADFI} = (\text{daily feeding} - \text{daily surplus material})/\text{test days}$$

The experimental cattle were weighed before midday feeding on days 0, 14, 28, 42, and 56, recorded in head units and the average daily weight gain (ADG) and feed-to-gain ratio (F/G) were calculated.

$$\text{ADG} = (\text{final weight} - \text{initial weight})/\text{test days}$$

$$\text{F/G} = \text{feed volume consumed during the test period/weight gain in the same period}$$

2.5 Determination of apparent digestibility of nutrients

The dry matter (DM), crude protein (CP), crude fat (EE), acid detergent fiber (ADF), and neutral detergent fiber (NDF) in feed and feces were determined. The determination steps refer to the "Feed Analysis and Feed Quality Detection Technology" and the determination was based on the following standards: water content: GB/T 20195-2006, GB/T 6435-2014 (22); crude protein: GB/T 6432-2018 (23); crude fat: GB/T 6433-2006 (24); neutral detergent fiber: GB/T 20806-2022 (25); acid detergent fiber: NY/T 1459-2022 (26).

Apparent digestibility of nutrients in feed (%) = [mass of nutrients ingested (g) – corresponding mass of nutrients in feces (g)]/mass of nutrients ingested (g) × 100%.

2.6 Serum indicators

2.6.1 Growth hormone in serum

The content of growth hormone (GH) and insulin-like growth factor-1 (IGF-1) in serum on days 0, 28, and 56 were determined using a one-step sandwich enzyme-linked immunosorbent assay (ELISA) kit from Shanghai Enzyme-linked Biotechnology Co., Ltd. (GH: ml610251; IGF-1: ml002483) The test was performed strictly according to the instructions of the kit.

2.6.2 Serum biochemical indicators

The contents of total protein (TP), alanine aminotransferase (ALT), aspartate aminotransferase (AST), creatinine (Cr), blood urea nitrogen (UREA), glucose (GLU), and calcium (Ca) in serum were measured using biochemical detection kits from Meikang Biotechnology Co., Ltd. (TP: MJ102; ALT: MJ001; AST: MJ002; Cr: MJ105; BUN: MJ106W; GLU: MJ109; Ca: MJ303). The tests were performed strictly according to the instructions of the kit.

2.6.3 Serum immunoglobulin and inflammatory factors

The serum levels of immunoglobulins IgA, IgG, and inflammatory factors IL-8 and IFN-γ were measured on days 0, 14, 28, 42, and 56 of the experiment using ELISA kits produced by Jiubang Biological Technology Co., Ltd. (IgA: QZ-11414; IgG: QZ-11416; IL-8: QZ-11413; IFN-γ: QZ-16216). These indicators were analyzed by Jiubang Biological Technology Co., Ltd.

2.6.4 Serum antioxidant indices

The total antioxidant capacity (T-AOC), superoxide dismutase (SOD), malondialdehyde (MDA), and glutathione peroxidase (GSH-PX) in serum were measured on days 0, 14, 28, 42, and 56 of the experiment using antioxidant-related kits produced by Nanjing Jiancheng Bioengineering Research Institute (T-AOC: A015-2-1; SOD: A001-3; MDA: A003-1; GSH-PX: A005-1). The experiments were performed strictly according to the instructions of the kit.

2.7 Analysis of fecal microbiota

The genomic DNA of the feces was extracted using a Stool DNA Kit (Tiangen Biotech (Beijing) Co., Ltd.) according to the manufacturer's instructions (DP705). The V1-V9 hypervariable regions of the 16S rRNA gene were amplified using primers (27F: AGRGTTTGATYNTGGCTCAG; 1492R: TASGGHTACCTTGTTASGACTT). The amplicons were quantified, and normalized equimolar concentrations of the amplicons were pooled and sequenced on the PacBio Sequel II platform (Beijing Biomarker Technologies Co., Ltd., Beijing, China).

The bioinformatics analysis of this study was performed with the aid of the BMKCloud (http://www.biocloud.net/). The raw reads generated from sequencing were filtered and demultiplexed using the SMRT Link software (version 8.0) with the minPasses ≥5 and minPredictedAccuracy ≥0.9, in order to obtain the circular consensus sequencing (CCS) reads. Subsequently, the Lima (version 1.7.0) was employed to assign the CCS sequences to the corresponding samples based on their barcodes. CCS reads containing no primers and those reads beyond the length range (1,200–1,650 bp) were discarded through the recognition of forward and reverse primers and quality filtering using the Cutadapt (version 2.7) quality control process. The UCHIME algorithm (v8.1) was used in detecting and removing chimera sequences to obtain clean reads. Sequences with similarity >97% were clustered into the same operational taxonomic unit (OTU) by USEARCH (v10.0), and the OTUs counts <2 in all samples were filtered.

Clean reads then were conducted on feature classification to output ASVs (amplicon sequence variants) by DADA2, and the ASVs counts <2 in all samples were filtered. Taxonomy annotation of the OTUs/ASVs was performed based on the Naive Bayes classifier in QIIME2 using the SILVA database (release 138.1) with a confidence threshold of 70%. The Alpha diversity was calculated and displayed by the QIIME2 and R software, respectively. Beta diversity was determined to evaluate the degree of similarity of microbial communities from different samples using QIIME. Principal coordinate analysis (PCoA), heatmaps, UPGMA and nonmetric multidimensional scaling (NMDS) were used to analyze the beta diversity. Furthermore, we employed Linear Discriminant Analysis (LDA) effect size (LEfSe) to test the significant taxonomic difference among groups. A logarithmic LDA score of 4.0 was set as the threshold for discriminative features. To explore the dissimilarities of the microbiome among different factors, a redundancy analysis (RDA) was performed in R using the package vegan.

The qualified sequences with more than 97% similarity thresholds were allocated to one operational taxonomic unit (OTU) using USEARCH (version 10.0). Taxonomy annotation of the OTUs/ASVs was performed based on the Naive Bayes classifier in QIIME2 using the SILVA database (release 138.1) with a confidence threshold of 70%. Alpha was performed to identify the complexity of species diversity of each sample utilizing QIIME2 software. Beta diversity calculations were analyzed by principal coordinate analysis (PCoA) to assess the diversity in samples for species complexity. One-way analysis of variance was used to

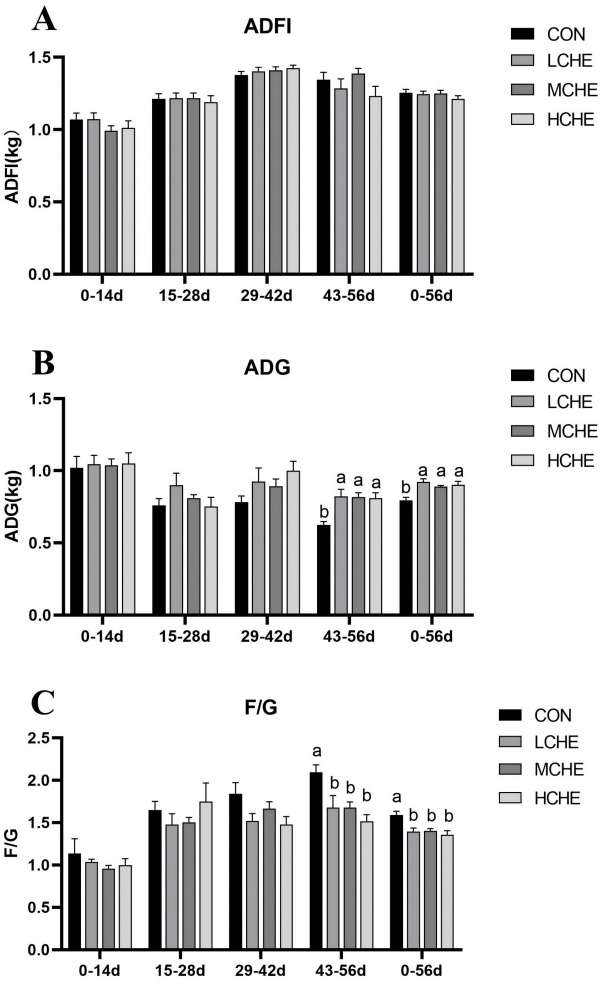

FIGURE 1
Effects of CHE on feed intake and body weight of calves. **(A)** calves average daily feed intake level. **(B)** calves average daily weight level. **(C)** the feed-to-weight ratios level. CON group (fed basal diet), LCHE (basal diet + 0.1% CHE), MCHE (basal diet + 0.2% CHE), HCHE (basal diet + 0.4% CHE). Mean ± SEM are shown ($n = 8$).Significant differences ($p < 0.05$) are denoted by the distinct letters a and b.

compare bacterial abundance and diversity. Linear discriminant analysis (LDA) coupled with effect size (LEfSe) was applied to evaluate the differentially abundant taxa. The online platform BMKCloud (https://www.biocloud.net) was used to analyze the sequencing data.

2.8 Statistical analysis

The experimental data were analyzed for significance of differences by One-WayANOVA using SPSS27.0 statistical software, and Duncan's method was used for multiple comparisons and tests, with $p < 0.05$ indicating a significant difference, and $p > 0.05$ indicating a non-significant difference. Correlation analysis between intestinal flora and growth performance was performed with Spearman and graphing was performed with GraphPadPrism software.

3 Results

3.1 Growth performance

3.1.1 Effects on feed intake and body weight

As shown in on Figure 1, there was no significant difference in average daily feed intake (ADFI) between the CHE groups and the CON group ($p > 0.05$) (Figure 1A). From day 0 to day 56 and from day 43 to day 56, the average daily weight gain of all CHE groups was significantly higher than that of the CON group ($p < 0.05$) (Figure 1B). During these periods, the feed-to-weight ratios were significantly lower in the CHE groups than in the CON group ($p < 0.05$). Additionally, there was no significant difference among the high, medium, and low dosage groups ($p > 0.05$) (Figure 1C).

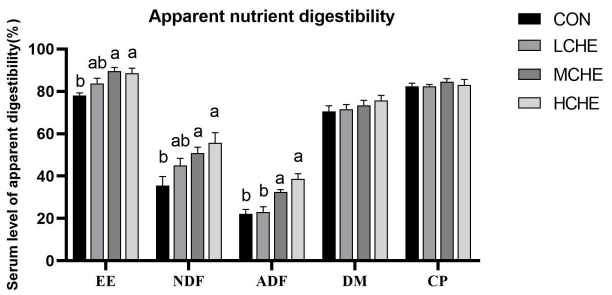

FIGURE 2
Effects of CHE on Apparent nutrient digestibility of calves. EE, crude fat. NDF, neutral detergent fiber;ADF, acid detergent fiber; DM, dry matter; CP, crude protein. CON group (fed basal diet), LCHE (basal diet + 0.1% CHE), MCHE (basal diet + 0.2% CHE), HCHE (basal diet + 0.4% CHE). Mean ± SEM are shown ($n = 5$).Significant differences ($p < 0.05$) are denoted by the distinct letters a and b.

3.1.2 Effect on apparent digestibility of nutrients

Figure 2 shows that there was no significant difference in the apparent digestibility of dry matter and crude protein between the CHE groups and the CON group ($p > 0.05$). However, the apparent digestibility of crude fat, neutral and acid detergent fibers was significantly higher in the HCHE and MCHE groups than in the CON group ($p < 0.05$). The MCHE group showed the best digestibility of crude fat, while the HCHE group showed the best digestibility for neutral and acid detergent fibers.

3.1.3 Effect on serum growth hormone

Figure 3 shows that the GH content in the serum of the MCHE group was significantly higher than that of the CON group on day 28 of the experiment ($p < 0.05$) (Figure 3A). The serum IGF-1 level of the HCHE group was significantly higher than that of the CON group ($p < 0.05$), while the low- and MCHE groups showed higher IGF-1 levels compared to the CON group, but the differences were not significant ($p > 0.05$) (Figure 3B). In addition, there were no significant differences between the high, medium and LCHE groups ($p > 0.05$).

3.2 Serum biochemical indicators

Table 2 shows that there were no significant differences between the CHE groups in the biochemical indicators in the blood on day 0 of the trial ($p > 0.05$). On trial day 28, the serum Ca content was significantly higher in the MCHE and HCHE groups than in the CON group and the LCHE group ($p < 0.05$). On day 56 of the trial, the serum GLU level was higher in the CHE groups than in the CON group, with the LCHE and MCHE groups being significantly higher than the CON group ($p < 0.05$).

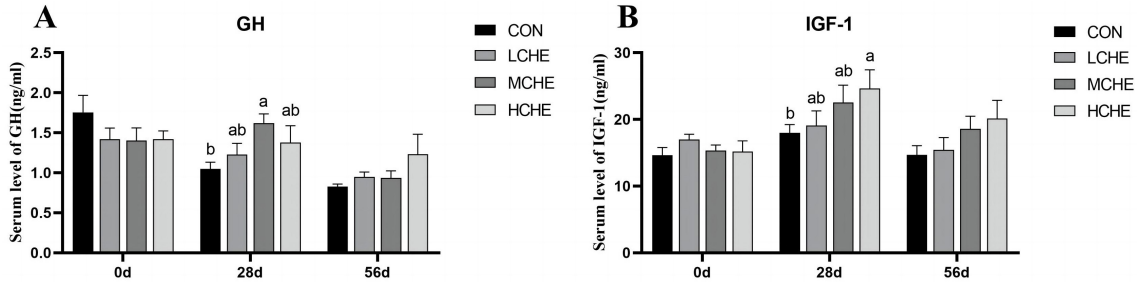

FIGURE 3
Effects of CHE on serum growth hormone of calves. **(A)** Serum GH level. **(B)** Serum IGF-1 level. CON group (fed basal diet), LCHE (basal diet + 0.1% CHE), MCHE (basal diet + 0.2% CHE), HCHE (basal diet + 0.4% CHE). Mean ± SEM are shown ($n = 8$). Significant differences ($p < 0.05$) are denoted by the distinct letters a and b.

TABLE 2 Results of blood biochemical indicators ($n = 8$).

Items	Treatments				SEM	*p*-value
	CON	LCHE	MCHE	HCHE		
0 days of age						
TP (g/L)	69.15	66.58	67.07	66.33	2.05	0.967
CREA (μmol/L)	38.88	43.20	41.45	43.98	1.26	0.517
GLU (mmol/L)	2.95	2.47	2.72	2.15	0.18	0.473
CA (mmol/L)	2.47	2.15	2.52	2.28	0.08	0.345
UREA (mmol/L)	3.07	3.18	3.08	3.37	0.15	0.904
ALT (U/L)	7.62	9.68	5.72	5.63	1.15	0.585
28 days of age						
TP (g/L)	57.08	55.23	60.75	61.70	1.06	0.091
CREA (μmol/L)	37.25	37.92	40.80	35.57	1.08	0.399
GLU (mmol/L)	2.87	3.08	3.60	2.95	0.15	0.302
CA (mmol/L)	2.07^{b}	2.10^{b}	2.33^{a}	2.33^{a}	0.04	0.012
UREA (mmol/L)	2.03	2.13	2.05	2.42	0.08	0.368
ALT (U/L)	7.27	6.40	4.92	5.63	0.62	0.598
56 days of age						
TP (g/L)	68.00	63.18	64.47	64.07	1.36	0.638
CREA (μmol/L)	101.03	107.25	97.20	100.32	3.12	0.741
GLU (mmol/L)	2.05^{b}	2.70^{a}	2.93^{a}	2.45^{ab}	0.11	0.025
CA (mmol/L)	2.07	2.18	2.12	2.10	0.02	0.384
UREA (mmol/L)	3.20	3.48	3.27	3.53	0.16	0.877
ALT (U/L)	5.65	8.37	12.23	5.58	1.18	0.146

Results of blood biochemical indicators. CON group (fed basal diet), LCHE (basal diet + 0.1% CHE), MCHE (basal diet + 0.2% CHE), HCHE (basal diet + 0.4% CHE). The means are shown ($n = 8$). Within the same row, data with different superscript letters indicate significant differences ($p < 0.05$); data with the same superscript letters or without any letters indicate no significant differences ($p > 0.05$).

There were no significant differences among the CHE groups ($p > 0.05$).

3.3 Immunoglobulin and inflammatory factors

Figure 4 shows that on day 28, the IFN-γ content in the serum of the HCHE group was significantly higher than that of the CON group. On day 56, the serum IgG level was significantly higher in the HCHE and MCHE groups than in the CON group ($p < 0.05$), while the MCHE group was higher than the blank CON group but without a significant difference ($p > 0.05$). Furthermore, there were no significant differences between the high, medium and LCHE groups ($p > 0.05$).

3.4 Antioxidant index

Figure 5 shows that on day 14 of the experiment, the T-AOC content in the serum of the MCHE group was significantly higher than that of the CON group ($p < 0.05$), and the MDA content in the serum of the HCHE group was significantly lower than that of the CON group and the LCHE group ($p < 0.05$). On day 28, the serum MDA level in the HCHE group was significantly lower than that in the CON group ($p < 0.05$), and the serum GSH-PX level in the HCHE group was significantly higher than that in the CON group ($p < 0.05$). On day 42, the serum SOD level in the HCHE group was significantly higher than that in the other groups ($p < 0.05$), and the serum MDA level in the HCHE and MCHE groups was significantly lower than that in the CON group ($p < 0.05$). In addition, the serum GSH-PX level was significantly higher in the HCHE group than in the empty CON group ($p < 0.05$). On day 56, the serum SOD level was significantly higher in the HCHE group than in the CON group and the LCHE group ($p < 0.05$), and the serum MDA level in the HCHE and MCHE groups was significantly lower than in the CON group ($p < 0.05$). In addition, the serum GSH-PX level was significantly higher in the HCHE group than in the CON group and the LCHE group ($p < 0.05$). Additionally, there was no significant difference between the HCHE and MCHE groups ($p > 0.05$).

3.5 Analysis of gut microbiota

In this study, the effects of adding different doses of CHE to the diet on the gut microbiota of calves before and after weaning were investigated by 16S rRNA amplification and sequence analysis. Upon reaching 30,000 sequences, the rarefaction curve reached a plateau, indicating that sampling was sufficient and appropriate for this experiment (Figure 6A). Figure 6B shows the Simpson index of alpha diversity, which measures species diversity and is influenced by both species richness and community evenness. A higher Simpson's index value indicates greater species diversity in the sample. There were no significant differences in microbiota between groups ($p > 0.05$). Figure 6C shows the beta diversity of the principal coordinate analysis (PCoA). There are differences in the composition of the diversity of the gut microbiota between the four calf groups. Principal component 1 (PC1) accounts for 43.27% of the total variance, while principal component 2 (PC2) accounts for 11.51%.

The LCHE group and the CON group are similar, while the HCHE group was different from the CON group and the MCHE group was more different from the CON group. The Venn diagram (Figure 6D) shows the number of common and unique OTUs in the samples. The CON group had a total of 1,511 OTUs, including

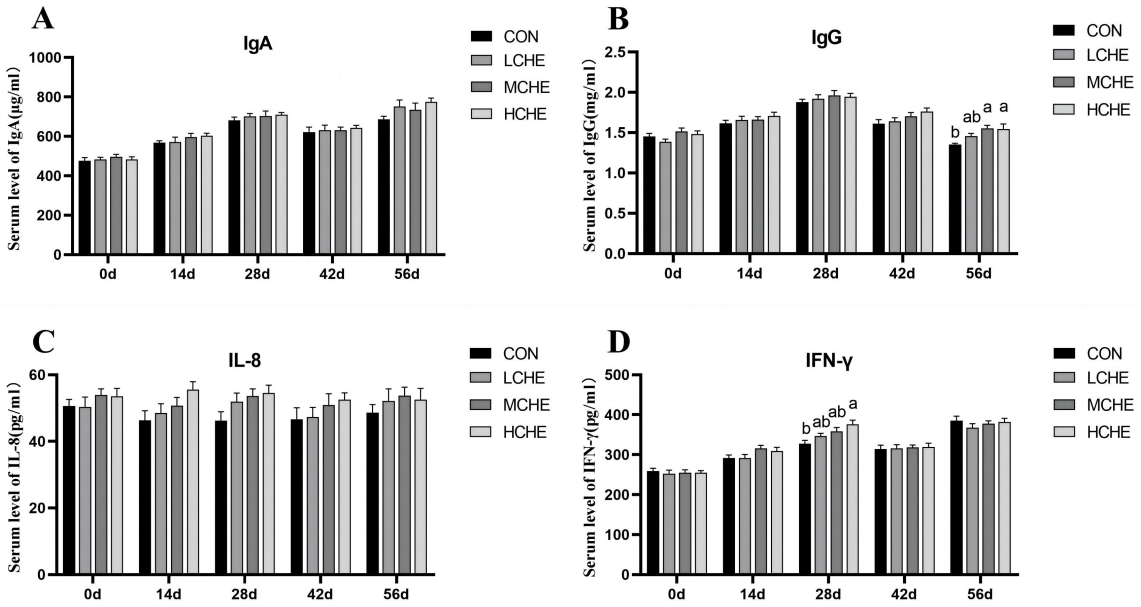

FIGURE 4
Effects of CHE on serum immunoglobulin of calves. **(A)** Serum IgA level. **(B)** Serum IgG level. **(C)** Serum IL-8 level. **(D)** Serum IFN-γ level. CON group (fed basal diet), LCHE (basal diet + 0.1% CHE), MCHE (basal diet + 0.2% CHE), HCHE (basal diet + 0.4% CHE). Mean ± SEM are shown ($n = 8$).Significant differences ($p < 0.05$) are denoted by the distinct letters a and b.

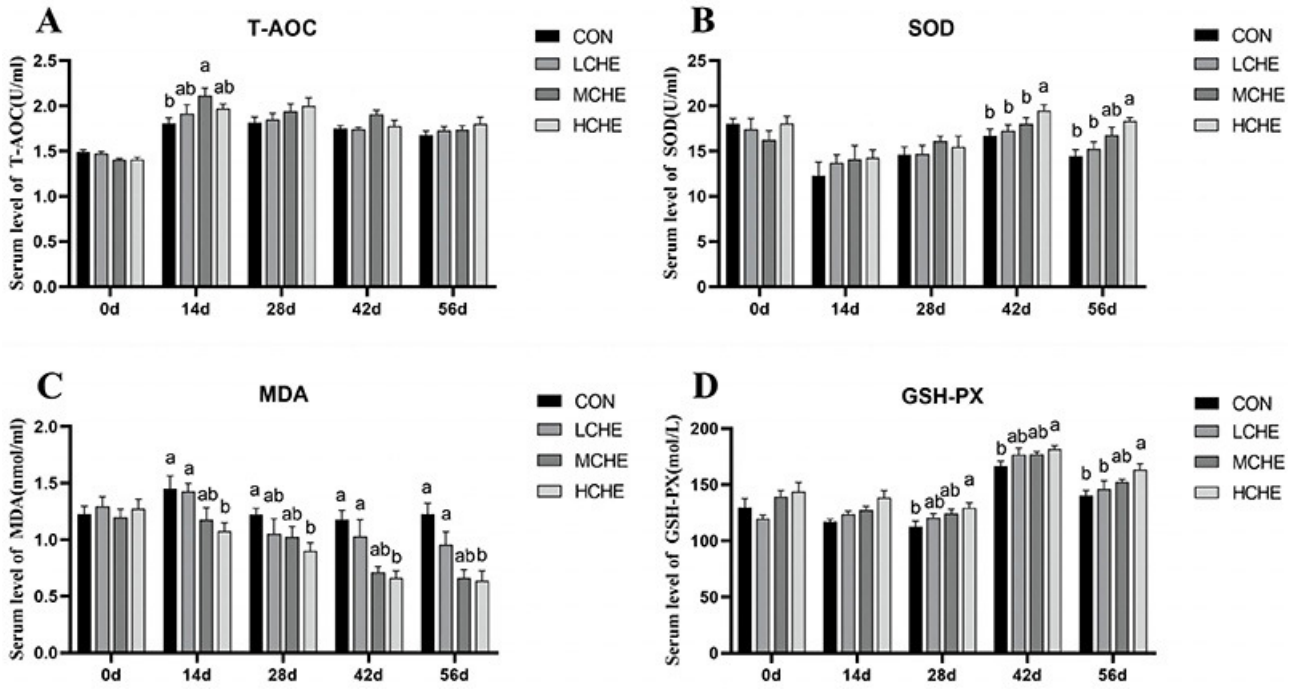

FIGURE 5
Effects of CHE on serum antioxidant levels of calves. **(A)** Serum T-AOC level. **(B)** Serum SOD level. **(C)** Serum MDA level. **(D)** Serum GSH-PX level. CON group (fed basal diet), LCHE (basal diet + 0.1% CHE), MCHE (basal diet + 0.2% CHE), HCHE (basal diet + 0.4% CHE).Mean ± SEM are shown ($n = 8$).Significant differences ($p < 0.05$) are denoted by the distinct letters a and b.

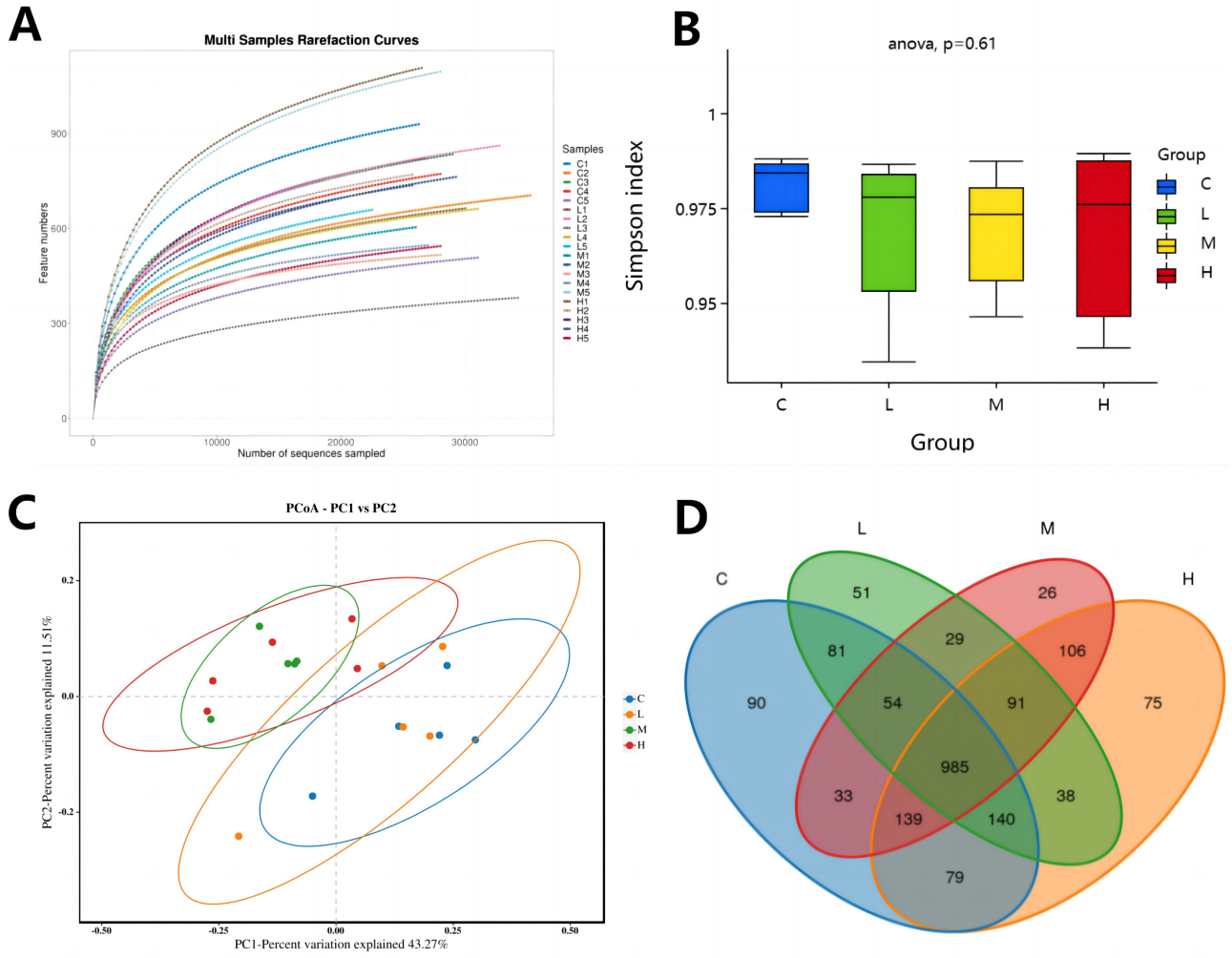

FIGURE 6
Effects of CHE on the fecal-like flora of calves **(A)** Sample dilution curve plot. **(B)** Grouped box plots of the AlPha diversity index. **(C)** Two-dimensional ordination plot of samples analyzed by Beta Diversity PCoA. **(D)** Venn diagram.C (fed basal diet). L (basal diet + 0.1% CHE), M (basal diet + 0.2% CHE), H (basal diet + 0.4% CHE). Mean ± SEM are shown ($n = 5$).

90 unique OTUs; the LCHE group had 1,418 OTUs, including 51 unique OTUs; the MCHE group had 1,437 OTUs, including 26 unique OTUs; and the HCHE group had 1,578 OTUs, including 75 unique OTUs.

Figure 7 shows the 10 most abundant gut microbiota at the phylum, genus and species level. At the phylum level, *Firmicutes* and *Bacteroidota* were the two predominant phyla in calf feces. The *Bacteroidota* content in the feces of the MCHE and HCHE groups was significantly higher than that of the CON group ($p < 0.01$). At the genus level, the *unclassified Muribaculaceae* content in the MCHE and HCHE groups was significantly higher thanin the CON group and the LCHE group ($p < 0.05$). The content of the genus *UCG-005* from the family *Ruminococcaceae* was higher in the LCHE, MCHE, and HCHE groups compared to the CON group, but the differences were not significant ($p > 0.05$). The content of the genus *RC9* from the family *Lachnospiraceae* was higher in the HCHE group compared to the CON group, though the difference was not significant ($p > 0.05$). At the species level, the *unclassified Muribaculaceae* content in the MCHE and HCHE groups was significantly higher than that in the CON group and the LCHE group ($p < 0.05$). The *UCG-005 species* from the family *Ruminococcaceae* was significantly higher in the MCHE group compared to the CON group ($p < 0.05$), while the LCHE and HCHE groups were higher than the CON group, but the differences were not significant ($p > 0.05$).

3.6 Correlation analysis between gut microbiota abundance and growth performance

To further explore the impact of gut microbiota abundance on calf growth performance, we conducted a correlation analysis between microbiota abundance at the phylum and species levels and growth performance (Figure 8). As shown in Figure 8A, *Bacteroidota* is highly negatively correlated with F/G and highly positively correlated with EE and NDF. Figure 8B shows that

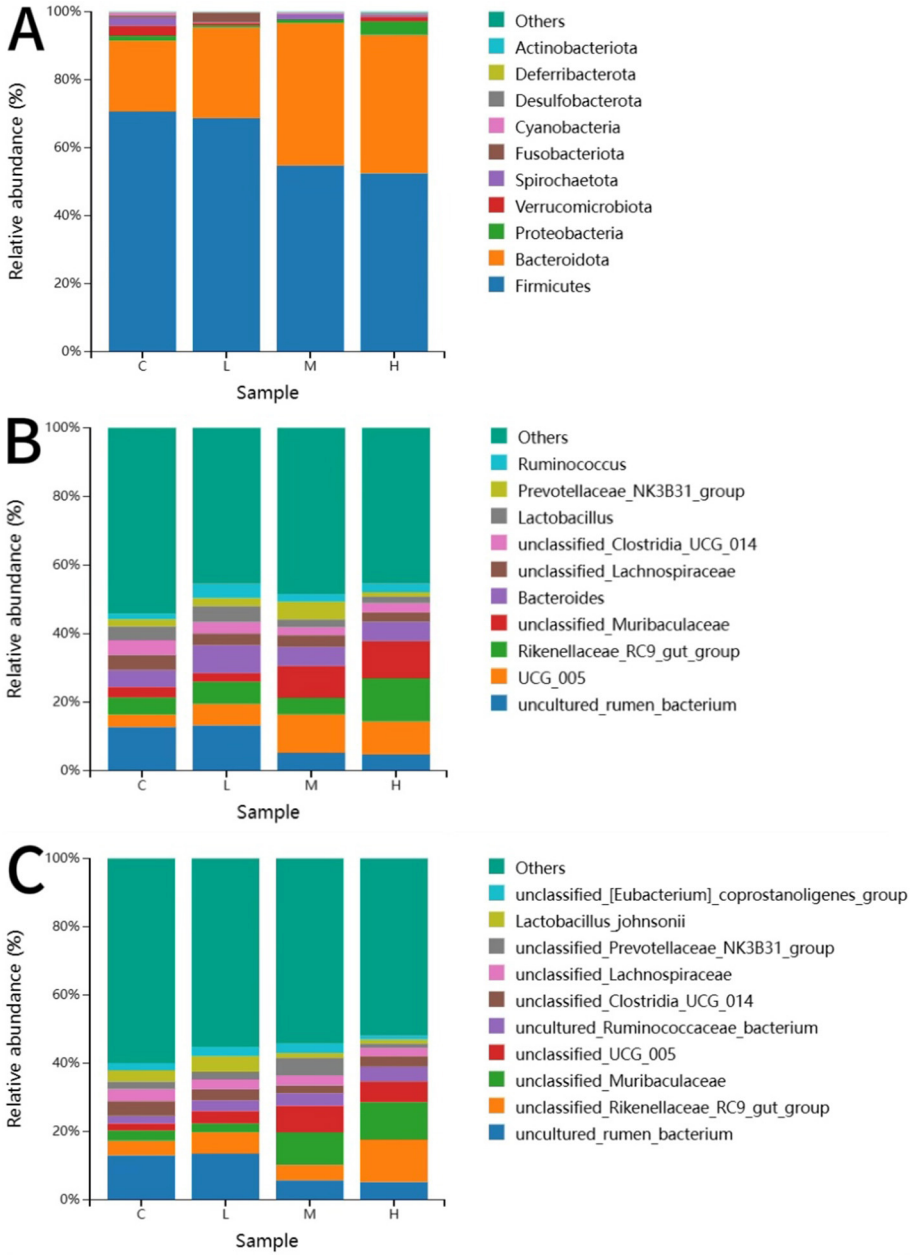

FIGURE 7
Effects of CHE on the fecal-like flora of calves. **(A)** Distribution of taxonomic composition at the Phyla level. **(B)** Distribution of taxonomic composition at the genus level. **(C)** Distribution of taxonomic composition at the species level. C (fed basal diet). L (basal diet + 0.1% CHE), M (basal diet + 0.2% CHE), H (basal diet + 0.4% CHE). Mean ± SEM are shown ($n = 5$).

unclassified-Muribaculaceae has a substantial positive correlation with EE and ADF, while *unclassified-UCG-005* shows a strong positive correlation with DM and *unclassified-Lachnospiraceae* has a strong negative correlation with CP.

4 Discussion

In this study, the CHE primarily contain several bioactive components, including chlorogenic acid, flavonoids, and polysaccharides, which exhibit significant antibacterial, anti-inflammatory, heat-clearing, detoxifying, and growth-promoting properties (27). So we investigated the potential of CHE as a feed additive in calf rearing, focusing on their effects on growth performance, serum immune indices and gut microbiota composition.

Growth performance, indicated by average daily gain and feed to gain ratio, reflects animal development and feed efficiency, respectively (28). Our results showed that there were no significant differences in dry matter intake between the CHE groups and the control group on days 0–56 and 43–56. However, the average daily gain was significantly higher in all CHE groups, with a

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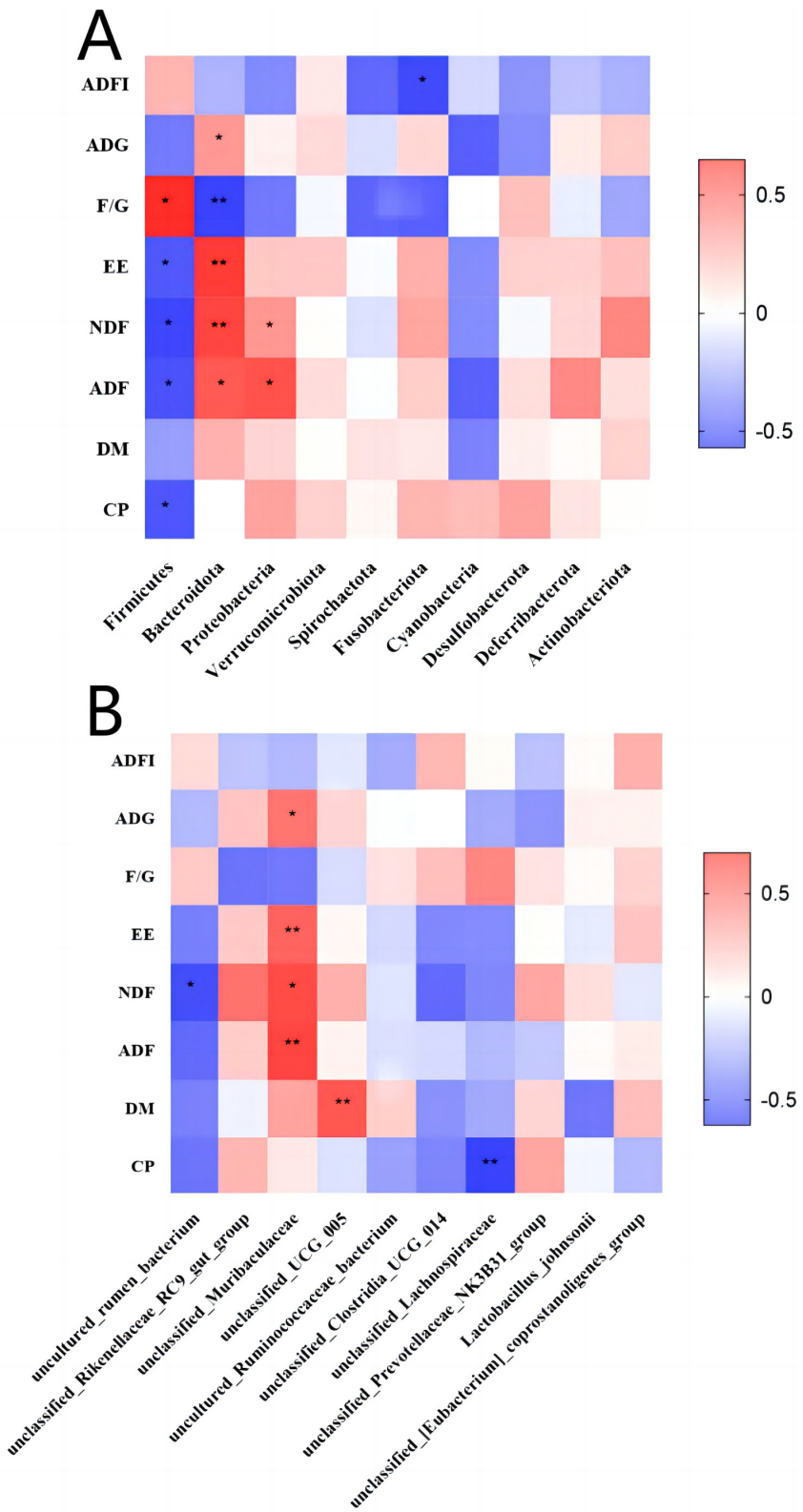

FIGURE 8
Correlation analysis chart. **(A)** Correlation analysis between phylum-level microbiota abundance and growth performance. **(B)** Correlation analysis between species-level microbiota abundance and growth performance. X-axis: microbial names. Y-axis: growth performance indicators. Red represents a positive correlation and blue represents a negative correlation. *$p < 0.05$, **$p < 0.01$.

correspondingly lower feed to gain ratio. The MCHE group had the best weight gain, while the HCHE group had the lowest feed to gain ratio, although these differences were not statistically significant. This indicated that the CHE additive improved calves' ADG, exhibited good palatability, and had no adverse effects on feed intake. Previous research suggests that the beneficial effects of herbal medicines are due to their polysaccharides, flavonoids and organic acids, which improve animal nutrition and growth. For example, the addition of traditional chinese herbal extract to calf diets can effectively improve growth performance this may be because the nutrients and some of the active ingredients of traditional Chinese medicine remaining in the residue promote the growth and development of animals, improve intestinal health, and thus promote the healthy growth of animals (29).

The apparent digestibility of nutrients reflects the ability of calves to digest and utilize nutrients from the diet and is an important indicator of feed quality (30, 31). In this trial, the apparent digestibility of EE, NDF and ADF was higher dose in the CHE groups than in the CON group, with the high dose and MCHE groups showing significant improvements. Among the dose groups, the MCHE group had the best digestibility of crude fat, while the HCHE group had the best digestibility of neutral and acid detergent fibers, although no significant differences were observed between the additive groups. The inclusion of plant residues in the diet has been shown to enhance nutrient digestion and absorption (32). Alkaloid compounds in herbal medicines can increase the activity of proteases and lipases, which facilitates the digestion and absorption of proteins and lipids (33). Therefore, it is likely that CHE improve the growth performance of calves by promoting nutrient digestibility and improving the gastrointestinal environment.

Growth hormone (GH) is an important indicator of growth promotion and metabolic regulation. GH, a peptide hormone secreted by pituitary cells, promotes tissue growth and increases protein and fat breakdown (34). IGF-1 in serum, a growth regulator, is closely associated with animal growth. It promotes cell proliferation, regulates protein synthesis and supports organ development and bone growth (35). The inclusion of herbal extracts in broiler diets has been shown to significantly increase serum GH and IGF-1 concentrations, leading to improved growth performance (36). Supplementation with chlorogenic acid at 1,000 mg/kg has been reported to enhance average daily gain and feed conversion efficiency in piglets, while also increasing serum albumin and IGF-1 levels (37). Additionally, flavonoids derived from herbal medicines have been demonstrated to positively modulate the hypothalamic-pituitary-adrenal axis, thereby enhancing GH and IGF-1 production and promoting growth performance in beef ewes (38). In this study, at day 28, serum GH and IGF-1 levels were higher in the CHE groups than in the CON group. Specifically, the MCHE group had the highest and significantly elevated GH levels, while the HCHE group had the highest and significantly elevated IGF-1 levels compared to the CON group. However, no significant differences were observed between the dose groups. It is therefore assumed that the flavonoids and chlorogenic acid components in CHE can increase the growth hormone level in calves and thus promote their growth and development.

Serum biochemical indicators are important indicators of nutrition, organ function and animal health (39). In this study, the effects of CHE feed additives on liver function (indicated by TP, ALT), kidney function (indicated by CREA, UREA), blood glucose level (GLU) and blood calcium level (CA) were investigated to determine the safe dosage of CHE. Numerous studies have evaluated the safety and efficacy of supplements based on biochemical indicators. For example, Previous studies have demonstrated that Artemisia leaves have no adverse effects on lamb health, as evidenced by serum levels of TP, ALT, and AST (40). Fathi et al. (41) found that administration of turmeric improved liver function of broiler chickens as shown by serum ALT and AST levels. In this study, serum TP and ALT levels in the CHE groups showed no significant differences compared to the CON group, indicating that the CHE does not impair protein synthesis in the liver or cause side effects on liver function. There were no significant differences in CREA and UREA levels between the groups, indicating that the CHE does not impair kidney function. Found in previous studies, chlorogenic acid in CHE could promote glucose uptake by up-regulating the expression of glucose transporter 2 (GLUT2) and phosphofructokinase (PFK), which activates the thermogenesis of brown adipose tissue and promotes the endocrine release of fibroblast growth factor-2 (42). It has been found that the addition of different concentrations of CHE to the diet of laying hens resulted in a higher trend in serum GLU levels with increasing herbal concentration (43). On day 56 of this trial, serum GLU levels in the CHE groups were within the normal range and significantly higher than in the CON group (44), with the low and high groups showing significant increases. The MCHE group had the highest value, although there were no significant differences between the CHE groups. This suggests that GLU levels may indirectly reflect weight gain in calves and that chlorogenic acid in CHE may improve weight gain while maintaining normal serum GLU levels. CA is an important component of teeth and bone, contributes to the transmission of neuromuscular excitability, regulates muscle contraction, and is essential for enzyme-catalyzed chemical reactions (45). At day 28, serum CA levels were within the normal range in all CHE groups and higher than in the CON group, with significantly higher levels in the medium and HCHE groups. Among the doses, the medium and high doses had the highest CA levels, with no significant differences between them. This indicates that the CHE can improve the growth performance of calves by increasing the CA content. Therefore, the MCHE and HCHE can improve serum GLU and CA levels without affecting liver or kidney functions.

Serum concentrations of IgA and IgG are important indicators of immune function, which play important roles in antibacterial and antiviral responses (46). IgA acts as an inhibitor of inflammatory responses, preventing pathogen adhesion to mucosal surfaces and thereby resisting mucosal infections (47). IgG, which accounts for ∼75% of the total immunoglobulins, activates complement system and neutralizes toxins, reflecting the overall immune status of the body (48). Many traditional Chinese herbs have immuno-modulatory effects, stimulating the immune system, activating immune cells, and enhancing the synthesis of antibodies, thus improving immune

function and promoting animal health (49). Numerous studies have demonstrated that *Astragalus* polysaccharides (APS) in *Astragalus* has significant immunomodulatory effects both *in vivo* and *vitro* in immunosuppression animal models and it also enhances the immune effects of normal animals (50). Dietary supplementation with 0.3% *Astragalus* significantly elevated serum IgA and IgM levels in lambs, indicating *Astragalus* improves immune function in early weaned lambs (51). Similarly, the inclusion of *Honeysuckle* extract in geese diets was shown to significantly increase serum IgA and IgG levels (52). In this study, although there were no significant differences in serum IgA levels between groups, the CHE groups had higher levels compared to the CON group, suggesting that the CHE promoted IgA expression. At day 56, serum IgG levels were higher in all CHE groups than in the CON group, with significant increases in the medium and HCHE groups. There were no significant differences between doses. IL-8 is a pro-inflammatory cytokine secreted by monocytes, macrophages, and endothelial cells and it recruits neutrophils to inflammatory sites, activates inflammatory cells and induces cell proliferation and the release of inflammatory substances (53). IFN-γ is a highly biologically active non-specific antiviral substance with immunoregulatory and differentiation-inducing functions, playing a key role in cell-mediated immune responses (54). The inclusion of a CHE compound in laying hen feed was shown to increase serum IFN-γ levels while reducing pro-inflammatory cytokine IL-8 levels, indicating that Chinese herbal compounds may enhance immune function and suppress inflammatory responses (8). This effect is presumed to be due to the stimulation of small intestine lymphocyte proliferation by the herbs. In this study, there were no significant differences in IL-8 levels among the groups, indicating similar levels across groups. On day 28, serum IFN-γ levels were higher in all CHE groups compared to the CON group, with the HCHE group showing the highest level, significantly higher than the CON group. There were no significant differences among the additive doses, but IFN-γ levels increased linearly with the dose. This may be due to chlorogenic acid in CHE can directly act on the NF-kB signaling pathway, regulating the expression of anti-inflammatory and pro-inflammatory factors. In addition, chlorogenic acid in CHE prevents the damage caused by inflammation by regulating the expression of related proteins and genes in the inflammatory response (55). In conclusion, CHE can promote the expression of IgG and IFN-γ in calves, with the 2% and 4% doses showing the most effective results.

The antioxidant capacity of dairy calves during the lactation period is closely related to their overall health. Increased antioxidant capacity strengthens the body's ability to resist oxidative damage (56, 57). The antioxidant capacity of animals can be determined by changes in serum GSH-PX and SOD activity and MDA levels. MDA, the end product of lipid peroxidation, can cause cell damage. In contrast, GSH-PX and SOD can neutralize free radicals and inhibit MDA formation, thereby maintaining the oxidative balance in the body (58–60). Numerous studies have shown that traditional chinese herbal feed additives can improve the antioxidant capacity of animals, Because chlorogenic acid in chinese herbal feed has a comprehensive antioxidant function. The molecular structure of chlorogenic acid contains five active hydroxyl groups and one carboxyl group, which gives chlorogenic acid its natural antioxidant properties (61). For example, *Honeysuckle* extract has been shown to enhance antioxidant capacity by increasing ferric reducing antioxidant power (FRAP) and 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radical scavenging activity (62). Dietary administration of APS was found to improve antioxidant capacity in broilers by elevating serum activities of SOD and GSH-PX, while reducing MDA concentrations (63). Similarly, supplementation with 10% *Astragalus* residues in the diet of fattening pigs significantly increased their antioxidant capacity (64). Furthermore, magnolol supplementation at varying doses enhanced antioxidant capacity in broilers, as evidenced by increased T-SOD activity in serum and intestinal mucosa, along with reduced MDA levels (65). In this study, serum T-AOC levels at day 14 were higher in the CHE groups than in the CON group, with the MCHE group having the highest and significantly higher levels than the CON group. Serum SOD levels on days 42 and 56 were higher in the CHE groups than in the CON group, with the HCHE group having the highest and significantly higher levels than the CON group. Serum MDA levels on days 14, 28, 42, and 56 were lower in the CHE groups compared to the CON group, with the MCHE and HCHE groups having the lowest and significantly lower levels than the CON group. Serum GSH-PX levels were higher in the CHE groups than in the CON group on days 28, 42, and 56, with the HCHE group having the highest and significantly higher levels than the CON group. These results are consistent with previous studies and suggest that CHE at different doses can improve the antioxidant capacity of dairy calves, with MCHE and HCHE groups doses showing better effects.

The gut microbiota plays a crucial role in the physiological processes of nutrient absorption, growth and development, and immune regulation in dairy calves (66, 67). The diversity and richness of microbial communities can lead to differences in digestive capacity (68, 69), which in turn affects growth performance. Studies have shown that chlorogenic acid, flavonoids, phenylpropanoids, alkaloids and terpenes in herbal supplements can inhibit the growth of harmful bacteria and prevent the formation of bacterial biofilms, thereby regulating gut health and improving growth performance (70). Regarding microbial diversity, although the addition of CHE to the diet had no significant effect on the Simpson index, the β-diversity results showed that the microbial community structure in the MCHE and HCHE groups differed from that of the CON group. Greater microbial diversity is associated with improved production performance, a robust gut microbiome and increased resistance to invasive species (71). Regarding the composition of the gut microbiota, the proportion of *Bacteroidota* in the feces of the MCHE and HCHE groups was significantly higher compared to the CON group. Previous studies have identified *Firmicutes* and *Bacteroidetes* as the two dominant phyla. Members of the *Bacteroidetes* are primarily responsible for protein hydrolysis and carbohydrate degradation (29). At the genus and species level, the abundance of the genus *Muribaculaceae* and *Muribaculaceae* species was significantly higher in the MCHE and HCHE groups than in the CON group. *Muribaculaceae* is a common gut symbiont in animals that is crucial for host health by maintaining intestinal

homeostasis through competitive relationships, production of antimicrobial substances and modulation of the immune system (72). Chlorogenic acid in chinese herbal feed can increase the relative abundance of *Bacteroidetes* and *Firmicutes* in the intestine, and reduce the relative abundance of *Proteobacteria*, and increase the diversity of intestinal microorganisms, indicating that the chlorogenic acid of CHE feed played an important role in regulating intestinal organisms in this study (73). In addition, *Muribaculaceae* bacteria contribute to the production of beneficial short-chain fatty acids by breaking down complex polysaccharides in the diet, which plays an important role in gut function and metabolism. At the species level, the relative abundance of *Ruminococcaceae-UCG-005* was significantly higher in the MCHE group compared to the CON group. The relative abundance of *Ruminococcaceae* in the bovine rumen is positively correlated with digestibility (74). *Ruminococcaceae-UCG-005* is involved in the breakdown of cellulose and the digestion of starch, converts complex polysaccharides into various nutrients required by the host and plays a crucial role in the metabolic functions of dairy calves (75). In summary, the results show that the MCHE and HCHE groups have better intestinal stability compared to the CON and LCHE groups. ncreasing the number of harmful microorganisms in the gut helps maintain normal physiological functions, thereby improving gastrointestinal digestion and increasing the growth performance of dairy calves (76).

Therefore, this study investigated the correlation between gut microbiota abundance and growth performance indicators in dairy calves to explore the effects of CHE on growth performance by modulating the abundance of the gut microbiota. Found that *Bacteroidota* was strongly negatively correlated with F/G and strongly positively correlated with EE and NDF. *Unclassified-Muribaculaceae* showed a highly significant positive correlation with EE and ADF, while *Ruminococcaceae-UCG-005* showed a highly positive correlation with DM. It has been demonstrated that *Ruminococcaceae* abundance exhibits a strong positive correlation with body weight and ADG (77). Additionally, a significant positive correlation was observed between the abundance of *norank_f_Bacteroidales_UCG-001* and ADF digestibility (74). Furthermore, the relative abundance of *Ruminococcaceae* was positively associated with feed efficiency and feed intake. Improved CF utilization in dairy calves, which is closely linked to higher DM digestibility.These findings suggest that *Ruminococcaceae* abundance plays a significant role in promoting nutrient utilization and growth performance (78). Thus, CHE intake may improve growth performance by increasing the number of beneficial gut bacteria, which in turn promotes more effective nutrient digestion. We also found that *unclassified-Lachnospiraceae* were strongly negatively correlated with CP. The relationship between *Lachnospiraceae* and CP is currently limited and further studies are needed to investigate the underlying mechanisms.

5 Conclusion

In conclusion, the addition of CHE to the feed significantly improves the average daily weight gain of Holstein calves, reduces the feed-to-gain ratio and improves the structure of the gut microbiota, thereby increasing growth performance. In addition, CHE can also enhance immune function and antioxidant function. Given the observed improvements in growth performance and health benefits, a clinical dosage of 0.2% CHE supplement is recommended for optimal results.

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 research protocol was approved by the Animal Experiment Ethics Committee of the Lanzhou Institute of Husbandry and Pharmaceutical Sciences, Chinese Academy of Agricultural Sciences (Approval No.: SYXK (Gan) (2024-054). The studies were conducted in accordance with the local legislation and institutional requirements. All procedures strictly adhered to animal use and ethical guidelines. Written informed consent was obtained from the owners for the participation of their animals in this study.

Author contributions

MZ: Data curation, Formal analysis, Validation, Writing – original draft. DS: Investigation, Methodology, Resources, Writing – review & editing. YW: Data curation, Investigation, Resources, Writing – review & editing. DD: Project administration, Supervision, Writing – review & editing. SD: Funding acquisition, Project administration, Writing – review & editing. JZ: Methodology, Resources, Writing – review & editing.

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Conflict of interest

DS was employed Gansu Qingliang Yuan Biological Medicine Co., Ltd. YW and DD were employed by Xian Caotan Animal Husbandry 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.

Generative AI statement

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Effect of biotin on growth performance, serum biochemical profiles, and histomorphological changes in acetate gossypol-treated mice

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The objective of this study was to determine the effects of biotin on growth performance, serum biochemical profiles, and morphology of acetate gossypol-treated mice. Seventy-five healthy male Kunming mice were randomly assigned to five treatment groups: the control group (C), the biotin control group (BC), the acetate gossypol control group (AGC), the biotin prevention group (BP) and the biotin treatment group (BT). The study examined the growth performance, visceral index, serum biochemical profiles and histomorphological changes of mice. The results showed that compared with AGC group mice, the average daily feed intake of BP group and BT group increased by 40.47 and 45.69% ($p < 0.05$). Biotin prevention reduced the elevated blood urea nitrogen (BUN) levels in acetate gossypol-treated mice, biotin treatment reduced the elevated serum aspartate aminotransferase (AST), total protein (TP) and BUN levels in acetate gossypol-treated mice ($p < 0.05$). Biotin increase the values of villus height/crypt depth in duodenum and jejunum ($p < 0.05$). In conclusion, biotin potentially increase the growth performance of acetate gossypol-treated mice, it also has a positive effect on serum biochemical profiles, and histomorphological of acetate gossypol-treated mice.

KEYWORDS

acetate gossypol, biotin, growth performance, serum biochemical profiles, histomorphological

1 Introduction

Cotton by-products such as cottonseed meal and cottonseed protein can be used as animal feed and are important protein supplements. However, cotton by-products contain anti-nutritional factors such as gossypol. Gossypol and it's derivatives accumulate in animal bodies and produce toxicity, which is not conducive to the growth and development of animals. Gossypol is widely distributed in the body with the blood circulation it damages the liver, kidney and other substantive organs, stimulates and destroys the gastrointestinal mucosa of animals, damages the digestive function of animals. It was found that gossypol could react with the ε-amino group of lysine to block the effect of trypsin, thereby reducing the utilization rate of lysine. When the gossypol content in the diet was 183 mg/kg, the malondialdehyde content in the liver tissue of the male geese decreased, with the increase of the free gossypol content in the diet, the serum triglyceride and low density lipoprotein content of the male geese

increased linearly (1), the study shows that gossypol affected the lipid metabolism of the organism. Feeding 400 mg/kg gossypol could reduce the feed intake and daily gain of 21-day-old and 42-day-old male broilers (2). Gossypol will accumulate in animal tissues, reduce animal growth performance, and cause certain damage to animal viscera and intestinal health.

Vitamins are involved in the body's biochemical reactions, and have the functions of regulating metabolism and enhancing immunity. Biotin also known as vitamin B_7 and vitamin H, is a water-soluble B vitamin. Biotin acts as an active carrier and a cofactor in the enzymatic reaction, by forming a covalent bond with the ε-amino group of the lysine residue on the protein, it binds to the enzyme to form a covalent binding coenzyme of five carboxylases in mammals. These enzymes are involved in physiological activities such as gluconeogenesis, fatty acid synthesis metabolism, amino acid catabolism and energy metabolism (3). Biotin is involved in the tricarboxylic acid cycle and fat metabolism in animals, and regulates glucose metabolism and lipid metabolism in animals. Studies have found that biotin can affect the morphology and development of intestinal mucosal epithelium, regulate intestinal flora, and improve the utilization of nutrients by animals, biotin can treat colitis induced by dextran sulfate sodium in mice, restore the colon length of mice caused by colitis, and maintain the integrity of the intestinal barrier in mice (4). Biotin regulates the production of pro-inflammatory cytokines in the immune system, affecting intestinal inflammation and intestinal permeability (5). Studies have shown that after feeding mice with biotin-deficient feed for seven weeks, the body weight of mice gradually decreased, and in the liver of mice lacking biotin, the specific activities of two biotin-dependent enzymes, pyruvate carboxylase and propionyl-CoA carboxylase, decreased by 75 and 80%, indicating that biotin deficiency has a certain effect on the immune system and function of mice (6).

Since gossypol is not stable, studies always use acetate gossypol to do the experiment. Studies have found that 5 mg/(kg·BW) gossypol can reduce the reproductive capacity of male rats, resulting in a decrease in mitochondrial ATP in rats, at the same time, 100 mg/(kg·BW) vitamin E can inhibit the decrease of mitochondrial ATP (7), as one of the vitamins, vitamin E has antioxidant effect and can reduce the toxicity of gossypol in animals. As a vitamin involved in the regulation of metabolism in the body, biotin has a certain antioxidant effect and protective ability to the animal body, we hypothesized that biotin has the potential to alleviate the damage of acetate gossypol to the animal body. However, the effects of biotin on the mice treated with acetate gossypol have not been fully characterized. To date, relatively few studies have investigated the application of biotin to alleviate and protect animals from damage caused by gossypol, this work might provide theoretical basis and value reference for the application of biotin to protect mice gavaged with acetate gossypol. Therefore, in this study, the growth performance, blood biochemical indexes, visceral and intestinal tissue morphology and function of mice were detected to explore the effect of biotin on mice gavaged with acetate gossypol.

2 Materials and methods

2.1 Animals and experimental design

All experimental procedures involving animals were approved (animal protocol number: 2024009) by the Animal Welfare and Ethics Committee of Xinjiang Agricultural University, Urumqi, Xinjiang, China. Seventy-five 4-week-old healthy male Kunming mice (BW, 19.37 ± 1.86 g) were purchased from Xinjiang Medical University, randomly assigned to five treatment groups (n = 15), each group was reared in a cage (540 × 395 × 200 mm) and then scheduled for a two weeks acclimation period. The mice were allowed access to food and water *ad libitum* throughout the study, and their daily food consumption was estimated by weighing the remaining food. The mice basal diet was from Jiangsu Medison Biopharmaceutical Co., Ltd., Catalog Number: MD17121. All the mice were housed in the Xinjiang Agricultural University Animal Center under controlled conditions (temperature, 20–24°C; humidity, 50–60%; 12 h light/dark cycle).

Biotin (Wuhan Servicebio Technology CO., Ltd., purity>99%) was dissolved in distilled water to make biotin solution at a dose of 1 mg/(kg·BW) of each group of mice, acetate gossypol (Medson Technology Co., Ltd., purity>98%) was dissolved in canola oil to make acetate gossypol solution at a dose of 80 mg/(kg·BW) of each group of mice. The feeding trial lasted for three weeks, biotin solution was administered to the BP group mice via oral gavage on a daily basis starting from in the first week, the remaining groups were administered equivalent volumes of distilled water and canola oil via oral gavage daily. In the second and third weeks, the control group mice were orally gavaged with distilled water and canola oil daily, the BC group mice were orally gavaged with biotin solution and canola oil daily, the AGC group mice were orally gavaged with distilled water and acetate gossypol solution daily, the BP group and the BT group mice were orally gavaged with biotin solution and acetate gossypol solution daily (Figure 1A).

2.2 Growth performance and visceral index

Body weight were recorded weekly and average daily feed intake of mice were recorded. Mice were euthanized by cervical dislocation, followed by immediate collection and weighing of hearts, livers, spleens, lungs, and kidneys, the visceral index was calculated according to the formula: (visceral weight/body weight) × 1,000.

2.3 Serum biochemistry

Blood was gained from the orbit of mice, let the blood stand at room temperature for one hour, centrifuge at 4000 r/min for 15 min, and take the supernatant as serum, store at −80°C, contents of serum urea (BUN), glucose (GLU), alanine aminotransferase (ALT), aspartate aminotransferase (AST), total bilirubin (TBIL), alkaline phosphatase (ALP), total protein (TP), albumin (ALB), triglyceride (TG), total cholesterol (TC), high density lipoprotein cholesterol (HDL-C) and low density lipoprotein cholesterol (LDL-C) were measured by Beckman au5821 Fully Automatic Biochemical Analyzer (Beckman Coulter Trading (China) Co., Ltd).

2.4 Histopathological observation

Following dissection of the liver, duodenum, jejunum, and ileum segments, the tissues were immediately fixed in 10% neutral buffered formalin. After fixation, specimens were systematically trimmed,

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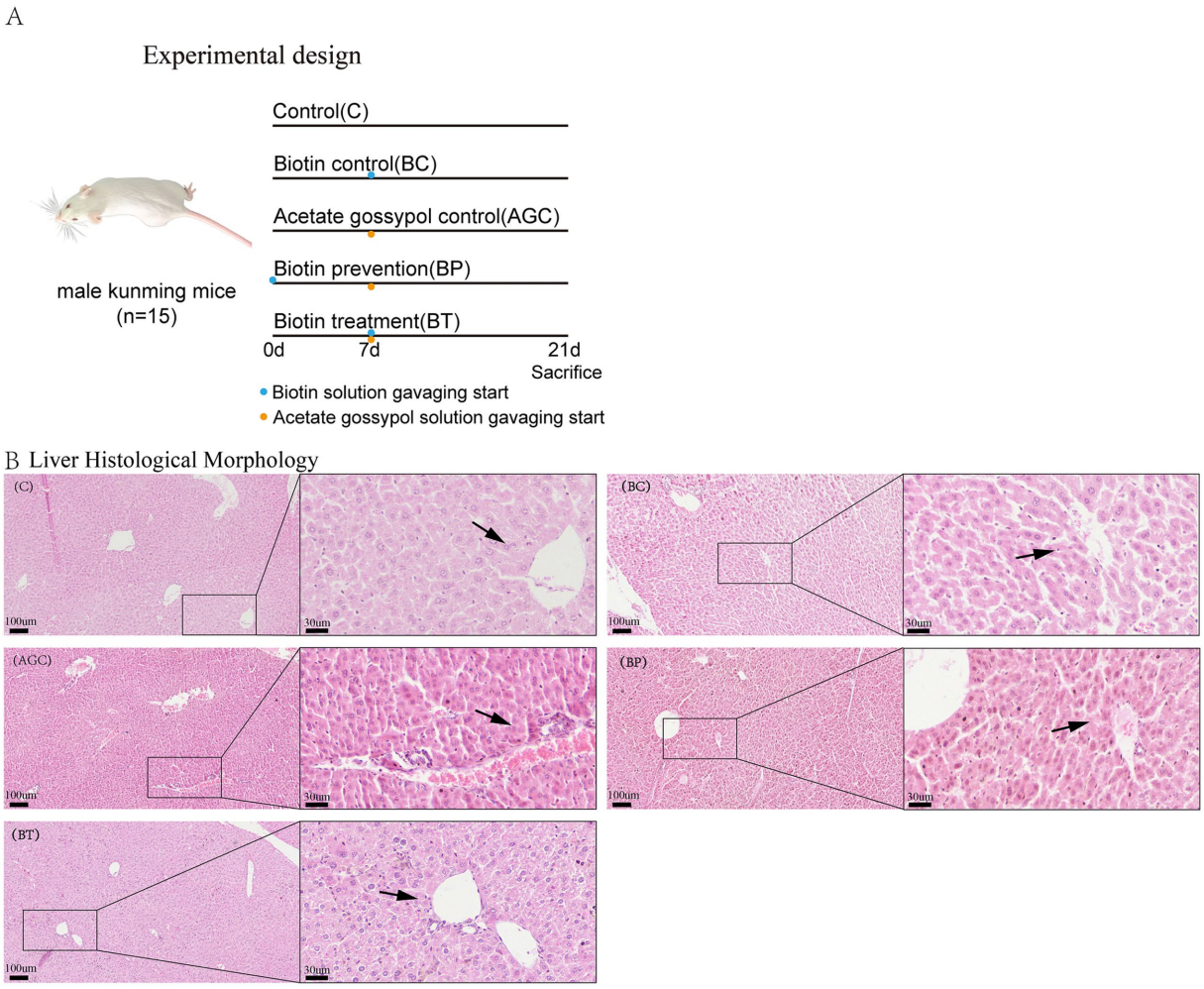

FIGURE 1
Effect of biotin on histomorphological of acetate gossypol-treated mice. **(A)** Experimental design. **(B)** Liver histological morphology (HE), magnification: 100× and 400×, the arrows indicate the liver cells surrounding the vein. C, the liver of control group mice; BC, the liver of biotin control group mice; AGC, the liver of acetate gossypol control group mice; BP, the liver of biotin prevention group mice; BT, the liver of biotin treatment group mice.

dehydrated through a graded ethanol series, cleared in xylene, and embedded in paraffin blocks. Serial sections of 5 μm thickness were then cut, mounted onto glass slides and air-dried. Finally, sections were stained with hematoxylin and eosin (H&E). Slide scanning and microscopic observation were performed using the MoticEasyScan Pro 6 digital slide scanning system. Blinded histological assessment was carried out by an experienced pathologist, for each sample, three intact villi were randomly selected, and the villus height and crypt depth was recorded.

2.5 Data analysis

The data was analyzed by a one-way ANOVA within the General Linear Model (GLM) framework in SPSS 28.0 software (SPSS Inc., Chicago, IL), followed by Duncan's multiple comparisons to investigate the differences between groups. All data were presented as means and standard error, and a p-values <0.05 was considered statistically significant.

3 Results

3.1 Growth performance and visceral index

As shown in Table 1, after two weeks of orally gavaging acetate gossypol, compared with the control group mice, the body weight of mice in AGC group, BP group and BT group reduced by 21.62, 17.84 and 13.64%, respectively ($p < 0.05$), and there was no significant difference in body weight among AGC group, BP group and BT group mice. In the first week of orally gavaging acetate gossypol, compared with the control group mice, the average daily feed intake of AGC group mice decreased ($p < 0.05$), in the second week of gavaging acetate gossypol, the average daily feed intake of AGC group mice was 47.25% lower than that of C group mice, compared with AGC group mice, the average daily feed intake of BP group and BT group increased by 40.47 and 45.69% ($p < 0.05$). The kidney index of mice in AGC group was lower than that in C group and BP group ($p < 0.05$), and the lung index of mice in AGC group, BP group and BT group was lower than that in the control group and BC group ($p < 0.05$).

TABLE 1 Effects of biotin on body weight, average daily feed intake, and visceral index in mice.

Project	C	BC	AGC	BP	BT	SEM	*p*-values
Body weight (g)							
0d	25.20	25.40	25.27	24.67	24.53	0.243	0.740
7d	31.87^{ab}	32.00^{ab}	33.20^{a}	30.67^{b}	30.53^{b}	0.299	0.025
14d	38.33	38.33	37.93	38.07	38.07	0.382	0.997
21d	41.21^{a}	42.59^{a}	32.30^{b}	33.86^{b}	35.59^{b}	0.709	<0.001
Average daily feed intake (g)							
1st wk	5.77	5.81	5.81	5.81	5.78	0.046	0.998
2nd wk	7.59^{a}	7.54^{a}	6.67^{b}	7.14^{ab}	7.48^{a}	0.104	0.005
3rd wk	7.26^{a}	7.37^{a}	3.83^{c}	5.38^{b}	5.58^{b}	0.237	<0.001
Visceral index							
Heart index	6.02^{a}	5.21^{b}	5.05^{b}	5.11^{b}	4.89^{b}	0.089	<0.001
Liver index	45.85	46.05	46.83	47.34	48.21	0.448	0.452
Kidney index	16.06^{a}	15.26^{ab}	13.24^{c}	14.63^{b}	13.51^{c}	0.208	<0.001
Spleen index	2.35	2.60	2.29	2.06	2.33	0.103	0.618
Lung index	6.61^{a}	5.56^{b}	5.10^{b}	4.86^{b}	5.27^{b}	0.172	0.009

$^{a-c}$Demonstrate a statistically significant distinction, $p < 0.05$.

3.2 Blood biochemistry

As shown in Table 2, that compared with the control group mice, the serum ALT, AST, TBIL, TP, LDL-C and BUN contents of AGC group mice ($p < 0.05$), and the contents of HDL-C and GLU were decreased ($p < 0.05$). Compared with the AGC group mice, the serum BUN content of BP group mice reduced by 15.38%, and the serum AST, TP and BUN contents of BT group mice reduced by 24.27, 7.39 and 10.73% ($p < 0.05$), respectively. Biotin had no significant effect on fluctuations of ALT, TBIL, HDL-C, LDL-C and GLU content in serum of acetate gossypol-treated mice.

3.3 Histopathological observation

As shown in Table 3, compared with the control group mice, the duodenum villus height of AGC group mice reduced by 19.94%, and the villus height/crypt depth of three intestinal segments of AGC group mice decreased significantly ($p < 0.05$). Biotin significantly increased the duodenum villus height and villus height/crypt depth in acetate gossypol-treated mice ($p < 0.05$). Biotin treatment significantly increased the jejunal villus height and the ratio of jejunal villus height to crypt depth of acetate gossypol-treated mice ($p < 0.05$). Compared with the control group mice, biotin reduced the jejunal crypt depth, and compared with the AGC group mice, biotin increased the jejunal villus height/crypt depth ($p < 0.05$).

As shown in Figure 1B, the arrows indicate the liver cells surrounding the vein. The sinusoids and portal triads of the control group and the BC group mice appear with no evidence of necrosis or inflammation. The liver cells of the AGC group were arranged in a disordered manner, the deposition of erythrocytes and plasma in the vein was obvious, indicating that acetate gossypol treatment caused certain liver damage in mice. The liver cells of the BP group and the BT group were arranged in a disordered manner, and there were blood cells between the liver cells. The liver cells in the BT group were arranged neatly, and there were blood cells between the liver cells, indicating that biotin treatment could alleviate the liver lesions caused by acetate gossypol to a certain extent.

4 Discussion

The continuous accumulation of low-dose gossypol in animals can cause poisoning, which is manifested as the decline of growth performance, damage of internal organs and reproductive performance. In this experiment, the body weight of mice was significantly reduced after one week of acetate gossypol administration, and the average daily feed intake of mice was significantly reduced after acetate gossypol administration. After two weeks of acetate gossypol administration, the feed intake of the AGC group mice was 47.25% lower than that of the control group. The body weight of grass carp treated with 400 mg/kg gossypol decreased significantly, the reason of this descent the intestinal physical barrier function was compromised (8). An experimental result indicated that dietary supplementation with gossypol at concentrations of 150 and 300 mg/kg in Nile tilapia significantly reduced the richness and diversity of the gut microbiota (9). Gossypol may exert inhibitory effects on animal growth performance, potentially by affecting intestinal metabolism.

Animal serum biochemical indicators can reflect the health of the body, and can understand whether animal glucose metabolism, lipid metabolism and liver function are normal. In this experiment, compared with the control group, the serum ALT, AST, TB, TP, ALP, LDL-C, and BUN content of mice fed with acetate gossypol were significantly increased, and the content of HDL-C and GLU was significantly decreased, indicating that acetate gossypol at a dose of 80 mg/(kg·BW) had an effect on glucose metabolism, lipid metabolism and liver and kidney function in mice. Compared with the control

TABLE 2 Effects of biotin on blood biochemical indices in mice.

Project	C	BC	AGC	BP	BT	SEM	*p*-values
ALT (U/L)	31.40^{b}	34.10^{b}	105.10^{a}	89.60^{a}	88.30^{a}	7.766	0.001
AST (U/L)	101.20^{c}	122.60^{bc}	177.20^{a}	157.30^{ab}	134.20^{bc}	6.338	<0.001
TBIL (μmol/L)	0.72^{b}	0.75^{b}	3.75^{a}	3.43^{a}	2.82^{a}	0.266	<0.001
TP (g/L)	58.61^{b}	57.09^{b}	63.90^{a}	62.12^{a}	59.18^{b}	0.552	<0.001
ALB (g/L)	21.61	25.04	27.62	28.20	26.96	0.960	0.185
ALP (U/L)	218.70	263.50	305.00	217.20	254.30	11.504	0.086
TG (mmol/L)	1.17	1.28	1.29	1.12	1.20	0.036	0.494
TC (mmol/L)	2.89	2.67	2.51	2.54	2.37	0.074	0.211
HDL-C (mmol/L)	2.52^{a}	2.25^{b}	1.58^{c}	1.61^{c}	1.55^{c}	0.069	<0.001
LDL-C (mmol/L)	0.30^{b}	0.24^{b}	0.60^{a}	0.60^{a}	0.50^{a}	0.036	<0.001
BUN (mmol/L)	6.96^{c}	8.66^{b}	9.69^{a}	8.20^{b}	8.65^{b}	0.192	<0.001
GLU (mmol/L)	6.66^{a}	5.61^{ab}	2.90^{c}	3.26^{c}	4.57^{bc}	0.359	0.002

$^{a-c}$Demonstrate a statistically significant distinction, $p < 0.05$.
ALT, alanine aminotransferase; AST, aspartate aminotransferase; TBIL, total bilirubin; TP, total protein; ALB, albumin; ALP, alkaline phosphatase; TG, triglyceride; TC, total cholesterol; HDL-C, high density lipoprotein cholesterol; LDL-C, low density lipoprotein cholesterol; BUN, urea; GLU, glucose.

TABLE 3 Effects of biotin on intestinal histomorphological in mice.

Project	C	BC	AGC	BP	BT	SEM	*p*-values
Duodenum							
Villus height (μm)	552.11^{a}	539.97^{a}	442.02^{b}	582.20^{a}	576.43^{a}	13.830	0.005
Crypt depth (μm)	109.37^{b}	112.69^{ab}	124.79^{ab}	129.93^{a}	129.86^{a}	2.732	0.032
Villus height/crypt depth	5.05^{a}	4.85^{ab}	3.57^{c}	4.47^{b}	4.50^{ab}	0.106	<0.001
Jejunum							
Villus height (μm)	528.00^{ab}	514.84^{ab}	465.50^{b}	540.69^{a}	522.21^{ab}	10.425	0.189
Crypt depth (μm)	105.41^{b}	114.85^{ab}	124.48^{a}	126.09^{a}	112.06^{ab}	2.678	0.071
Villus height/crypt depth	5.07^{a}	4.51^{b}	3.77^{c}	4.30^{bc}	4.75^{ab}	0.102	<0.001
Ileum							
Villus height (μm)	495.99^{a}	448.99^{ab}	444.4^{ab}	376.76^{c}	398.72^{bc}	10.772	0.002
Crypt depth (μm)	120.78^{ab}	112.93^{bc}	132.02^{a}	93.82^{d}	101.23^{cd}	2.790	<0.001
Villus height/crypt depth	4.09^{a}	4.00^{a}	3.38^{b}	4.04^{a}	3.94^{a}	0.067	0.02

$^{a-d}$Demonstrate a statistically significant distinction, $p < 0.05$.

group, mice treated with acetate gossypol suspension at the dosage of 25 mg/kg body weight for two weeks have higher serum ALT content and lower HDL-C content (10). When the free gossypol content in the feed was 103.1 mg/kg, the daily weight gain of meat ducks was significantly reduced, and the serum ALT and AST activities of meat ducks increased linearly with the increase of the level of gossypol in the feed, with the increase of the content of gossypol in the diet and the prolongation of the intake time of gossypol, the damage degree of the liver of meat ducks was significantly deepened (11). Gossypol can cause damage to animal liver and we hope that biotin could reduce these damages.

In this study, compared with the control group, the content of BUN in the biotin control group was significantly increased, and the content of HDL-C was significantly decreased. Feeding 5-week-old male rats with diets containing 0.05, 0.10 and 0.20% biotin for three weeks, there was no significant difference in plasma ALT and AST levels among the experimental groups, indicating that the intake of biotin below 0.20% had no significant effect on the liver of mice (12). It was found that the serum GLU content of diabetic rats was significantly reduced after 12 weeks of treatment with 15 mg/kg gossypol, which was 64% lower than that of untreated diabetic rats, gossypol reduced serum glucose content by inhibiting liver glucose production through down-regulation of related genes, such as the messenger mRNA levels of phosphoenolpyruvate carboxykinase and glucose-6-phosphatase (13). Treated mice with oral glucose 1 g/kg and oral gossypol 1 mg/kg and 2.5 mg/kg at the same time, compared with the mice treated with oral glucose, gossypol treatment groups showed significantly reduced blood GLU levels, and 2.5 mg/kg gossypol treatment group had stronger ability to reduce blood GLU levels (14). Gossypol can reduce serum GLU levels in hyperglycemic animals, which is consistent with the trend of acetate gossypol in reducing the blood glucose content in mice in this experiment. The effect of biotin supplementation on animal islets have been studied and it was found that compared with the control group, the insulin secretion of islets in

biotin-supplemented mice increased, the mRNA abundance of glucokinase, acetyl-CoA carboxylase and insulin increased, glucose tolerance increased, and glucose-stimulated serum insulin levels increased (15), indicating that biotin can be involved in the regulation of blood glucose.

Biotin may affect the content of HDL-C in serum of mice by regulating lipid metabolism. Biotin also affects the level of BUN in animal body. BUN is the main product of nitrogen metabolism, which is usually produced in the urea cycle of liver. Biotin may also indirectly affect the production of urea by affecting the islets of animals. Insulin not only promotes GLU uptake, but also promotes the synthesis and utilization of amino acids. When the level of insulin increases, the metabolism of amino acids accelerates, and the nitrogen produced also increases, eventually increasing the synthesis of urea (16). The study of Sawamura H et al. fed weaned rats with diets containing 0.01, 0.1 and 1.0% biotin for six weeks, no significant differences in serum TG, ALT, AST and BUN levels were observed among the groups. Intake of 1.0% and below biotin for six weeks did not lead to a significant increase in serum TG, ALT, AST and BUN levels, but the serum BUN content in the 1.0% biotin group was 21% higher than that in the control group (17), which was consistent with the trend of this test.

Through the experiments on rats and mice, it was found that biotin could reduce the increase of blood ammonia level caused by acute ammonia poisoning induced by ammonium acetate (18), indicating that after ammonia poisoning, biotin in animal blood could be rapidly detoxified, which was consistent with the fact that the BP group and the BT group could significantly reduce the increase of serum BUN content in acetate gossypol-treated mice. Biotin as a cofactor for biotin-dependent urea carboxylase, catalyzes urea decomposition into ammonia and carbon dioxide, facilitating nitrogen source utilization (19). Biotin has the effect of regulating BUN level in animal body. It shows that biotin can reduce the liver and kidney function damage caused by acetate gossypol to a certain extent, enhance the body's resistance, and protect the liver and kidney of the body.

The histological morphology of animal intestine is closely related to the function of intestine. The ratio of intestinal epithelial villus height to crypt depth is directly related to the digestion and absorption ability of intestine, which is an important index to evaluate intestinal health and absorption ability. In this experiment, biotin significantly increased the duodenum villus height and villus height/crypt depth in acetate gossypol-treated mice, and biotin treatment significantly increased the jejunal ratio of jejunal villus height to crypt depth of acetate gossypol-treated mice. The villus height and the ratio of villus height to crypt depth in duodenum, jejunum and ileum of goslings treated with 50 mg/(kg·BW) gossypol were significantly decreased on day 7 and 14 (20). Studies have found that the intestinal barrier permeability of Nile tilapia fed a 300 mg/kg gossypol diet increased, the intestinal epithelial cells at the top of the intestinal villi fell off, and the villus height, villus width and basal layer thickness of the intestine were significantly reduced (21). Compared with the control group, when soybean meal was replaced by 10 and 20% cottonseed meal, the villus height and crypt depth of duodenum and jejunum were significantly reduced, and the ratio of villus height to crypt depth of duodenum and jejunum was also significantly reduced (22). Compared with the control group, the villus height and villus height/crypt depth of mice gavaged with 0.2 mL solution with free gossypol = 100.06 mg/kg every other day for ten weeks are decreased significantly, and crypt depth increased, this result has the same trend with our experiment (23). These results were consistent with the results of this experiment, indicating that gossypol damaged the intestinal morphology and digestion and absorption capacity of mice.

Biotin may mitigate acetate gossypol-induced hepatic and intestinal damage in mice by modulating immune factors and antioxidant capacity. Biotin deficiency impacts intestinal health, potentially exacerbating inflammation and contributing to inflammatory bowel disease pathogenesis (24, 25). Biotin-deficient mice exhibit elevated serum TNF-α levels compared to controls (26). *In vitro* studies demonstrate biotin's inhibitory effect on IL-1β production (27), while cellular experiments reveal 4.3 and 5.6 fold increases in IFN-γ and IL-1β mRNA abundance with biotin supplementation (28). Biotin treatment reduces inflammatory cytokine expression, normalizing IL-6 levels in mice (29). Dietary biotin (1.62 mg/kg) restores hepatic/intestinal morphology and alleviates inflammation in turbot (30). Grass carp show minimal antioxidant enzyme activity and glutathione concentration at the lowest biotin dose (0.012 mg/kg) (31), whereas 0.33 mg/kg biotin significantly enhances superoxide dismutase activity in carp tissues and serum (32). These results suggest that biotin may have a certain effect on the integrity of intestinal morphology and function in animals. Combined with the results of our experiment, it was found that biotin can improve the intestinal and liver damage caused by acetate gossypol in mice.

5 Conclusion

In this study, the use of biotin potentially increased the average daily feed intake of acetate gossypol-treated mice, reduce the increase of serum AST, TP and BUN levels in acetate gossypol-treated mice, and increase the values of villus height/crypt depth in duodenum and jejunum in acetate gossypol-treated mice. Biotin has the potential to protect the morphological and functional integrity of the liver and intestine in acetate gossypol-treated mice. This experiment found that biotin has a certain protective effect on the liver and intestinal health of acetate gossypol-treated mice. In the future, we can continue to study the effect on growth performance, serum biochemical profiles, and morphology of acetate gossypol-treated mice.

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

All experimental procedures involving animals were approved (animal protocol number: 2024009) by the Animal Welfare and Ethics Committee of Xinjiang Agricultural University, Urumqi, Xinjiang, China. The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

QL: Data curation, Formal analysis, Investigation, Methodology, Writing – original draft, Writing – review & editing. TX: Data curation, Investigation, Writing – original draft. ZA: Data curation, Writing – original draft. WF: Data curation, Writing – original draft. CW: Data curation, Funding acquisition, Project administration, Supervision, Writing – review & editing.

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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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Immunological response enhancement in cows with subclinical mastitis fed diet supplemented with *Macleaya cordata*

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Introduction: The present study explored the immune response, milk production and health status of mastitis-infected lactating cows fed diets supplemented with Macleaya cordata extract.

Methods: Twenty-four Holstein and Jersey cows were equally assigned to two experimental groups: the first group was fed a control diet (control), and the second experimental group was fed a control diet plus Macleaya extract at 8 g/head/d (Macleaya). The experiment was conducted for 60 days. The daily milk yield was recorded, and the milk samples were analyzed for total solids, fat, protein, and lactose contents.

Results: Blood samples were analyzed for different blood constituents, biochemical parameters, antioxidant capacity and immune indices. Compared with the control, the addition of Macleaya improved immune indices ($p < 0.05$). No significant differences ($p > 0.05$) were recorded between the two groups for different rumen liquor parameters, antioxidant capacities, milk yields or compositions. However, supplementing the diet with Macleaya significantly decreased SCC, SAA, and endotoxin.

Conclusion: This study suggested that supplementing the diets of lactating cows with Macleaya extract potentially improved their immune competence without adversely impacting their productive performance.

KEYWORDS

antioxidant capacity, immune indices, *Macleaya cordata*, mastitis, milk yield

Introduction

Mastitis in cows is an inflammatory reaction of the mammary gland udder tissue caused by either physical injury or microbial infection. It is regarded as the most prevalent disease that causes financial losses for the dairy industry because of lower milk quality and output (1, 2). On average, the overall failure cost caused by bovine mastitis is projected to be $147 per cow per year, with milk production losses and culling accounting for 11% to 18% of the gross margin per cow per year (3). Seventy percent of the overall losses are due to mammary tissue injury, which causes reduced milk output (4). Clinical, subclinical, and chronic mastitis are

the three categories into which bovine mastitis can be divided according to the severity of inflammation. A fever and a red, swollen udder are two obvious signs of a clinical case of bovine mastitis that are simple to identify. The cow milk seems watery, with flakes and clots (5).

In dairy cows, mastitis is caused by bacteria that enter the teat canal and mammary glands and cause inflammation of the udder. These bacteria grow and create toxins that injure milk-secreting tissue, in addition to causing physical damage and chemical irritation. These factors lead to an increase in leukocytes, or somatic cells, in milk, which decreases their amount and degrades the quality of both the milk and its byproducts. Seventy percent of the losses are attributable to injury to the mammary tissue, which results in reduced milk production (4). The incidence of mastitis in a herd can be reduced, but mastitis cannot be completely eradicated. Good husbandry and sanitation techniques, teat dipping after milking, treating mastitis during the nonlactating phase, and culling chronically sick animals are the main components in the management of mastitis (5).

The two main strategies for controlling mastitis are to either reduce the teat's exposure to possible pathogens or increase the ability of dairy animals to fight infection. Widely prevalent in southern China, *Macleaya cordata* (MC) is a perennial herb belonging to the group *Papaveraceae* that is used in traditional Chinese medicine (6). The primary bioactive components of MC extract (MCE) are alkaloids, primarily sanguinarine and chelidonine, which have been shown to have antibacterial (7), anti-inflammatory (8), antitumor (9), and antioxidant properties (10).

In the European Union (EU) and China, sanguinarine derived from MC has been authorized for use as a feed supplement. Research has shown that this substance enhances the immune system and growth performance of livestock (11, 12). The majority of MCE research to date has been conducted on monogastric animals. For example, weaning piglets fed with 50 mg/kg MCE improved growth performance and resulted in a lower rate of diarrhea because the treatment increased the serum IgG concentration, antioxidant activity, and profile of beneficial bacteria in the intestine (13). Yang et al. (14) reported that adding 0.3 g/d MCE to the meals of early-weaned goats reduced the inflammatory response in their lower guts. Adding MCE to the sheep diet enhances their immune system, antioxidant levels, and growth performance (15). To the best of our knowledge, little research has been conducted on the consequences of feeding lactating cows MCE. Consequently, the aim of the current study was to examine the effects of supplementing the diets of lactating cows with dietary *Macleaya cordata* on the immune response, rumen fermentation, and milk yield and composition.

Materials and methods

All the experimental protocols for the use of animals and the experimental procedures in this study were approved by the Laboratory Animal Welfare and Animal Experimental Ethical Inspection Committee, Institute of Subtropical Agriculture, the Chinese Academy of Sciences, Changsha, China.

Twenty-four Holstein and Jersey cows with similar parities (2 parities), calving dates, daily milk yields, mid-lactation rates and recessive mastitis (somatic cell count (SCC) greater than 200×10^3/mL) were selected for the current experiment. Using a completely randomized block design, the experimental animals were divided into two groups with 12 animals (three Holstein and nine Jersey cows) in each group, and the milk SCCs in the control and experimental groups were 480×10^3/mL and 492×10^3/mL, respectively. The experimental diet was offered to meet their nutrient requirements according to NRC (16) recommendations. The cows in the control group were fed a basic diet, as shown in Table 1 (control), and the cows in the experimental group (Macleaya) were fed a basic diet supplemented with Macleaya extract at 8 g/head/d. The active ingredient of macleaya is benzenoid alkaloids (sanguinarine content of 1.5% and chelidonine content $\geq 0.75\%$).

The experiment lasted for 60 days, including 10 days for adaptation and 50 days as the experimental phase. The animals were raised in individual pens, fed twice daily (06:00 and 13:30), and had free access to clean drinking water.

Sample collection

Feed sample collection

Approximately 500 g of TMR sample was collected and dried in an oven at 65°C for 48 h. Feed samples were stored at −20°C until further analysis.

TABLE 1 Ingredients and chemical composition of the control diet.

Item	Basic diet
Ingredients, g/kg of DM	
Silage corn	487.80
Spanish alfalfa	48.70
Domestic oat grass	91.50
Timothy	24.40
Beet pulp	24.40
Brewer's grains	146.30
Molasses	24.40
Corn	79.70
Soybean meal	27.40
Extruded soybean	1.10
Cottonseed meal	15.20
DDGS	15.20
Sodium bicarbonate	1.50
Premix	17.50
Chemical composition, g/kg of DM	
Dry matter (DM)	568.70
Crude protein (CP)	135.20
Ether extract (EE)	55.90
Neutral detergent fiber (NDF)	308.10
Acid detergent fiber (ADF)	176.20
NE_L (MJ/kg)	6.70

Each kg of premix contained 9,000 mg CuSO4, 300 mg FeSO4·H2O, 300 mg $ZnSO_4 \cdot H_2O$, 31,000 mg $CoCl_2$, 90 mg Na_2SeO_3, 130 mg VA 900,000 IU, 180,000 IU VE 25,000 mg, and 22,000 mg niacin. The nutrient contents were measured, except for the NE_L.

Rumen liquor sample collection

Representative samples of rumen liquor were collected from the animals at 60 days of the experiment. The samples were collected from the central rumen at 4 h after the morning feeding through the oral cavity via an oral stomach tube as reported by Wang et al. (17). In summary, the first 150 mL of collected rumen mixture was discarded, and then the remaining 150 mL was collected and filtered through four gauze layers while being continuously exposed to carbon dioxide.

Blood sample collection

On the last day of the experiment, blood samples were collected from the jugular vein of the cows at 4 h post morning feeding. Tubes containing the anticoagulant heparin sodium and a venous blood collection needle were used to collect blood samples from the cows. The tubes of collected blood were inverted gently many times to ensure that the blood sample and anticoagulant were fully mixed. The blood samples were centrifuged for 15 min at 3000× g to quickly separate the plasma. After that, the plasma was separated into sterile 1.5 mL Eppendorf tubes and stored at −80°C.

Milk yield recording and sampling

The daily milk yield of each experimental cow was recorded during the 60-day data collection period. Cows were milked three times daily at 05:30, 13:00, and 19:00 h. Milk samples were collected at 60 days, and morning and afternoon milk samples were stored at 4°C until the evening samples were collected. The samples were subsequently pooled according to morning, afternoon, and evening milk yields.

Sample analysis

Feed sample analysis

The feed samples were analyzed for DM (method 930.15), crude protein (CP; method 954.01) and ether extract (EE; method 920.39) content according to the AOAC official methods (18). Neutral detergent fiber (NDF) content was determined according to the procedure of Van Soest et al. (19) without the use of alpha-amylase but with sodium sulfite. Acid detergent fiber (ADF) was analyzed according to AOAC (18) (method 973.18).

Rumen liquor sample analysis

pH and the concentrations of ammonia nitrogen (NH_3-N) and total volatile fatty acid and their proportion (VFA) in the rumen liquor were measured via methods described by Wang et al. (17).

Blood sample analysis

Blood samples were used to analyze biochemical parameters, antioxidant parameters, and immune indices. Blood cells [such as the white blood cell (WBC), neutrophil (Neu), lymphocyte (Lym), monocyte (Mon), eosinophil (Eos), basophilic granulocyte (Bas), red blood cell (RBC), hemoglobin (HGB), hematocrit (HCT), mean corpuscular volume (MCV), mean corpuscular-hemoglobin (MCH), mean corpuscular-hemoglobin concentration (MCHC), red blood cell distribution width variable coefficient (RDW-CV), red blood cell distribution width (RDW-SD), platelet count (PLT), mean platelet volume (MPV), platelet distribution width (PDW), and thrombocytocrit (PCT)], and EDTAK 2 anticoagulant whole blood collected within 2 h were tested via an automatic veterinary blood cell analyzer (BC-5000, Mindray, China), and the specific operation was carried out according to the operation procedure of the automatic veterinary blood cell analyzer.

The plasma total protein (TP), albumin (ALB), alanine transaminase (ALT), aspartate transaminase (AST), alkaline phosphatase (ALP), gamma glutamyl transpeptidase (GGT), cholinesterase (CHE), direct bilirubin (DBIL), total bilirubin (TBIL), triglyceride (TG), total cholesterol (CHOL), low-density lipoprotein cholesterol (LDL-c), high-density lipoprotein cholesterol (HDL-c), glucose (GLU), amylase (AMS), D-lactic acid (LAC), lactate dehydrogenase (LDH), blood urea nitrogen (BUN), creatinine (CRE), and calcium (Ca) and phosphorus (P) concentrations were measured on a Mindray BS-230 automatic chemistry analyzer (Shenzhen, China) (20).

For the immune indices, the plasma concentrations of interleukin 1β (IL-1β), interleukin 2 (IL-2), interleukin 6 (IL-6), interleukin 8 (IL-8), tumor necrosis factor (TNF-α), immunoglobulin G (IgG), immunoglobulin A (IgA), and immunoglobulin M (IgM) were determined with a Multimode Microplate Reader (Infinite M200 PRO Multimode, Tecan, Männedorf, Switzerland) and a commercial ELISA kit (CUSABIO, Wuhan, China) according to the manufacturer's protocols.

The samples were analyzed for different antioxidant parameters via assay kits (ELISAs) according to the manufacturers' instructions. The total antioxidant capacity (TAC), malondialdehyde (MDA), superoxide dismutase (SOD), and glutathione peroxidase (GPx) contents were determined as described by Khattab et al. (21).

Milk sample analysis

Milk samples were analyzed for fat, lactose, protein, nonfat solid (NFS), and total solids (TS) contents via a Foss Milko Scan FT1 Analyzer (Foss Electric A/S, Hillerod, Denmark). The average yield (g/d) of each milk component was calculated for individual animals by multiplying the milk yield by the component concentration (g/kg) of milk. The serum amyloid A (SAA) concentration in milk samples was determined via specific ELISA kits, and the endotoxin concentration was determined with a Multimode Microplate Reader (Infinite M200 PRO Multimode, Tecan, Männedorf, Switzerland) and a commercial ELISA kit (CUSABIO, Wuhan, China) according to the manufacturer's protocols. Energy-corrected milk (ECM) was calculated according to Sjaunja et al. (22).

Statistical analysis

All the data were subjected to analysis using ANOVA via the MIXED procedure of SAS (version 9.4; SAS Inc.). The fixed effects of breed, treatment, and the interaction effect between breed and treatment were included in the model. The cows represented a random effect. The results are expressed as the means. A p-value < 0.05 was considered to indicate a statistically significant difference.

Results

Rumen fermentation profile

The effects of supplementation of lactating cows' diets with *Macleaya cordata* on rumen fermentation profile are presented in

TABLE 2 Effect of supplementing the diets of lactating cows with *Macleaya cordata on* ruminal fermentation.

Item	Control	Macleaya	±SE	*p*-value
pH	6.50	6.56	0.037	0.322
NH_3-N (mM/L)	6.17	5.39	0.364	0.276
Acetate %	66.40	66.90	0.22	0.318
Propionate %	19.00	18.30	0.14	0.211
A/P ratio	3.51	3.68	0.040	0.202
Iso-butyrate %	0.98	0.94	0.040	0.615
Butyrate %	11.80	11.90	0.15	0.804
Iso-valerate %	0.98	1.03	0.049	0.243
Valerate %	1.33	1.32	0.022	0.886
TVFA mM/L	102.40	86.20	2.26	0.176

Control, control group fed a basic diet; Macleaya, Macleaya group fed a basic diet supplemented with Macleaya extract at 8 g/head/d; ±SE, standard error.

Table 2. All values of the different parameters were within the normal range for each parameter (23). The acetate, A/P ratio, isobutyrate, butyrate, and isovalerate concentrations were not significantly greater ($p > 0.05$) in the Macleaya group than in the control group. Additionally, insignificant ($p > 0.05$) reductions in the NH_3-N, propionate and TVFA concentrations were detected in the Macleaya group compared with those in the control group.

Plasma biochemical values

Table 3 shows the values of the plasma biochemical parameters of the experimental groups. All values of the different parameters were within the normal range of each parameter. No significant differences ($p > 0.05$) in different plasma biochemical parameters were noted between the experimental groups. Compared with the control group, the Macleaya group tended to decrease ($p = 0.09$) the concentration of CHE and slightly increased ($p > 0.05$) the globulin, ALT, AST, GGT, LDH, BUN, CRE, CHOL, HDL-c, amylase, LDL-c and phosphorus concentrations.

Blood constituents

Table 4 shows the values of blood constituents for the experimental groups. No values of different blood constituents were outside the normal range. The white blood cell count, neutrophil count, lymphocyte count, monocyte count, basophil count, RBC count, HGB, HCT, MCHC, RDW-CV, PLT, MPV, and PCT did not significantly differ between the Macleaya and control groups ($p > 0.05$). Compared with the control group, the Macleaya group presented a significant reduction in Eosinophil. However, compared with the control diet, supplementation with the Macleaya diet significantly increased the MCV, MCH, RDW-SD, and PDW.

TABLE 3 Effects of supplementing the diets of lactating cows with *Macleaya cordata on* plasma biochemical parameters.

Item	Control	Macleaya	±SE	*p*-value
TP (g/L)	79.30	79.10	0.95	0.343
ALB (g/L)	45.40	44.00	0.57	0.791
Globulin (g/L)	33.80	35.10	0.57	0.190
A/G ratio	1.36	1.27	0.024	0.357
ALT (IU/L)	24.50	32.00	0.63	0.209
AST (IU/L)	80.90	89.10	2.46	0.742
ALP (IU/L)	51.20	49.80	3.50	0.814
GGT (IU/L)	36.80	36.80	1.12	0.957
LDH (IU/L)	1,186	1,193	19.0	0.467
BUN (mmol/L)	5.23	5.82	0.095	0.570
CRE (μmol/L)	51.90	55.40	1.21	0.641
GLU (g/L)	3.71	3.78	0.059	0.637
TG (mmol/L)	0.29	0.26	0.007	0.316
CHOL (mmol/L)	4.25	5.16	0.122	0.231
HDL-c (mmol/L)	3.51	4.37	0.109	0.146
AMS (U/L)	31.10	31.80	0.74	0.433
LDL-c (mmol/L)	1.30	1.83	0.071	0.584
LACT (mmol/L)	2.24	1.89	0.276	0.301
CHE (g/L)	183	164	4.00	0.099
Ca (mmol/L)	2.48	2.15	0.023	0.390
P (mmol/L)	1.88	2.08	0.028	0.667

Control, control group fed a basic diet; Macleaya, Macleaya group fed a basic diet supplemented with Macleaya extract at 8 g/head/d; ±SE, standard error.

Plasma antioxidant capacity

The antioxidant capacity data are shown in Table 5. The levels of the antioxidant parameters total antioxidant capacity (TAC), superoxide dismutase (SOD), malondialdehyde (MDA) and glutathione peroxidase (GPx) did not significantly ($p > 0.05$) differ between the Macleaya group and the control group.

Immune indices

The immune indices of the different experimental animal groups are listed in Table 6. The results revealed significant decreases in the levels of IL-1β ($p = 0.020$), IL-6 ($p = 0.0004$), IL-8 ($p = 0.008$), and TNF-α ($p = 0.007$) in the Macleaya group compared with those in the control group. However, ceruloplasmin ($p = 0.015$) was greater in the Macleaya group than in the control group. IL-2, heptoglobin, IgA, IgG, and IgM levels were not significantly different ($p > 0.05$) between the experimental groups.

TABLE 4 Effect of supplementing the diets of lactating cows with *Macleaya cordata on* blood constituents.

Item	Control	Macleaya	±SE	*p*-value
White blood Cells (10^9/L)	7.32	7.31	0.261	0.983
Neu (10^9/L)	3.13	3.09	0.157	0.905
Lym (10^9/L)	3.46	3.60	0.131	0.605
Mon (10^9/L)	0.37	0.38	0.044	0.837
Eos (10^9/L)	0.34	0.22	0.044	0.009
Bas (10^9/L)	0.02	0.02	0.006	1.000
Neu (%)	42.00	42.30	1.17	0.903
Lym (%)	48.00	49.50	1.14	0.513
Mon (%)	5.13	5.06	0.237	0.881
Eos (%)	4.54	2.85	0.261	0.001
Bas (%)	0.34	0.29	0.092	0.589
RBC (10^{12}/L)	5.81	5.73	0.150	0.776
HGB (g/L)	106	111	2.6	0.355
HCT (%)	28.50	30.20	0.76	0.266
MCV (fL)	49.10	53.00	0.58	0.0003
MCH (pg)	18.20	19.50	0.21	0.002
MCHC (g/L)	371	367	1.6	0.260
RDW-CV (%)	21.90	23.10	0.33	0.065
RDW-SD (fL)	38.20	43.60	0.85	0.001
PLT (10^9/L)	424	314	29.6	0.063
MPV (fL)	5.84	5.81	0.069	0.822
PDW	15.10	15.40	0.07	0.007
PCT (%)	0.25	0.18	0.038	0.068

Control, control group fed a basic diet; Macleaya, Macleaya group fed a basic diet supplemented with Macleaya extract at 8 g/head/d; ±SE, standard error.

TABLE 5 Effects of supplementing the diets of lactating cows with *Macleaya cordata on* plasma antioxidant parameters.

Item	Control	Macleaya	±SE	*p*-value
TAC (U/mL)	0.96	0.96	0.019	0.898
SOD (U/mL)	71.20	73.10	2.04	0.637
MDA (nmol/mL)	4.20	3.67	0.278	0.339
GPx (U/L)	442	461	32.9	0.766

Control, control group fed a basic diet; Macleaya, Macleaya group fed a basic diet supplemented with extract at 8 g/head/d; ±SE, standard error.

Milk yield and composition

The milk yield did not significantly ($p > 0.05$) differ between the Macleaya and control groups (Table 7). However, the ECM was significantly greater in Macleaya than in the control ($p = 0.0203$). Additionally, no significant differences in pH, milk total solids content, milk fat content, milk protein content, milk lactose content, or milk NFS were recorded between the experimental groups. The same trend was observed for milk total solids yield, milk fat yield, milk protein yield, milk lactose yield, and milk NFS yield.

Compared with the control diet, the diets supplemented with Macleaya significantly decreased the SCC ($p = 0.048$), SAA ($p = 0.001$) and endotoxin content ($p = 0.001$).

TABLE 6 Effects of supplementing the diets of lactating cows with *Macleaya cordata on* immune indices.

Item	Control	Macleaya	±SE	*p*-value
IL-1β (pg/mL)	53.40	35.50	7.42	0.020
IL-2 (pg/mL)	257	357	174.4	0.570
IL-6 (pg/mL)	145.40	96.40	12.92	0.0004
IL-8 (pg/mL)	139	104	7.40	0.008
TNF-α (pg/mL)	95.30	58.20	6.91	0.007
Haptoglobin (μg/mL)	691	657	34.71	0.317
Ceruloplasmin (μg/mL)	815	1,087	107	0.015
IgA (μg/mL)	1837	1859	56.9	0.793
IgG (μg/mL)	12,811	12,969	41.1	0.740
IgM (μg/mL)	952	921	146.6	0.540

Control, control group fed a basic diet; Macleaya, Macleaya group fed a basic diet supplemented with Macleaya extract at 8 g/head/d; ±SE, standard error.

TABLE 7 Effect of supplementing the diets of lactating cows with *Macleaya cordata on* milk yield and composition.

Item	Control	Macleaya	±SE	*p*-value
Milk Yield (kg)	20.27	20.34	0.29	0.22
ECM (kg)	21.80	22.50	0.29	0.0203
pH	6.59	6.60	0.788	0.797
SCC (10^3/ml)	446	391	85.2	0.048
Milk composition (%)				
TS	13.90	14.40	0.187	0.246
Fat	4.73	5.37	0.241	0.120
Protein	3.99	4.01	0.172	0.768
Lactose	4.51	4.38	0.083	0.297
NFS	9.31	9.20	0.070	0.617
Urea (mg/dL)	19.40	20.00	0.19	0.520
SAA (μg/ml)	9.70	5.84	0.430	0.001
Endotoxin (EU/ml)	12.40	8.04	0.535	0.001
Milk yields (g/d)				
TS	2,383	2,447	88.3	0.713
Fat	797	880	35.4	0.242
Protein	693	685	27.9	0.879
Lactose	796	786	36.9	0.898
NFS	1,625	1,607	66.2	0.889

Control, control group fed a basic diet; Macleaya, Macleaya group fed a basic diet supplemented with Macleaya extract at 8 g/head/d; ±SE, standard error.

Discussion

The most expensive disease that affects dairy cattle is still mastitis. When the udder becomes inflamed and germs infiltrate the teat canal and mammary glands, dairy cows develop mastitis. These bacteria

grow and release toxins, in addition to physical stress and chemical irritants, which harm milk-secreting tissue.

These factors lead to an increase in leukocytes, or somatic cells, in milk, which decreases its volume and has a negative effect on the quality of milk and its byproducts (5). Mastitis in dairy cattle can be decreased by monitoring somatic cell levels and quickly diagnosing and treating the condition.

As previously stated, the emergence of antibiotic-resistant pathogen strains has become a significant obstacle in antibiotic treatment, and if the use of antibiotics as the primary treatment strategy is still in place, its efficacy is restricted (24, 25). Moreover, the milk industry is being pressured to use fewer antimicrobial medications due to growing concerns about antibiotic resistance in public health issues. Consequently, it is necessary to look for alternatives to antibiotic therapy, particularly those originating from natural sources such as plants and animals (26–28). Researchers are becoming more interested in the use of plants to treat bovine mastitis since they are rich sources of chemicals for traditional medicine. The benefit of plant-derived chemicals over antibiotics is that they do not cause resistance, even after extended use. The low toxicity of chemicals originating from plants is another benefit (2).

Chinese people have long utilized plants of this genus for their diverse medicinal properties. Traditionally, the entire plant of *Macleaya cordata* has been used as a popular medicine to cure inflamed wounds and stings and to temporarily reduce muscular discomfort. *M. cordata* leaves are often used in traditional Chinese medicine to treat rheumatism, scabies, bruises, ulcers, and other skin conditions (29). Both Macleaya species are used primarily for the treatment of inflammation and certain skin conditions. Currently, Trichomonas vaginalis, rheumatoid arthritis, wounds, and other conditions are treated with *M. cordata* in traditional Chinese medicine (30). The anticancer, antiseptic, anti-inflammatory, and insecticidal properties of several extracts and isolated parts of the Macleaya species have been assessed (31). According to previous studies (32–35), quaternary benzo[c]phenanthridine alkaloids are the main bioactive substances. Numerous pathophysiological mechanisms that respond to tissue injury and the host's defense against invasive microorganisms depend on inflammation. An increase in cytokines and nitric oxide production is indicative of inflammation. Numerous investigations have indicated that the Macleaya species has an anti-inflammatory effect (36). According to Vrba et al. (37), the anti-inflammatory properties of sanguinarine and chelerythrine may also be linked to their capacity to prevent the production of superoxide radicals by phagocyte NADPH oxidase. However, research on the direct effects of Macleaya extracts on mastitis in dairy cows is limited. These findings suggest that Macleaya has potential effects on oxidative stress control and that it has immune-boosting capabilities, antioxidant properties, antibacterial effects, and anti-inflammatory properties. However, the specific impact on mastitis remains to be fully explored.

The current study showed no noticeable effect of *Macleaya cordata* extract on rumen fermentation profile of cows. These findings might be due to the bioactive alkaloids in *Macleaya cordata*, such as sanguinarine and chelerythrine, which known for their antimicrobial and anti-inflammatory properties (38). While these compounds may affect certain microbial populations, they might not target the specific microbes involved in producing key fermentation metabolites (e.g., VFAs, NH_3-N) (39). Also, the rumen microbiome is remarkably adaptable and robust.

Leucocyte settling is the mammary gland's main defensive function during an inflammatory response. These typically have fewer than 200,000 cells per milliliter of milk. The cell count increases when mastitis is present, mostly due to leukocyte infiltration. Inflamed neutrophils and macrophages eliminate cellular aggregates and phagocytize ingested bacteria (40, 41). An aspect of nonspecific resistance, the acute phase response is characterized by a rapid rise in protein synthesis, which is known to be a hallmark of inflammation in animals (42, 43).

The findings of the present study revealed that the addition of macleaya extract to the diet decreased the IL-1β, IL-6 and IL-8 levels. A reduction in the IL-1β, IL-6 and IL-8 levels in cows fed Macleaya extract may indicate the resolution of inflammation or successful treatment for mastitis. Cows with naturally occurring and experimentally induced mastitis, as well as those injected with bacterial LPS, have been shown to have relatively high levels of IL-6 in their blood and milk (44, 45). The fact that IL-6 may stay in the bloodstream longer than other proinflammatory cytokines is a very significant benefit (46).

Both clinical and subclinical diseases may develop from mastitis. According to recent research, the host's reaction during the early stages of illness, including the impacts of cytokines released, influences the consequences of infection (47–49). Small molecule proteins called cytokines are crucial for intercellular communication. Numerous processes, including cell differentiation, proliferation, remodeling, degeneration, regeneration, and even cell death, are either driven or hindered by cytokines. The SCC and levels of generated cytokines (interleukin (IL)-1, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, and IL-12) in milk increase in both naturally occurring and experimentally induced mastitis (50). One of the cytokine families that is most commonly represented is the IL-1 family (51). Through a number of signaling pathways, IL-1 family cytokines interact with immune competent tissue cells (52), which mostly carry out inflammatory, autoimmune, fracture-initiating, and mitogenic tasks. Interleukin-1 alpha (IL-1α) locally regulates first-line defense mediators in experimental acute bovine mastitis, including prostaglandin (PG-F2α) and leukotriene (LT-B4), indicating its participation in early inflammation (53). T and B lymphocytes, eosinophils, basophils, mast cells, plasma cells, and epithelial cells are the primary producers of IL-4 in the mammary glands of cows (54, 55). These cells also serve as the foundation for type 2 immunity (56). IL-1 has an indirect effect on immunity by interacting with other messenger molecules through a number of signaling pathways (52). Macrophages, lymphocytes, monocytes, endothelial cells, and fibroblasts in the mammary gland generate IL-1. Furthermore, IL-1 promotes the synthesis of TNF-α, IL-6, IL-8, and IL-12 in addition to its own (55).

Numerous experimental investigations have demonstrated the importance of inflammatory components in the initial inflammatory response. Upon identifying microbial components, bovine mammary epithelial cells can trigger an innate immune response through the release of proinflammatory cytokines (IL-1β, TNF-α, IL-6, and IL-8) and antimicrobial molecules (cathelicidin and defensins) (57, 58). The primary source of the proinflammatory cytokine tumor necrosis factor (TNF)-α is produced by macrophages. It may carry out a variety of tasks, including inducing inflammatory responses and encouraging the manufacture of other cytokines, regulating essential cell activities,

and preserving tissue homeostasis, depending on where it is released and which receptor it binds to (59–61). The acute-phase cytokine TNF-α is generated in the early phases of infection (62, 63). The TNF-α concentration significantly decreased as the cows recovered (64). Consequently, the significance of TNF-α can extend beyond its proinflammatory function. However, with endotoxin infusion, the TNF-α concentration only slightly increased (65). TNF-α levels in serum are undetectable, but they increase in milk when the effects of intramammarily administered lipopolysaccharide on the acute-phase response in early and late lactation are examined (66).

Serum amyloid A, an apoprotein that is a component of high-density lipoprotein, is an acute phase protein (APP) (67). SAA functions by preventing platelet aggregation, phagocytosis, and the proliferation of lymphocytes and endothelial cells. Additionally, it promotes the production of prostaglandins and metalloproteinases as well as the migration of neutrophils and monocytes (68). Tumor necrosis factor-α, IL-1α, and IL-6 impact the transcription of SAA mRNAs (SAA1 and SAA2 isoforms), primarily in hepatocytes (69). Research on mastitis-affected cows has demonstrated that acute mammary infections cause a large (>200-fold) increase in SAA concentrations. In cows, SAA is thought to be the most sensitive APP, but it is not correlated with subclinical mastitis (70).

A variety of humoral and cellular immune response mechanisms interact through the transmission of stimulatory or inhibitory cytokine signals. An especially significant function is played by interleukin-6, which is thought to be the greatest stimulant of APP synthesis and secretion (46). Numerous cell types, including lymphocytes, monocytes, macrophages, endothelial cells, epithelial cells, and fibroblasts, express this cytokine, which is stimulated by bacteria. The concentrations of haptoglobin, ceruloplasmin, and transferrin are influenced by interleukin-6. Furthermore, it plays a role in T-cell antigen recognition activation, cytotoxic T-cell differentiation, and B-cell differentiation into distinct classes of Ig-releasing cells by acting as a stimulant (71).

Conclusion

The findings of the present study suggested that supplementing lactating cows infected with mastitis improved immune indices without any adverse effects on rumen fermentation, blood chemistry, antioxidant capacity, and milk yield or milk composition. Additionally, further experiments are urged to examine the impacts of different doses of these supplements on ruminal microbial diversity and animal productive performance.

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 studies were approved by the Laboratory Animal Welfare and Animal Experimental Ethical Inspection Committee, Institute of Subtropical Agriculture, the Chinese Academy of Sciences, Changsha, China. The studies were conducted in accordance with the local legislation and institutional requirements. Written informed consent was obtained from the owners for the participation of their animals in this study.

Author contributions

MK: Writing – original draft, Writing – review & editing. LH: Data curation, Formal analysis, Methodology, Writing – review & editing. TS: Conceptualization, Funding acquisition, Project administration, Writing – review & editing. YQ: Data curation, Formal analysis, Methodology, Writing – review & editing. LY: Data curation, Investigation, Writing – review & editing. TZ: Funding acquisition, Project administration, Writing – review & editing. LQ: Formal analysis, Methodology, Writing – review & editing.

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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.

Generative AI statement

The authors declare that no Gen AI was used in the creation of this manuscript.

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Glossary

ALB - Albumin

ALP - Alkaline phosphatase

ALT - Alanine transaminase

AMS - Amylase

AST - Aspartate transaminase

Bas - Basophilic granulocyte

BUN - Blood urea nitrogen

CHE - Cholinesterase

CHOL - Total cholesterol

CRE - Creatinine

DBIL - Direct bilirubin

ECM - Energy corrected milk

Eos - Eosinophils

GGT - Gamma glutamyl transpeptidase

GLU - Glucose

GPx - Glutathione peroxidase

HCT - Hematocrit

HDL-c - High-density lipoprotein cholesterol

HGB - Hemoglobin

IgA - Immunoglobulin A

IgG - Immunoglobulin G

IgM - Immunoglobulin M

IL-1β - Interleukins 1β

IL-2 - Interleukin 2

IL-6 - Interleukin 6

IL-8 - Interleukin 8

LAC - D-lactic acid

LDH - Lactate ehydrogenase

LDL-c - Low-density lipoprotein cholesterol

Lym - Lymphocytes

MC - *Macleaya cordata*

MCE - *Macleaya cordata* extract

MCH - Mean corpuscular-hemoglobin

MCHC - Mean corpuscular-hemoglobin concentration

MCV - Mean corpuscular volume

MDA - Malondialdehyde

Mon - Monocytes

MPV - Mean platelet volume

Neu - Neutrophils

NFS - Milk nonfat solid

PCT - Thrombocytocrit

PDW - Platelet distribution width

PLT - Platelet count

RBC - Red blood cell

RDW-CV - Red blood cell distribution width variable coefficient

RDW-SD - Red blood cell distribution width standard deviation

SCC - Somatic cell count

SOD - Superoxide dismutase

TAC - Total antioxidant capacity

TBIL - Total bilirubin

TG - Triglycerides

TNF-α - Tumor necrosis factor

TP - Plasma total protein

TS - Milk total solids

WBC - White blood cell

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Biochemical impact of ALAEm supplementation in late gestation on the reproductive performance of sows

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Adding plant extracts to diets to enhance sow performance and health is widely regarded as a healthy and sustainable practice. In promoting antibiotic-free farming, plant extracts have emerged as a leading solution for enhancing sow fertility through nutritional strategies. The aim of this study was to investigate the biochemical impacts of supplementation of sows with ALAEm (composed of nine plant extracts) on blood and placental indices of sows in late gestation. The components of ALAEm were determined by UPLC-MS/MS. 196 normal gestation parturient sows were randomly allocated into two groups (n = 98 per group): the control group and the test group fed 20 g/d ALAEm supplementation at 74–114 d of gestation. The study examined the various clinical indexes in the blood, the expression of genes and proteins and metabolomics in the placenta. Dietary ALAEm supplementation improved sow reproductive performance (total number of piglets born alive, number of piglets weaned, wean weight), serum biochemical indices, placental structure and increased gene and protein expression of ZO-1, Claudin-1 and other placental junction-associated factors. ALAEm attenuated placental tissue oxidation, inflammation, and apoptosis, promoted placental growth (EGF and IGF-1) and angiogenesis factors (VEGFA, PIGF and other factors), and increased the nutrient transport in placental (GLUT1 and SNAT2). Dietary ALAEm supplementation decreased the number of metabolites associated with lipid metabolism through alpha-linolenic acid metabolism. Therefore, dietary supplementation of ALAEm in the late gestation may improve fertility by reducing the levels of inflammation, oxidation and apoptosis in placental tissues via the EGFR/VEGFR2-PI3K-AKT1 pathway, promoting placental growth, angiogenesis and nutrient transport, and altering the levels of placental lipid metabolites via α-linolenic acid metabolism.

KEYWORDS

Angelica sinensis **(Oliv.) Diels, blood, metabolomics, placenta, sow**

1 Introduction

The reproductive performance of sow is influenced by various factors, including breed, feeding practices, environment, reproductive techniques, dietary metabolic levels, and nutrient availability (1). Sows in the late gestation period (74–114 d) require a high level of nutrients to support fetal bone and muscle development. The porcine placenta consists of a maternal part (the maternal vascular endothelial cell layer, uterine connective tissue layer, and endometrial epithelial layer) and a fetal part (the placental chorionic epithelial layer, placental stromal connective tissue layer, and placental vascular endothelial cell layer) and serves as a link between the mother and the fetus (2). The placenta maintains maternal gestation and

regulates the growth and development of the fetus, facilitates the exchange of substances and gases, and serves as an immune barrier to safeguard the health of fetal pigs. Nutrients in the maternal circulation accumulate in the placenta through specific transporters. Some of these nutrients are metabolized in the placenta to provide energy for organ maintenance and development or are converted into other active components for the regulation of placental functions (3) and to provide nutrition for fetal development during gestation (4). Thus, placental nutritional regulation is critical for maintaining pregnancy and healthy fetal development (5).

Studies have shown that changes in the uterine environment can disrupt the development and function of the placenta, affecting vascularization (6), structural integrity, and material exchange between the sow and fetus (7). Impaired placental angiogenesis leads to intrauterine growth retardation (IUGR) of the fetal pig and serious pregnancy complications, fetal loss and cessation of gestation (8), ultimately causing a decline in the reproductive performance of the female animal (9–11). Vaginal infection with bacteria in sows during gestation may be caused by poor barn environment, an imbalanced diet, improper pre-breeding practices, and mechanical injuries caused during herd transfer (12, 13). These can lead to inflammation environment, dysregulation of the endometrium and vaginal microbiota and affect reproduction (14). It has been shown that LPS-induced neuroinflammation is associated with the phosphatidylinositol 3-kinase-serine–threonine kinase (PI3K-AKT) pathway (15), which can activate downstream pathways related to oxidation, inflammation, apoptosis, and angiogenesis. To treat reproductive disorders in sows in the late gestation period, nutritional regulation is generally administered, and plant extracts have been used as feed additives. These extracts exhibit homologation, antibacterial, and antioxidant activities, in addition to the capacity to regulate reproductive hormone levels and enhance immunity and production performance (16). In accordance with the theory of traditional Chinese veterinary medicine, the ALAEm is a natural plant complex composed of nine plants: *Angelica sinensis* (Oliv.) Diels, *Glycyrrhiza uralensis* Fisch., *Astragalus membranaceus* (Fisch.) Bge., *Eucommia ulmoides* Oliv., *Atractylodes macrocephala* Koidz., *Populus tomentosa* Carr., *Perilla frutescens* (L.) Britt., Gynostemma pentaphyllum and *Leonurus japonicus* Houtt. (ALAEm). In the preceding research, it was established that the extract mixture of *Angelica sinensis* (Oliv.) Diels, *Glycyrrhiza uralensis* Fisch., *Astragalus mongholicus* (Fisch.) Bge., and *Eucommia ulmoides* Oliv. could enhance the reproductive function of sows (17). In order to provide a higher nutritional value and a more effective formulation, ALAEm was created based on the previous research, adding several plant extracts with antioxidant effects such as Gynostemma pentaphyllum (18) and treating obstructed labour, such as *Leonurus japonicus* Houtt. (19). *Angelica sinensis* (Oliv.) Diels (20), *Glycyrrhiza uralensis* Fisch. (21), *Astragalus mongholicus* (Fisch.) Bge. (22), *Eucommia ulmoides* Oliv. (23), *Atractylodes macrocephala* Koidz (24), and Gynostemma pentaphyllum (25) have the effect of replenishing blood. *Eucommia ulmoides* Oliv., *Atractylodes macrocephala* Koidz, and *Perilla frutescens* (L.) Britt. (26) are known to tranquilize the fetus, and *Leonurus japonicus* Houtt. (27) invigorates blood circulation. *Angelica sinensis* (Oliv.) Diels and *Astragalus mongholicus* (Fisch.) Bge. have anti-inflammatory, immunomodulatory, antioxidant, antiviral, and hepatoprotective effects (22, 28). *Eucommia ulmoides* Oliv. improved the reproductive and lactation performance of sows and promoted the growth of piglets (29).

Under the environment of intensive antimicrobial-free farming, the reproductive performance of sows is facing serious challenges, and the development of new green feed additives can improve the litter size and piglet survival rate of sows, and promote the healthy development of pig farming. The aim of this study was to investigate the mechanism of ALAEm supplementation to improve the reproductive performance of sows in the late gestation period. We hypothesized that ALAEm would cause significant positive changes in the related factors in the blood and placenta of sows, thereby improving the reproductive performance of sows and laying the experimental foundation for the popularization of the use of ALAEm in production.

2 Materials and methods

2.1 Analysis of extracts

The extracts that compose ALAEm were purchased from Sichuan Hengruitongda Bio-technology Co., China. All the ingredients of ALAEm were in compliance with the National Standard General Requirements for Natural Plant Feed Ingredients of the People's Republic of China (GB/T 19,424-2018).

ALAEm was subjected to ultra-performance liquid chromatography coupled with tandem mass spectrometry (UHPLC-Q Exactive HFX, Thermo, United States) (30). The analytical conditions were as follows: UPLC: column, Waters HSS T3 (100 × 2.1 mm, 1.8 μm); column temperature, 40°C; flow rate, 0.3 mL/min; injection volume, 2 μL; solvent system, phase A was Milli-Q water (0.1% formic acid), phase B was acetonitrile (0.1% formic acid); gradient program, 0 min phase A/phase B (100:0, v/v), 1 min phase A/phase B (100:0, v/v), 12 min phase A/phase B (5:95, v/v), 13 min phase A/phase B (5:95, v/v), 13.1 min phase A/phase B (100:0, v/v), and 17 min phase A/phase B (100:0, v/v). The ESI source parameters were set as follows: sheath gas pressure, 40 arb; aux gas pressure, 10 arb; spray voltage, +3,000 v/−2,800 v; temperature, 350°C; and ion transport tube temperature, 320°C. The scanning range of the primary mass spectrometry was 70–1,050 Da (scan m/z range), with a primary resolution of 70,000 and secondary resolution of 17,500. The raw data were pre-processed using Progenesis QI software (Waters Corporation, Milford, MA, United States) for baseline filtering, peak identification, peak matching, retention time correction, and peak alignment to obtain a data matrix containing retention time, mass-to-charge ratio, and peak intensity. The peaks containing secondary mass spectrometry data were identified using San Shu Biotechnology's secondary mass spectrometry database (San Shu Biotechnology Inc., Shanghai, China), and the corresponding cleavage patterns and the metabolites in the biological samples were analyzed qualitatively and quantitatively (31).

2.2 Experimental animals, diets, design, and management

The Institutional Animal Care and Use Committee of Guangdong Ocean University determined the guidelines and approved all animal

experiments (ethics approval number 2022-scuec-021). All animal experiments were carried out in compliance with the National Research Council's Guide for the Care and Use of Laboratory Animals. Based on initial body weight, parity, and backfat thickness, 196 gestating sows (Long White × Large White, Body weight of 200 ± 10 kg, Parity of 2, Backfat thickness of 18–22 mm) were randomly allotted to two experimental groups namely; the control and 20 g/d ALAEm supplementation ($n = 98$). The experimental duration was between the 74th d of gestation to farrowing from March to May 2023 at a pig farm in Yulin City, Guangxi Province, China.

The ALAEm group was fed the basal diet supplemented with ALAEm 20 g/d, while the sows in the control group were fed a normal basal diet. The formulation of the experimental diet was conducted in accordance with the nutrient requirements for late-gestation sows as stipulated by the National Research Council (NRC, 2012). Specifically, the ALAEm group followed a dosage regimen based on its classification as a plant-derived feed additive, with optimal dosage determined to be 100 mg/kg–200 mg/kg through comprehensive review of published literature (32–36). Given that the average weight of a sow in the late gestation period (74–114 d) is approximately 200 kg, the calculated dose would be 20–40 g per sow per day. However, due to the cost—optimization, considerations in practical applications, a dose of 20 g/d was selected as the optimal dose. During the trial, sows were fed twice a day (7:30 and 16:00) with free access to water.

2.3 Data collection

Birth weight was recorded at birth (Day 0), while within 24 h of parturition, the numbers of total piglets born, live piglets, healthy piglets, weak piglets, stillbirths, and mummified fetuses and the weight of piglets at birth were recorded. Weaning outcomes (number of weaned piglets and their weight) were recorded at 21 days post-farrowing.

Placenta and blood samples were collected from 10 randomly selected sows from each group. Blood samples were collected from the jugular vein in sterile tubes, stored at 37°C for 1 h, centrifuged at 3000 g for 15 min at 4°C, and stored at −80°C until analysis. The 20 sows were closely observed on the day of parturition, and cord blood was collected during parturition to measure lactic acid (LA) and total bile acid (TBA) levels. When the placenta was discharged at the end of parturition, the placental tissue was collected using 75% alcohol for surface disinfection. The whole placental tissue was divided into sampling bags, quickly frozen in liquid nitrogen for quick freezing, and transferred to −80°C for storage until analysis.

2.4 Blood components and biochemical indicators

Blood samples were analyzed using a three-category automatic blood cell analyzer (URIT-5180, Guilin Unite Medical Electronics Co., Ltd., China). Biochemical indicators were measured by automatic biochemical analyzers (Chemray 240, 420, 800, Shenzhen Radiometer Life Science and Technology, China). The reagent kits were purchased from Radiometer/Changchun Huili (China), and all steps were performed following the manufacturer's instructions. Normal ranges were as follows: red blood cell (RBC) (5.0–9.5 × 10^{12}/L), white blood cell (WBC) (11.0–22.0 × 10^{9}/L), platelet (PLT) (150–700 × 10^{9}/L), total protein (TP) (60–80 g/L), albumin (ALB) (34–48 g/L), globulin (GLO) (15–35 g/L), aspartate aminotransferase (AST) (45–125 U/L), alanine aminotransferase (ALT) (5–40 U/L), and alkaline phosphatase (ALP) (45–125 U/L).

2.5 Serum analyses

Enzyme-linked immunosorbent assay (ELISA) was used to evaluate IgA (MM-090501), IgM (MM-040201), IgG (MM-040301), IL-6 (MM-041801), ROS (MM-120501), SOD (MM-045001), and porcine progesterone (P)(MM-120501). All kits were purchased from Jiangsu Meimian Industry Co., Ltd., China. The Detection of Lactic Acid Levels in Cord Blood by Lactic Acid (LA) Content Assay Kit (Lot. No. 1012304171) was purchased from Beijing Box Biotechnology Co., Ltd., China. All steps were performed following the manufacturer's instructions.

2.6 Hematoxylin & eosin staining

The placental tissue specimens in paraformaldehyde fixative were dehydrated in gradient ethanol, paraffin embedded after xylene transparency, sectioned, and subjected to hematoxylin (G1001, Servicebio Biotechnology Co., Ltd., Wuhan, China) and eosin (G1004, Servicebio Biotechnology Co., Ltd.) staining. The samples were dehydrated and sealed, and the histologic changes of the placenta were observed by orthopantomography (Sony, Tokyo, Japan).

2.7 qPCR assay

Total RNA was extracted from placental tissue samples using TRIzol reagent (TransGen Biotechnology Co., Ltd., China) following the manufacturer's instructions. The RNA concentration was determined using a NANODROP 2000 (Thermo Fisher Scientific, United States), and the purity of RNA was determined by the A260/A280 ratio. Complementary DNA (cDNA) was synthesized from 1 μg of RNA by reverse transcription using All-in-One-First-Strand Synthesis MasterMix (with dsDNase) (F0202, Beijing Lablead Biotechnology Co., Ltd., China). Real-time fluorescence qPCR of cDNA was performed using Taq SYBR Green qPCR Premix (R0202, Beijing Lablead Biotechnology Co., Ltd.) and DNA-specific primers (Sangon Biotech, Shanghai, China) with β-actin (Sangon Biotech) as the reference gene. The primers are listed in Table 1. PCR amplification was performed on the Fluorescent Quantitative Polymerase Chain Reaction (PCR) Detection System (FQD-96X; Hangzhou Bori Technology Co., Ltd., China) following the manufacturer's instructions. The qPCR conditions were 95°C for 30 s and 40 cycles of 95°C for 10 s and 60°C for 30 s. The melting curve conditions were 95°C for 15 s, 60°C for 1 min, and 95°C for 15 s. Relative mRNA expression was calculated by the $2^{-\Delta\Delta CT}$ method.

2.8 Western blotting

Proteins were extracted from placental tissue with a Protein Extraction Kit (Lot 01408/22,222; Kangwei Century Technology Co.,

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TABLE 1 Primers for real-time quantitative PCR.

Categories	Gene	Primer sequence	Product length	Gene sequence number
Inflammatory factors	IL-6	F:GACGGATGCTTCCAATCTGGGTTC R:TAATCTGCACAGCCTCGACATTTCC	142	NC_010451.4
	IL-10	F:AGCCAGCATTAAGTCTGAGAACAGC R:GGTCAGCAACAAGTCGCCCATC	139	NC_010451.4
	IL-1β	F:TGGTGTCTGTGATTGTGGCAAAGG R:TTTCAAGGACGATGGGCTCTTCTTC	120	NW_018085011.1
	TNF-α	F:GCACTGAGAGCATGATCCGAGAC R:CGACCAGGAGGAAGGAGAAGAGG	120	NC_010449.5
	STAT3	F:TGGAGAAGGACATCAGCGGTAAG R:ACCAGCGGAGACACAAGGATG	142	NC_010454.4
Oxidation factors	SOD	F:GAAGCACAGCCTCCCCGATTTAC R:TCCTGGTACTTCTCCTCAGCGATG	141	NC_010455.5
	CAT	F:GCTGGTTAATGCGAGTGGAGAGG R:AGTCGTGCTGCGTCTTCAACAG	96	NC_010444.4
	HO-1	F:GCACACCCAGGCAGAGAACAC R:GCGGCATAGATGTGGTACAGTGAG	108	NT_176279.1
Apoptosis factors	caspase-3	F:TGTGGGATTGAGACGGACAGTGG R:GCCAGGAATAGTAACCAGGTGCTG	112	NC_010457.5
	BAX	F:CATCTACCAAGAAGTTGAGCGAGTG R:ACGGCTGCGATCATCCTCTG	88	NC_010448.4
	Fasl	F:CCACCAACACTCCTGCCATCAAG R:ATCCCCAGCCCCAATCCAACC	143	NC_010451.4
Angiogenesis-related factors	VEGFA	F:GCCTTGCCTTGCTGCTCTACC R:CAGGACGGCTTGAAGATGTACTCG	103	NC_010449.5
	MMP9	F:GCTCCGACGACATGCTCTGG R:GAATGGAAACACGCAGGGCTTG	131	NC_010459.5
	PPARγ	F:TCTGTGGACCTGTCGGTGATGG R:TCAGCTCTCGGGAATGGGATGTC	122	NC_010455.5
	PIGF	F:TCAAGAGACTGCTGTATGCCCATC R:ACAACCATGTCAAGTGCGTTTCC	117	NC_010445.4
	VEGFR1	F:GGATTCTGCTGCGGACGGTTC R:GAGGCTGAGGGTCACGGAGTAG	98	NC_010453.5
	VEGFR2	F:CCAGATGACAGCCAGACAGACAG R:GGAGCCTTCAGATGCCACAGAC	141	NC_010450.4
Growth and receptor factors	EGF	F:GGTCCTGCTGCTGCTCCTG R:ACTCACATCTCTGCCTGACTCTTC	109	NC_010450.4
	IGF-1	F:CACATCCTCTTCGCATCTCTTCTAC R:GTCTCCGCACACGAACTGAAG	127	NC_010447.5
	EGFR	F:GCCTGGACATAGCGTCCTTG R:CAGATGTGGCCCATGGCTTT	193	NC_010451.4
	IGF1R	F:AGAAGCAGGCAGAGAAGGAGGAG R:CCGTTCAGGTCTGGGCACAAAG	89	NC_010443.5
	TGFβR1	F:TGCCATAACCGTACAGTCATTCACC R:AAGCCTGATCCAGAACCTGATGTTG	137	NC_010443.5
	FGFR1	F:TCCGACAAGGGCAACTACAC R:CTGTCTTGTTGGCTGGCAAC	133	NC_010457.5
	FGFR2	F:CACTGTCCTGCCAAAACAGC R:CAGAATGACCGTCACCACCA	130	NC_010456.5

(Continued)

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TABLE 1 (Continued)

Categories	Gene	Primer sequence	Product length	Gene sequence number
Tight junction factors	occludin	F:CAGCAGCAGTGGTAACTTGGAG R:CGTCGTGTAGTCTGTCTCGTAATG	109	NC_010458.4
	claudin1	F:ACTCCTACGCTGGTGACAACATTG R:CGACACGCAGGACATCCACAG	71	NC_010455.5
	claudin3	F:TTCATCGGCAGCAGCATTATCAC R:CCAGCAGAGAGTCGTACACTTTG	112	NC_010445.4
	ZO-1	F:CTGACAGTATCCACTCTGCTAATGC R:AGAAGGCTCTGACCGCTGATC	146	NC_010443.4
	TJP1	F:TGATGATCGTCTGTCCTACCTGTC R:CCGCCTTCTGTATCTGTGTCTTC	105	NC_010443.5
	CDH1	F:TCTGCTGCTCCTGCTCCTTATTC R:GTCCTCTTCTCCACCTCCTTCTTC	121	NC_010448.4
	CTNNB1	F:CCATCTGTGCTCTCCGTCATCTG R:GGTAGTCCGTAGTGAAGGCGAAC	85	NC_010455.5
Function-related factors	GLUT1	F:CGCTTCCTGCTCATCAACC R:CCTTCTTCTCCCGCATCAT	136	NC_010448.4
	SNAT1	F: GCAGGTCTTCGGCACCACAG R: GGTAGCTCAGCATTGCTCCAGTG	80	XM_003355629.4
	SNAT2	F: GCCGCAGCCGTAGAAGAATGATG R: AAGCAATTCCGTCTCAACGTGGT	125	NM_001317081.1
	PLET1	F:TCAAGAATCCAGGTCAACCCAAAGG R:TTGCCAGAAGCCAATGACTGAGTG	135	NC_010451.4
	ESR1	F:AGGAACCAGGGCAAGTGTGTC R:CATACGGAAGCGAGATGATGTAGC	78	NC_010443.5
	CCND1	F:GCTCGCCCTCCGTGTCCTAC R:TCGCAGACCTCCAGCATCCAG	94	NC_010444.4
Reference	β-actin	F: GATCTGGCACCACACCTTCTACAAC R: TCATCTTCTCACGGTTGGCTTTGG	107	NC_010445.4

Ltd., China). The bicinchoninic acid (BCA) Protein Assay Kit (Lot 28,523; Kangwei Century Technology Co., Ltd.) was used to assess protein concentrations. Protein samples were separated on 8% sodium dodecyl sulfate-polyacrylamide gels (SDS-PAGE, Lot: P6301010; Yeasen Biotechnology [Shanghai] Co., Ltd. China) and transferred to polyvinylidene difluoride membranes. After blocking in rapid closure solution (Lot: F6310030; Yeasen Biotechnology [Shanghai] Co., Ltd.), the membranes were incubated with primary antibody for 30 min at 4°C. The following primary antibodies were used: VEGFR2 (1:1000, #WL02294), EGFR (1:1000, #WL0682a), Akt1 (1:1000, #WL01652), P-Akt1 (Ser 473) (1:1000, #WLP001), PI3K (1:1000, #WL03380), and eNOS (1:1000, #WL01789), all from Wanleibio Biotechnology Ltd., China. Antibody against Claudin-1 (1:1000, Lot #ab15098) was obtained from Abcam Ltd., United Kingdom. Antibody against P-PI3K (p85α/*β*/p55γ) (1:500, #SC-374534) was from Santa Cruz Biotechnology, United States. Antibody against β-actin (1:1000, #R10602) and horseradish peroxidase-labeled goat anti-mouse IgG (1:1000, #R20619) and goat anti-rabbit IgG (1:1000, #R10327) were from TransGen Biotechnology Co., Ltd., China. Bands were visualized using ECL chemiluminescent solution from Tanon Science & Technology Co., Ltd., China (Cat. No. 180–5001). Blots were scanned using a Tanon infrared fluorescence imaging system (Tanon-5200; Tanon Science & Technology Co., Ltd.). ImageJ software (Imager software, Inc., AL, United States) was used to quantify the protein bands, and normalized protein expression levels were calculated.

2.9 Immunofluorescence analysis

Paraffin sections were deparaffinized for antigen repair; after antigen recovery, the samples were blocked in normal serum. Samples were incubated with primary antibodies against ZO-1 (1:200, GB111981) and Claudin1 (1:500, GB15032), both from Servicebio Biotechnology Co. Ltd., overnight at 4°C. The samples were then simultaneously incubated with CY3-labeled goat anti-rabbit IgG (1:300, GB21303) and Alexa Fluor 488-labeled goat anti-mouse IgG (1:400,GB25301) (both purchased from Servicebio Biotechnology Co., Ltd.). DAPI (G1012, Servicebio Biotechnology Co., Ltd.) was used to label the nuclei, followed by treatment with quenching autofluorescence (G1221, Servicebio Biotechnology Co., Ltd.) and antifluorescence-quenching sealer (G1401, Servicebio Biotechnology Co., Ltd.). Images were captured by an upright fluorescence microscope (Nikon, Tokyo, Japan) and processed using ImageJ software (National Institutes of Health, United States).

2.10 Non-targeted metabolomics assays

Metabolites were determined in two groups of placenta samples by ultra-performance liquid chromatography coupled with tandem mass spectrometry (UHPLC-Q Exactive HFX, Thermo). The placental tissue was thawed slowly at 4°C, and 50–100 mg was placed into a centrifuge tube. Next, 200 μL of pre-cooled water was added, and the sample was vortexed for 60 s, followed by the addition of 0.8 mL of pre-cooled methanol-acetonitrile mixture (1:1, v/v). The samples were analyzed following the methods described in a previous section. Qualitative, quantitative, and bioinformatics analysis of metabolites was performed in placental tissues by matching information on retention time, molecular mass (molecular mass error within 10 ppm), and secondary fragmentation spectra of metabolites in self-constructed and commercial databases.[1]

2.11 Data processing for non-targeted metabolomics

Principal component analysis (PCA) and orthogonal partial least squares discriminant analysis (OPLS-DA) were used to process the UHPLC-Q Exactive data. Differential metabolites were determined by FC > 1.5 or FC < 0.67 with a p value < 0.05 and VIP > 1 as the criteria for statistically significant differences. Metabolic pathway enrichment analysis was performed using the integrated HMDB database, KEGG compound database, and LIPID MAPS database.

2.12 Statistical analysis

Data were analyzed using SPSS 25 software (IBM Corporation, Armonk, NY, United States) and GraphPad Prism 8.0 software (GraphPad, CA, United States). Numerical value is expressed as means ± standard error. For data that conformed to normal distribution, a two-sided t-test was used to compare the means of two groups. The Mann–Whitney U test was used when data did not conform to normal distribution. Correlation was determined using Pearson's correlation analysis, and a p value < 0.05 was deemed to indicate a statistically significant difference. Graphs were prepared using GraphPad Prism 8.0 software.

3 Results

3.1 Chemical components of ALAEm

The active components in ALAEm were identified using UPLC-MS/MS. A total of 803 active components in ALAEm (Supplementary material 1) were identified by matching with secondary mass spectrometry. All components were analyzed by secondary mass spectrometry. Supplementary material 2 shows the secondary mass spectra of the top 30 components in ALAEm, which were classified into 10 major groups: phenylpropanoids and polyketides, lipids and lipid-like molecules, benzenoids, lignans, neolignans and related compounds, alkaloids and derivatives, organic nitrogen compounds, organic acids and derivatives, organic oxygen compounds, and organoheterocyclic compounds. The total ion flow diagram of ALAEm is shown in Figure 1. Information on the top 20 active ingredients in terms of relative content is shown in Table 2.

3.2 Effect of ALAEm on the reproductive performance of sows

The sows fed dietary ALAEm had significantly higher number of total piglets born alive, healthy piglets, newborn litter weight, number of weaned head, and weaned litter weight at 21 days ($p \leq 0.001$) (Table 3). Further analysis of the healthy piglets revealed that the percentages of ≥14 and ≤11 piglets in the control group were 12 and 63%, respectively, while the percentages in the ALAEm group were 24 and 39%, respectively, suggesting that ALAEm increased the number of healthy piglets born to low-producing sows (number of healthy piglets ≤11) (Figure 2).

3.3 Effect of ALAEm on the levels of factors in sow serum and cord blood

RBC, WBC, and PLT counts were within the normal range in the control and ALAEm groups; however, the RB counts were significantly higher in the ALAEm compared with the control group ($p < 0.01$) (Figure 3A). ALT ($p < 0.01$), TP and ALP ($p < 0.001$) were highly significant increased (Figures 3D,H–I). IgA levels increased significantly after ALAEm treatment ($p < 0.05$) (Figure 3K). There were no significant differences in the other factors (Figure 3).

3.4 Effect of ALAEm on the levels of placental tissue factors in sows

3.4.1 Placental connection function

3.4.1.1 Structure of the placenta

Hematoxylin & eosin staining of longitudinal section of placenta showed that the placenta of both groups had a generally normal structure; however, because of multiple births, the placenta showed intervillous fibrosis and fibrinoid necrosis of chorionic interstitium, nodularity of syncytiotrophoblasts, and reduction of chorionic vasculature. In the ALAEm group, the structure of the placenta was improved, and the chorionic barrier was intact without obvious edema and abnormal fibrosis (Figure 4).

3.4.1.2 Placental connection factors

Tight junction-associated factors are expressed in placental tissue. Abnormalities in these factors can affect endometrial tolerance, placental implantation, and corpus luteum function during pregnancy (37). Tight junction-associated factors are involved in the physiological processes and pathology of pregnancy and can be used as markers of uterine tolerance (38). The mRNA levels of tight junction factors in placental tissues were detected using qPCR assay; the expression levels of claudin1, claudin3,

1 http://chemrich.fiehnlab.ucdavis.edu/

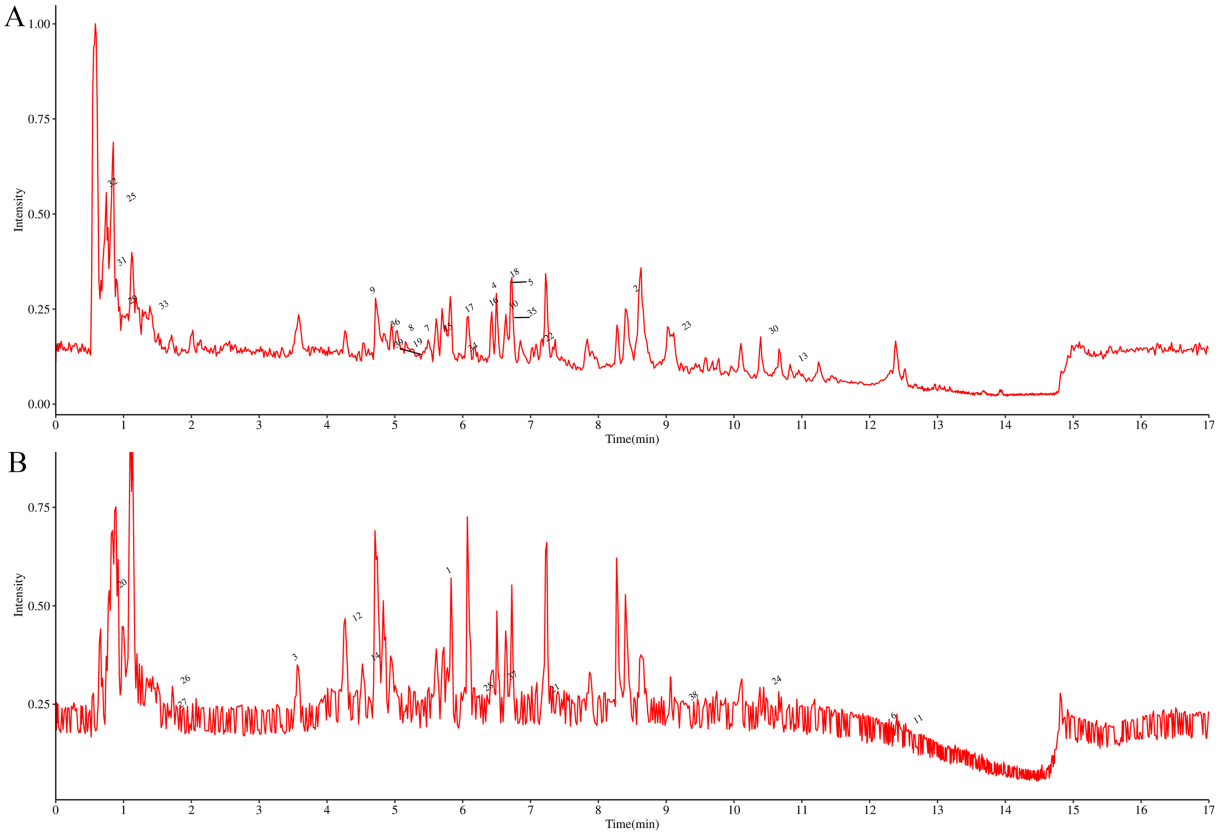

FIGURE 1
Total ion flow chromatogram of ultra-performance liquid chromatography coupled with tandem mass spectrometry of ALAEm. **(A,B)** Total ion flow chromatogram in positive **(A)** and negative ion **(B)** modes.

CDH1 ($p < 0.01$), occludin, ZO-1 and TJP1 ($p < 0.05$) mRNAs were significantly elevated in the ALAEm group compared with the control group; there was a tendency for CTNNB1 mRNA to be elevated, but not significant ($p > 0.05$) (Figure 5A). The expressions of ZO-1 and claudin1 proteins in placental tissues were examined using immunofluorescence and western blotting. Both proteins were detected in placental tissues, and the expression levels of ZO-1 and claudin1 were significantly elevated in the ALAEm treatment group ($p < 0.05$) (Figures 5B–F).

3.4.2 Oxidation-, inflammation- and apoptosis-related factors

Oxidation levels in serum and placental tissues of sows were detected using ELISA. ALAEm highly significantly reduced ROS levels in serum and placental tissues of sows ($p < 0.01$) (Figure 6A) and significantly increased serum levels of the antioxidant enzyme SOD ($p < 0.05$) (Figure 6B). The mRNA expression levels of antioxidant enzymes SOD, CAT and HO-1 in placental tissues were examined using qPCR assay; all mRNA levels were all significantly elevated ($p < 0.05$) (Figure 6C). ELISA showed that ALAEm decreased IL-6 levels ($p > 0.5$) (Figure 6D).

The mRNA expression levels of inflammation-related factors in placental tissues were detected by qPCR assay. ALAEm treatment significantly increased the mRNA expression of the anti-inflammatory factor IL-10 ($p < 0.05$) and highly significantly decreased the mRNA expression of the pro-inflammatory factor IL-1β and STAT3, which promotes the infiltration of inflammatory cells ($p < 0.01$). There were no significant differences in the other factors (Figure 6E).

ALAEm significantly reduced the mRNA expression levels of the apoptosis-associated factors Caspase-3 ($p < 0.05$), Bax ($p < 0.001$), and Fasl ($p < 0.05$) in placental tissues (Figure 6F).

3.4.3 Placental growth, angiogenesis-related factors and other factors

Examination of the mRNA expression of growth factors and their receptors in placental tissues by qPCR assay revealed a highly significant increase in the expression of EGF and IGF-1 mRNAs in the ALAEm group ($p < 0.01$) (Figure 7A) and a highly significant decrease in the mRNA expression of growth factor TGFβR1 ($p < 0.05$), EGFR IGF1R FGFR1 and FGFR2 ($p < 0.01$) (Figure 7B). Evaluation of angiogenesis-related factors in the placental tissues of sows showed that ALAEm treatment highly significantly increased the mRNA expression of VEGFA and PIGF ($p < 0.01$) and significantly increased the mRNA expression of MMP9 and PPARγ ($p < 0.05$). There were no significant differences in the other factors (Figure 7C).

In the late gestation period, placental nutrient transport is critical, and the expression of nutrient transport-related proteins, such as GLUT1, SNAT1, and SNAT2, which affects nutrient transfer between the mother and fetus, leading to pregnancy outcome. Placenta expressed transcript 1 (PLET1) may affect the

TABLE 2 The top 20 active components in ALAEm in terms of relative content.

Number	Name	Class	PubchemID	Formula	m/z	RT (min)	Group area	Adducts
1	Liquiritin	Flavonoids	503,737	C21H22O9	417.119280781248	5.82818333333333	15765556.3901665	M-H, 2 M-H
2	Glycyrrhizic acid	Prenol lipids	14,982	C42H62O16	823.409563180559	8.5933	12856389.8521106	M + H
3	Geniposidic acid	Prenol lipids	443,354	C16H22O10	373.114499971138	3.56058333333333	11406459.7275438	M-H, 2 M-H, M + Cl, M + Na-2H
4	Lamiidoside	Prenol lipids	23,815,404	C26H32O14	551.174912602924	6.49601666666667	10629888.3182296	M + H-H2O
5	Ononin	Isoflavonoids	442,813	C22H22O9	431.132712307734	6.70691666666667	10551070.1587453	M + H
6	Glycyrrhetinate	Prenol lipids	6,857,363	C30H46O4	469.33259627824	12.3872333333333	6318909.85052584	M-H
7	Daidzein-4′,7-diglucoside	Isoflavonoids	171,292	C27H30O14	579.170051005654	5.48851666666667	5770642.93219325	M + H-H2O, M + H, M + H-2H2O
8	Musk ketone	Organooxygen compounds	6,669	C14H18N2O5	312.154494036942	5.01748333333333	5572106.11914579	M + NH4
9	3 Hydroxycoumarin	Coumarins and derivatives	13,650	C9H6O3	163.038631225614	4.71428333333333	5113679.51850721	M + H-H2O, M + H
10	9-Deoxygoniopypyrone	Organooxygen compounds	126,233	C13H14O4	257.080291166898	6.63603333333333	4362775.61593968	M + Na
11	Colubrinic acid	Prenol lipids	21,672,700	C30H46O4	469.332672875787	12.5243833333333	4160198.8858633	M-H, 2 M-H, M + Cl
12	1-Caffeoylquinic acid	Organooxygen compounds	10,155,076	C16H18O9	353.088130555977	4.25008333333333	3906192.45687982	M-H, M + K-2H, M + Na-2H
13	1-Linoleoyl-sn-glycero-3-phosphorylcholine	Glycerophospholipids	11,005,824	C26H50NO7P	520.338940452058	10.8269333333333	3296792.03531289	M + H, M + Na, M + H-H2O
14	Osmanthuside H	Organooxygen compounds	192,437	C19H28O11	431.156274816091	4.52078333333333	3270766.53814627	M-H, M + Cl
15	RUTIN	Flavonoids	5,280,805	C27H30O16	611.159645735714	5.59286666666667	3203725.07177923	M + H
16	Isoliquiritin	Flavonoids	5,318,591	C21H22O9	419.132848014932	6.42846666666667	3144862.99565176	M + H
17	Isoferulic acid	Cinnamic acids and derivatives	736,186	C10H10O4	195.064864268543	6.08091666666667	3063778.09347184	M + H, M + ACN + H, M + H-H2O
18	Formononetin	Isoflavonoids	5,280,378	C16H12O4	269.08024015293	6.70691666666667	3032084.7624773	M + H
19	Vicenin III	Flavonoids	185,958	C26H28O14	565.15436175854	5.22445	2972598.67260747	M + H-H2O, M + H
20	Raffinose	Organooxygen compounds	439,242	C18H32O16	503.161127217343	0.78785	2888583.35021924	M-H

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TABLE 3 Effect of ALAEm on the reproductive performance of sows.

Items		Control group	ALAEm group	*p*
The number of (*n*)	Total piglets born	11.32 ± 0.31	13.06 ± 0.31	<0.001
	Live piglets born	10.42 ± 0.32	11.96 ± 0.28	0.001
	Healthy piglets[a]	10.13 ± 0.33	11.74 ± 0.28	<0.001
	Weak piglets	0.29 ± 0.07	0.21 ± 0.06	0.517
	Stillbirths	0.46 ± 0.1	0.44 ± 0.09	0.595
	Mummies	0.18 ± 0.06	0.46 ± 0.09	0.005
The weight of (kg)	Piglets weight at birth	15.86 ± 0.50	18.13 ± 0.40	0.001
The number of (*n*)	Weaned piglets	10.38 ± 0.2	11.59 ± 0.15	<0.001
The weight of (kg)	Litter weight at weaning	76.72 ± 1.42	83.77 ± 0.93	<0.001

Data are shown as mean ± standard error ($n = 98$).
[a]Healthy piglets were defined as newborn piglets weighing ≥0.75 kg.

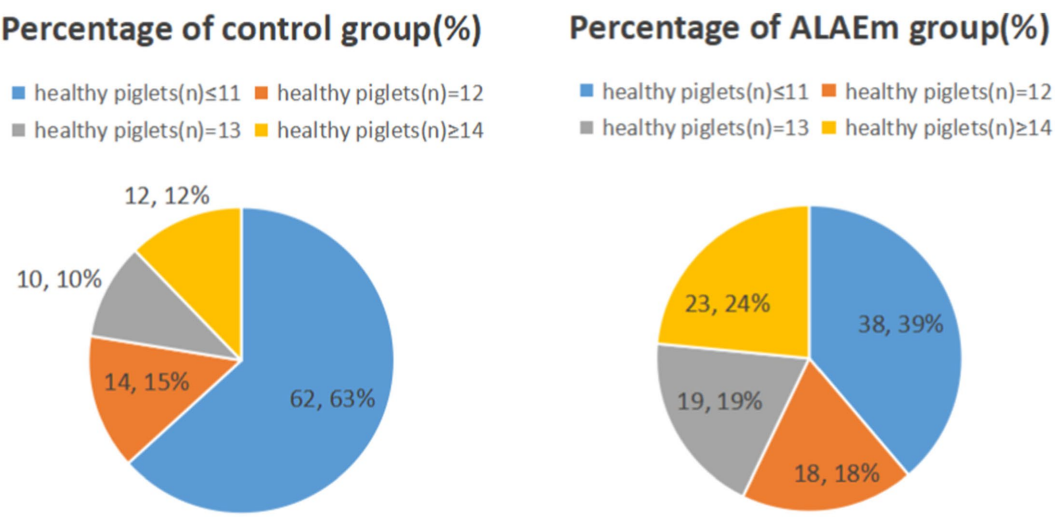

FIGURE 2
Distribution of healthy piglets as a percentage.

interaction of trophoblast cells with maternal endometrial cells and be associated with placental development (39). Targeting CCDN1 to CCND1 blocks cell cycle progression and promotes apoptosis in uterine smooth muscle tumor cells (40). ESR1, which encodes an estrogen receptor implicated in the development of endometrial cancer, has been linked to recurrent miscarriages (41). The expression levels of SNAT2 ($p < 0.05$), GLUT1, PLET1 and CCND1 genes were highly significantly increased ($p < 0.01$) and the expression of the ESR1 gene was significant decreased ($p < 0.05$) after ALAEm treatment (Figure 7D).

3.5 Mechanistic studies on the effect of ALAEm on sow reproduction

Compared with the control group, the ALAEm treatment group showed significantly decreased protein expression of EGFR ($p < 0.01$), VEGFR2 ($p < 0.05$), AKT1 ($p < 0.01$) and PI3K ($p < 0.05$) (Figures 8B,C,E,H), significantly increased expression of P-PI3K, P-AKT1 and eNOS ($p < 0.05$) (Figures 8D,F,I), and significantly increased ratio of P-AKT1/AKT1 ($p < 0.01$) and P-PI3K/PI3K ($p < 0.05$) (Figures 8G,J). ALAEm regulation of sow reproduction may be accomplished by modulating the expression levels of key proteins in the EGFR/VEGFR2-PI3K-AKT1-eNOS signaling pathway in placental tissues (Figure 8A).

3.6 Metabolomics profiling

A total of 1,532 metabolites were identified by categorizing all metabolites in the control and ALAEm groups (Supplementary material 3). The pie chart statistics of the number of metabolites in each category as a proportion of the number of all metabolites were presented as class B classification results (Figure 9A). As shown in the PCA score plot (Figure 9B), there was a clear separation between the control and ALAEm groups, indicating that the QC samples were highly aggregated, the sample reproducibility was good, the analytical system was stable, and the obtained data were reliable. Fold change (FC) is the ratio of the mean of the quantitative values of all the biological replicates of each metabolite in a comparison group. A metabolite was considered to be a differential

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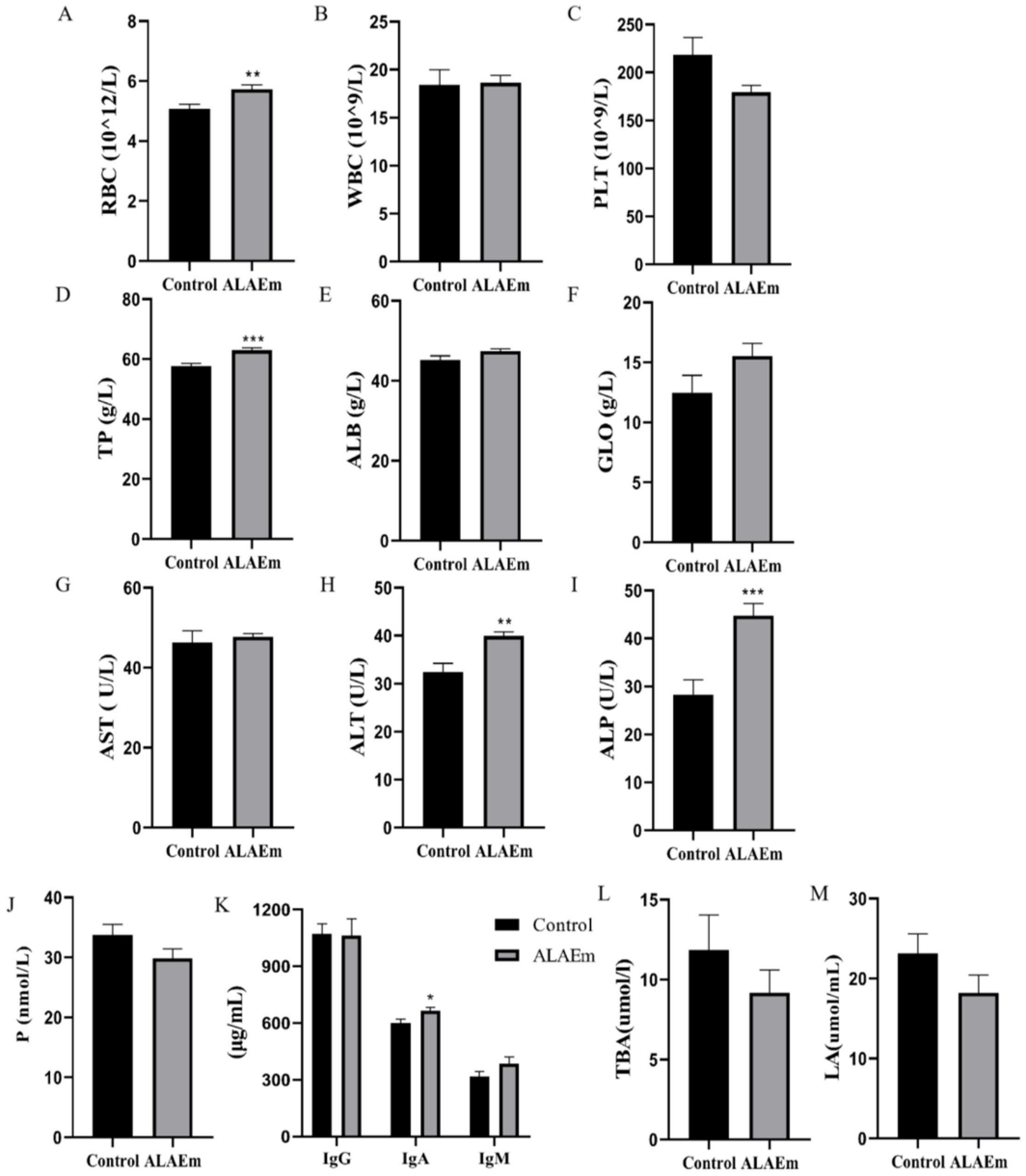

FIGURE 3
Effect of ALAEm on factors in sow serum and umbilical cord blood. **(A)** Red blood cell (RBC). **(B)** White blood cell (WBC). **(C)** Platelet (PLT). **(D)** Total protein (TP). **(E)** Albumin (ALB). **(F)** Globulin (GLO). **(G)** Aspartate aminotransferase (AST). **(H)**Alanine aminotransferase (ALT). **(I)** Alkaline phosphatase (ALP). **(J)** Progesterone (P). **(K)** IgG, IgA, and IgM. **(L)** Total bile acid (TBA). **(M)** lactic acid (LA). * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

metabolite in the volcano diagram (shown in Figure 9C) when FC > 1.5 or FC < 0.67 and p value <0.05. A total of 16 differential metabolites were identified and are listed in Table 4.

$R^2Y(cum)$ and $Q^2(cum)$ are the parameters for evaluating the goodness of the model; generally, when $Q^2(cum) > 0.5$, the OPLS-DA model is stable and reliable with good fitting and prediction ability. Further analysis using OPLS-DA revealed a marked difference in endogenous metabolites between the two groups (Figure 9D). Potential biomarkers were screened by the VIP value in the VIP value graph (Figure 9E); biomarkers with a VIP value greater than 1 were considered qualified. *T*-test combined with multivariate analysis of OPLS-DA was used to add the condition of VIP > 1 to the screening results of differential metabolites; the classification pie charts are shown in Figure 9F, and the information of the substances is shown in Table 5. Five differential metabolites were screened (three down-regulated and two up-regulated), belonging to fatty acids and acrylolipids; box line diagrams of the five substances are shown in Supplementary material 4.

The KEGG ID numbers of the differential metabolites were obtained from the database. The metabolic pathway maps obtained from the pathway annotation analysis of the differential metabolites through the KEGG database were enriched to the alpha-linolenic acid metabolism pathway (Figure 9G), which was consistent with the relatively high content of lipid active ingredients in ALAEm. This indicates that the regulation of sow reproduction may be accomplished by lipids through alpha-linolenic acid metabolism.

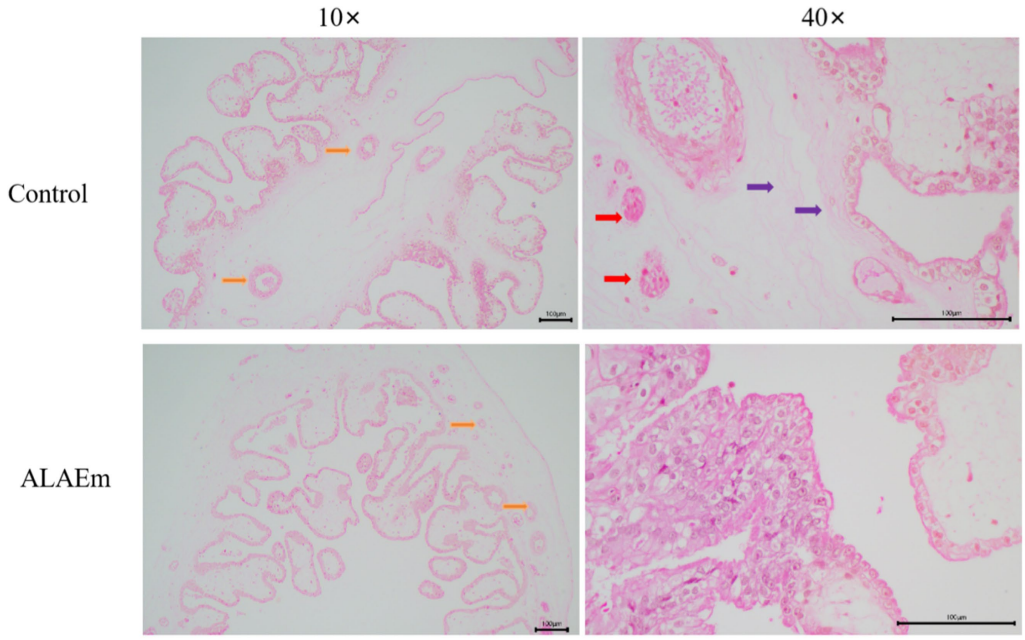

FIGURE 4
Effect of ALAEm on the placental structure. Yellow arrows: chorionic vasculature; red arrows: syncytiotrophoblast nodules; purple arrows: chorionic interstitial fibrosis.

4 Discussion

In this study, the main active components of ALAEm were determined by UPLC-MS/MS. Among the top 20 active ingredients in terms of relative content, nine were classified as phenylpropanoids and polyketides, which were further classified as flavonoids and isoflavonoids. Liquiritin, a flavonoid, was also identified and the highest relative content was observed for this component. Subsequently, a higher relative content of prenol lipids and Fatty Acyls belonging to the lipids and lipid-like molecules classes was noted, including Glycyrrhizic acid, Geniposidic acid, and alpha-linolenic acid. Therefore, we hypothesize that the regulation of sow reproductive performance may be mainly related to the flavonoids and lipids in ALAEm.

Our results also showed ALAEm modulation of sow reproductive performance by increasing the number of weaned piglets and average weaning weight. ALAEm has been found to improve placental status, facilitating increased nutrient delivery to the fetal pig and resulting in increased piglet birth weight. This is achieved by regulating various factors including sow serum factors, placental structure, growth, and angiogenesis, which in turn results in higher weaned piglet weights. Studies have shown that there is an important relationship between piglet birth weight and pre-weaning mortality, and that the pre-weaning mortality rate of low-birth-weight piglets was 34.4%, which accounted for 43% of the total pre-weaning mortality (42), therefore ALAEm also promoted the weaning weight of piglets. *Angelica sinensis* (Oliv.) Diels, *Glycyrrhiza uralensis* Fisch., *Eucommia ulmoides* Oliv. and *Astragalus membranaceus* (Fisch.) Bge., as the main components of ALAEm, have important roles in regulating reproduction. Adding plant extracts during gestation can improve sow reproduction indexes, mainly by improving the inflammatory response and hormone levels and regulating placental function (43, 44). For example, feeding 2% Radix Astragali Extracts compound preparation every day from 70 d of gestation to 21 d of weaning can regulate the state of sows before and after farrowing, which plays a role in increasing the total protein, albumin content, and estradiol level in the plasma of the sows and the immunoglobulin content in the serum and colostrum and decreasing the level of progesterone (45). The herb combination Dang Gui-Yi Mu improved reproductive performance of sows by preventing abortion through regulating T helper (Th)1/Th2 cells (46).

The umbilical cord, as one of the key organs connecting the sow and the fetus during late pregnancy, affects the outcome of piglets through the maternal—fetal integrated system. Sows are prone to LA and TBA metabolic abnormalities during late pregnancy. Some studies have found that piglets born in the posterior quarter are more likely to develop lactic acidemia and are more likely to be stillborn (47). Sows with significantly elevated TBA levels in late pregnancy compared to mid—pregnancy had a stillbirth rate as high as 76.2%, and the performance of piglets after birth was also reduced to varying degrees (48). This abnormality is mainly caused by an oxidative stress response due to liver injury and can lead to decreased fetal piglet survival (49) and altered newborn piglet survival and litter weight at weaning (50). We found that ALAEm treatment reduced LA and TBA levels in cord blood, increased piglet survival, decreased ROS accumulation, and increased antioxidant enzyme levels in the serum and placental tissues of sows with elevated oxidative levels. Many blood indices and biochemical indices change during the gestation of sows, and blood volume increases gradually during gestation until the end of gestation. Thus, the effect of ALAEm on blood indices in sows was also investigated in this study. We found that ALAEm had the effect of reducing anemia and platelet accumulation, which may be through its regulation of PI3K-AKT1 signaling and the subsequent

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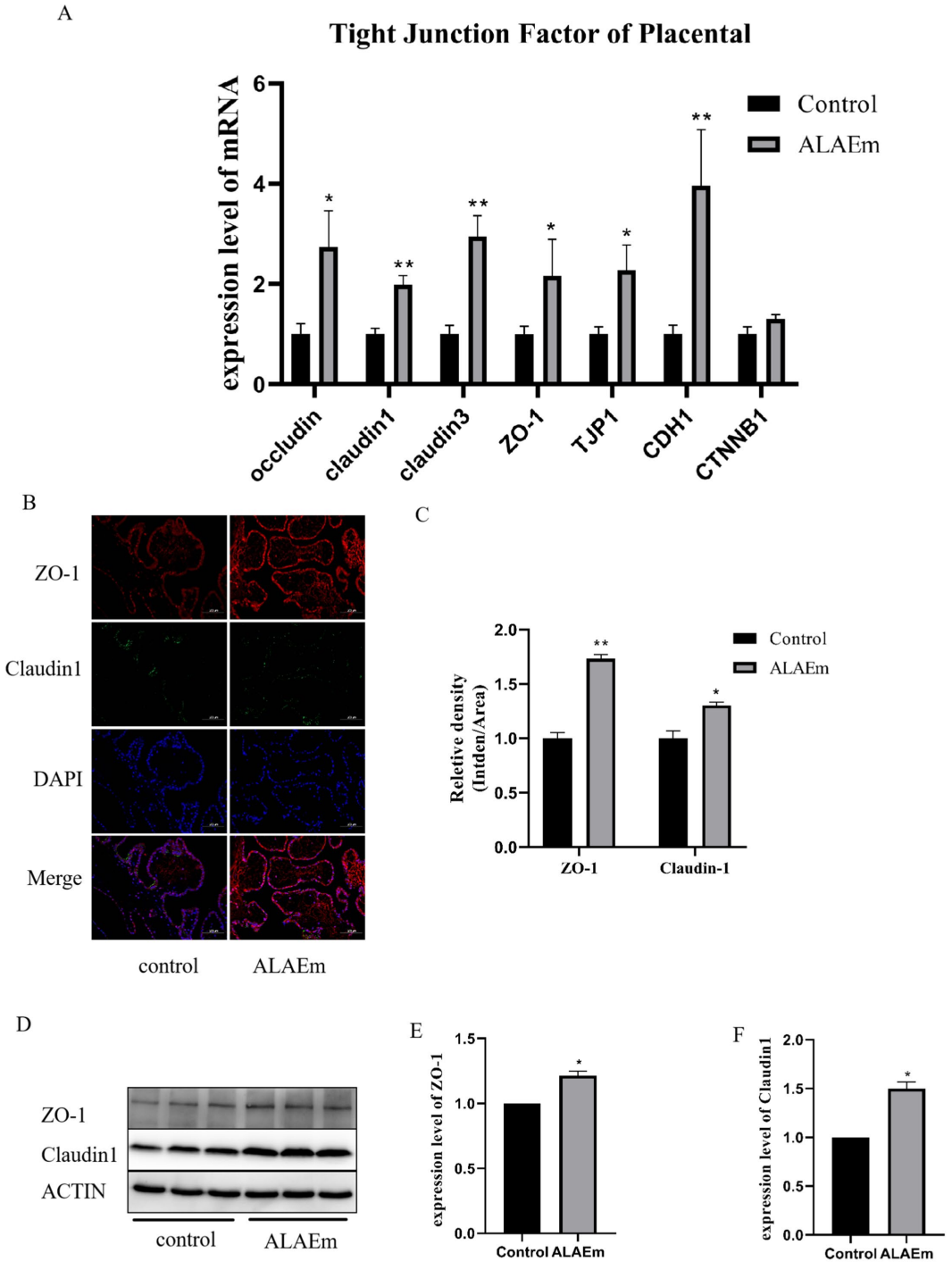

FIGURE 5
Effect of ALAEm on placental tight junction factors. **(A)** mRNA expression levels of tight junction factors in placental tissues of control and ALAEm. **(B)** Immunofluorescence analysis of ZO-1 and claudin1 levels in placental tissues. **(C)** Statistical analysis of ZO-1 and claudin1 levels. **(D)** Western blotting of ZO-1 and claudin1. **(E,F)** Quantification of protein expression levels of claudin1 **(E)** and ZO-1 **(F)** in placental tissue.

anti-thrombosis effects (51). ALAEm increased the levels of TP, AST, ALT, and ALP, reflecting improved hepatic synthesis and reduced occurrence of protein loss because of renal pathology. ALAEm also significantly elevated IgA levels, which may represent enhanced mucosal immunity, indicating improved immune function of the experimental sows. ALAEm shows a potential tendency to shorten the days to return to estrus after weaning as inferred from the changes in progesterone levels (52, 53). Therefore, ALAEm improves sow reproduction by affecting the levels of related factors in sow serum and cord blood.

In epithelial cells, tight junctions (TJs) and adhesion junctions (AJs) integrate to form a protein complex called the cell adhesion apical junctional complex (AJc), which constitutes the placental barrier. Tight junction structures and tight junction-associated factors (Claudin,

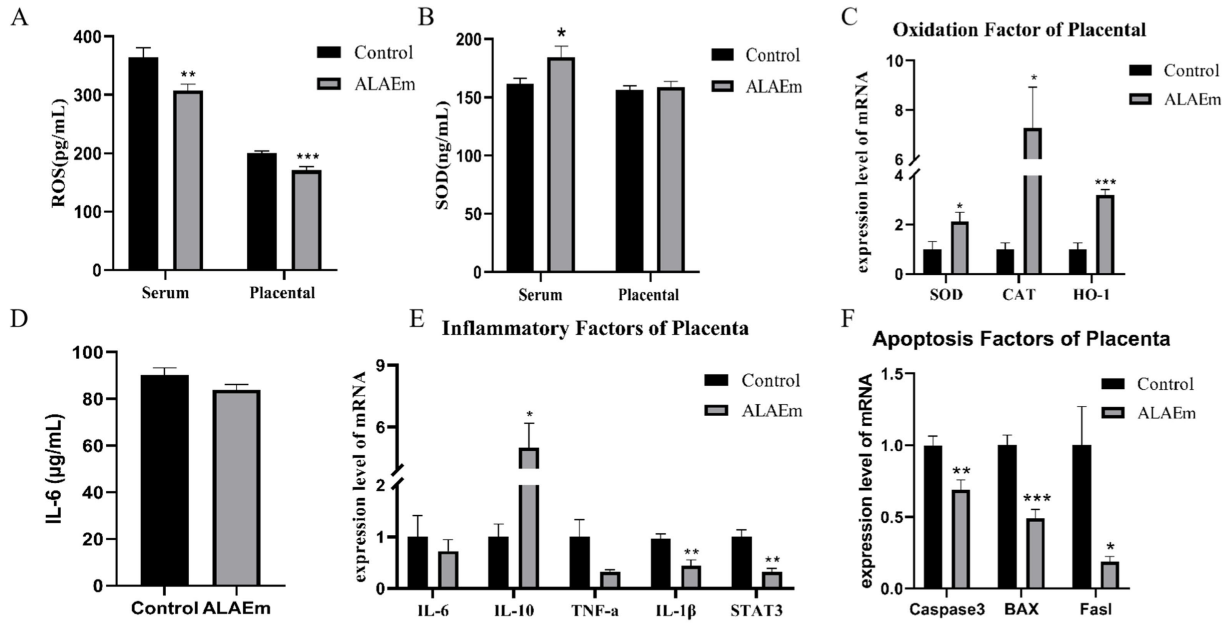

FIGURE 6
Effect of ALAEm on oxidation, inflammation, and apoptosis factors in placenta. **(A,B)** The content of ROS **(A)** and SOD **(B)** in serum and placenta. **(C)** mRNA expression levels of oxidation factors. **(D)** IL-6 levels in serum. **(E,F)** mRNA expression levels of inflammatory **(E)** and apoptosis factors **(F)** in placenta.

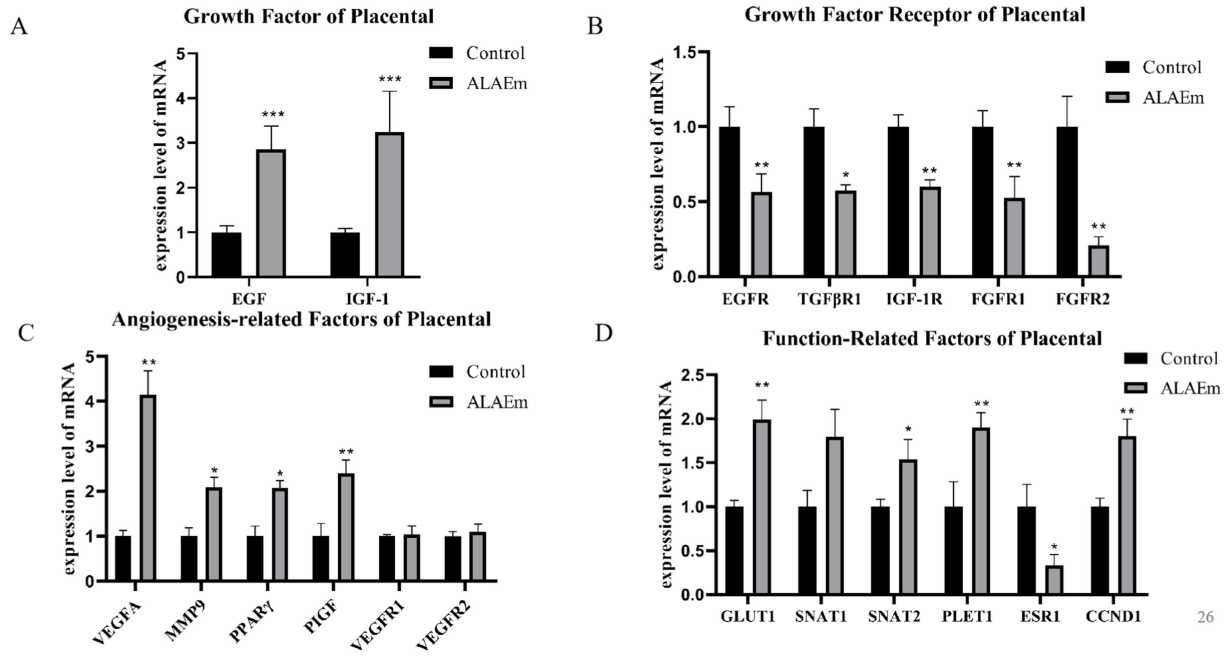

FIGURE 7
Effect of ALAEm on the levels of growth-related factors, angiogenesis and other factors in placenta. **(A–D)** Levels of mRNA expression of the growth-related **(A)**, growth receptor **(B)**, angiogenesis-related **(C)**, and other factors **(D)**.

Occludin, ZO-1, TJP1) regulate biological processes during placenta formation that affect endometrial tolerance and placental implantation during pregnancy (54). Reduced CTNNB1 expression is observed in chorionic and fetal membranous tissues in recurrent miscarriages (55) and inhibits trophoblast cell proliferation and invasion (56); studies showed an involvement of ZO-1 protein in trophoblast cell differentiation (57) and reduction of occludin and claudin1 expression in the placenta in pre-eclampsia (58). We infer that ALAEm may potentially play a role in promoting trophoblast cell functions similar to the beneficial effects associated with higher levels of these factors.

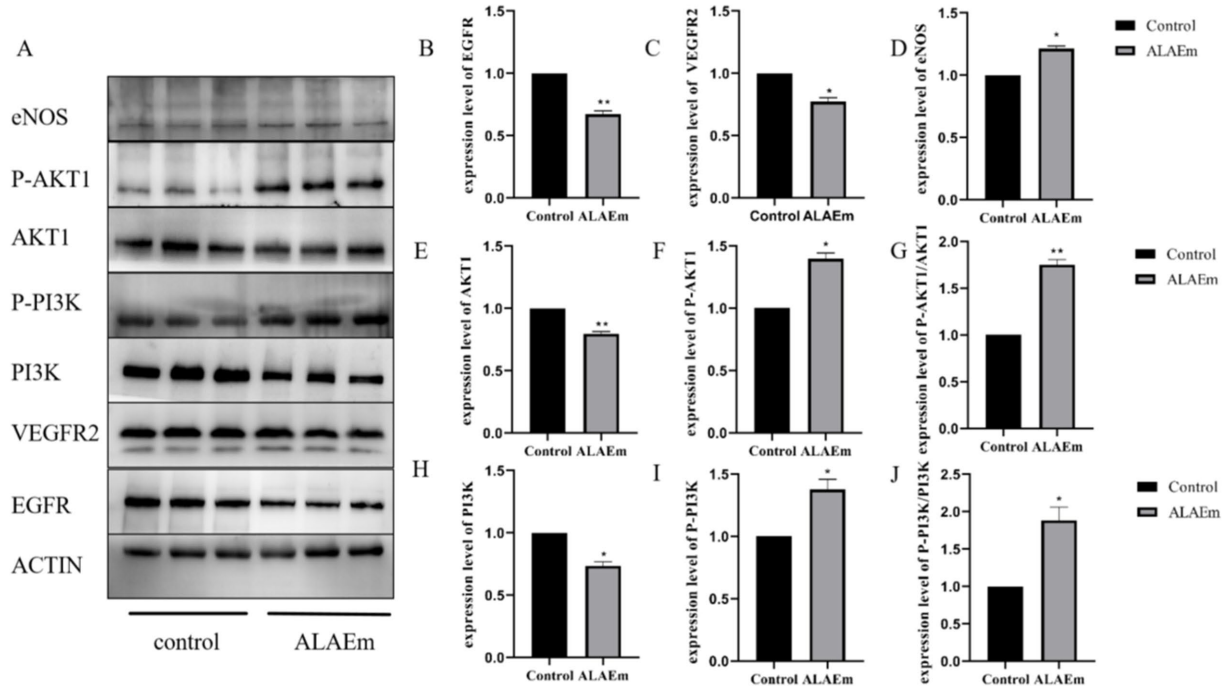

FIGURE 8
Effect of ALAEm on the expression levels of key proteins in the EGFR/VEGFR2-PI3K-AKT1-eNOS signaling pathway in placenta. **(A)** Western blotting of EGFR, VEGFR2, eNOS, and the unphosphorylated and phosphorylated (P) forms of PI3K and AKT1 after treatment with ALAEm. **(B–J)** Protein expression levels of EGFR, VEGFR2, eNOS, AKT1, P-AKT1, PI3K, and P-PI3K and ratios of P-AKT1/AKT1 and P-PI3K/PI3K after treatment with ALAEm.

However, further functional studies are needed to confirm this speculation. ALAEm treatment increased the expression of TJ and AJ-related factors in placental tissues, thereby improving placental structure and strengthening the placental barrier. Placental structure directly affects placental angiogenesis and placental growth, and factors regulating placental angiogenesis mainly include PPARγ, MMP9, and PIGF. PPARγ affects trophoblast invasion and placental angiogenesis by regulating VEGF (59, 60) and regulates progesterone content (61); its deficiency leads to placental abnormalities and fetal death (62). MMP9 plays a role in angiogenesis and remodeling (63) and is downregulated in the placenta in IUGR (64). PIGF is involved in the activation of STAT3 signaling, which is closely related to angiogenesis (65). EGF and IGF-1 are key factors that promote placental growth, and the interaction of EGF and EGFR activates the tyrosine-protein kinase C system, which is involved in placental trophoblast cell infiltration, proliferation, differentiation, and endocrine regulation. EGFR is also associated with the growth of tumor cells and functions as an immune-suppressor factor to promote cervical tumor growth through the STAT3 pathway (66). The vivo increase in IGF-1 concentration may enhance placental nutrient transport, thereby stimulating fetal IGF-1 production and further promoting fetal growth (67). ALAEm treatment increased the expression of VEGFA, PPARγ, MMP9, PIGF, and EGF and decreased the expression of their receptors in placental tissues. So, it is inferred that the improvement of placental connectivity, growth and angiogenesis may be the key to the regulation of placental function by ALAEm in sows.

Abnormalities in placental structure lead to a reduced exchange area, limiting nutrient uptake and transport, which becomes the morphological basis that leads to placental hypofunction, ultimately leading to fetal growth restriction, the production of weak or stillborn piglets, and an impact on the body weight of weaned piglets (5, 68). GLUT1, SNAT1, and SNAT2 are responsible for the transport of glucose and neutral amino acids (e.g., glutamine, alanine, and proline) from the sow to the fetus (69). The addition of ALAEm caused an increase in the gene expression levels of all three nutrient transporters in the placenta, which may facilitate the supply of nutrients from the sow to the fetus, and may also be related to the improvement in placental structure observed in histomorphometric observations. PLET1 expression is upregulated with the progression of pregnancy (70). Upregulated ESR1 was associated with recurrent miscarriages (41). Our results suggest that ALAEm treatment increased PLET1 and decreased ESR1 expression, which may promote placental development nutrient transport and reduce the occurrence of recurrent miscarriage. In this paper, the newborn weight of fetal pigs in the ALAEm group was significantly higher than that of the control group, which could may be attributed to the improvement of placental structure and nutrient transportation function.

Incorrect husbandry and management practices in the second trimester of gestation and multiple deliveries can lead to changes in the sow uterus, and accumulation of microbial products and host inflammatory mediators (e.g., cytokines and chemokines) can lead to apoptosis of the trophoblast cells, resulting in the expulsion of the embryo or fetus (71). In late gestation, rapid fetal development, increased energy intake of sows, and accelerated maternal metabolism all lead to the increased production of ROS (72). Accumulation of ROS passes through the placenta and causes fetal death (73) and the development of pregnancy complications, such as IUGR (74), which ultimately impairs fertility. In this paper, ALAEm treatment reduced serum and placental tissue levels of inflammatory factors IL-6, IL-1β,

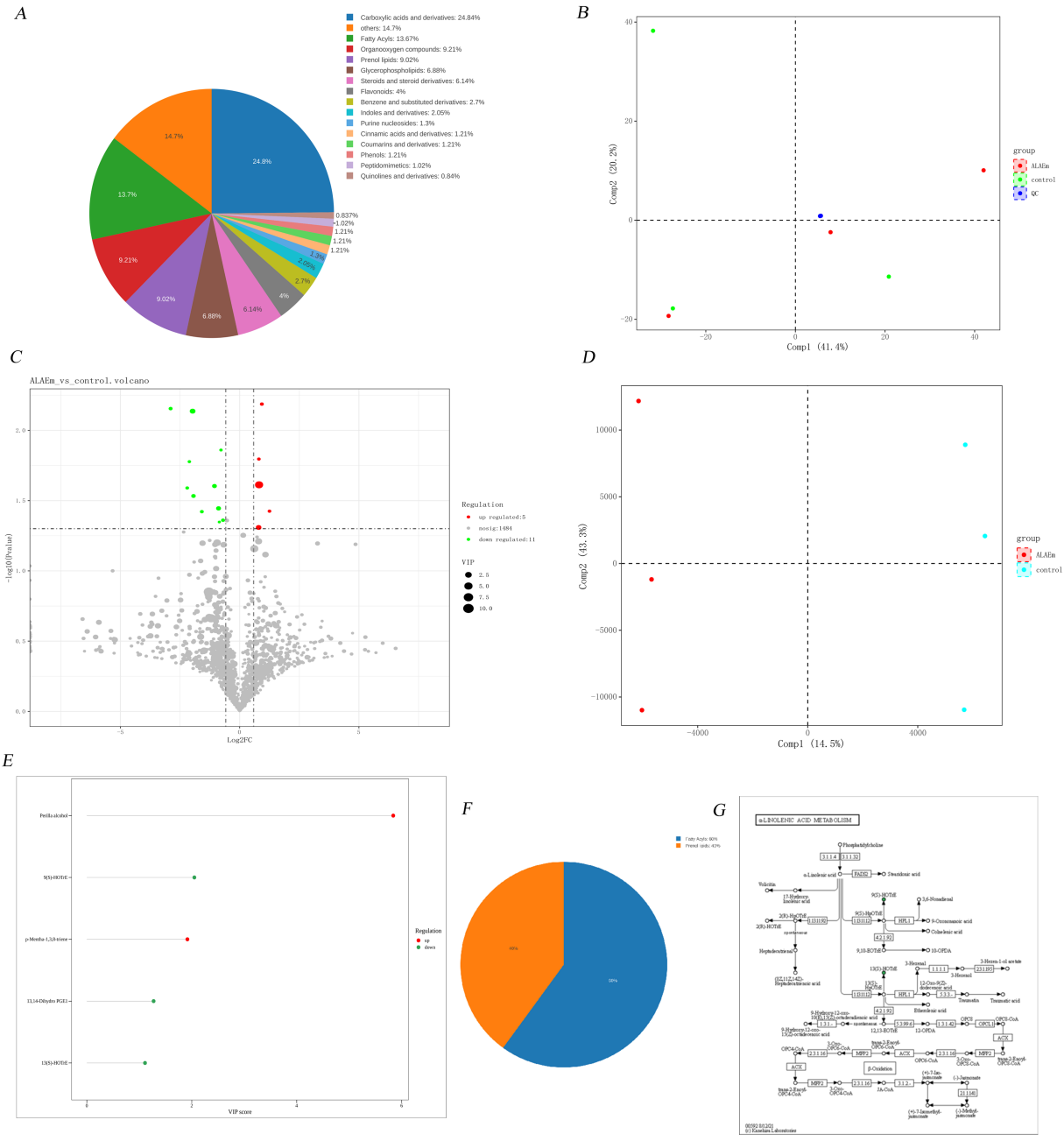

FIGURE 9
Metabolome profiling. **(A)** Chemical taxonomy. **(B)** PCA score plot. **(C)** Volcano plots. The color of the dots indicates the changes in metabolite content: red indicates upregulated, green indicates downregulated, and gray indicates no significant difference. The size of the dot indicates the size of the metabolite VIP value. **(D)** OPLS-DA score plots. The horizontal coordinate Comp1 represents the first principal component, the vertical coordinate Comp2 represents the second principal component, and the numerical percentage in parentheses represents the variance explained for the principal component. **(E)** VipScore plot. **(F)** Pie class plot. **(G)** KEGG annotation plot. Green indicates a relative downregulation.

and TNF-α, increases levels of the proinflammatory factor IL-10, reduces ROS levels, and increases antioxidant enzyme activity. VEGFA inhibits ROS production and downregulates the NF-kB pathway to exert an anti-inflammatory effect (75); it also inhibits uterine necrosis and apoptosis (62). Flavonoids are indeed the primary bioactive components in ALAEm. Studies have shown that flavonoids can inhibit apoptosis in mitochondria and improve the function of the antioxidant system by enhancing SOD activity and scavenging ROS (76, 77), and also have hepatoprotective effects (78). For example, Daidzein is an isoflavonoid that improves embryo survival by enhancing the levels of estrogen and GSH-Px in the amniotic fluid of sows and participating in the arginine and proline metabolic pathways (35). Quercetin is another flavonoid that reduces P release and promotes IGF-1 secretion (79). So, it is inferred that the relatively high content of flavonoids in ALAEm may be a key factor influencing inflammation, oxidation, and apoptosis in placental tissues. VEGFA is a pro-angiogenic active factor that stimulates placental angiogenesis and growth, resulting in a greater flow of nutrients from the sow to the

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TABLE 4 Differential metabolites.

Metabolite	A_mean	B_mean	FC	VIP	*p* value	Sig
13,14-Dihydro PGE1	119213.430	220377.433	0.541	1.273	0.036	Down
Linalool 3,7-oxide beta-primeveroside	2689.356	12358.119	0.218	0.404	0.026	
9(S)-HOTrE	79602.477	312607.963	0.255	2.049	0.007	Down
Homovanillic acid	30895.766	16219.664	1.905	0.508	0.007	
3-Aminoisobutanoic acid	18590.638	10649.391	1.746	0.373	0.016	
Ribose 1-phosphate	16241.223	62191.018	0.261	0.880	0.029	
4-Hydroxystyrene	5122.814	8818.327	0.581	0.253	0.014	
Austalide K	1444.794	6236.306	0.232	0.290	0.017	
Leu Pro	2603.309	19385.169	0.134	0.554	0.007	
Perilla alcohol	4904323.917	2791217.682	1.757	5.839	0.024	Up
p-Mentha-1,3,8-triene	580857.325	335002.143	1.734	1.916	0.049	Up
Ile Val Gly	7456.669	22389.734	0.333	0.476	0.038	
13(S)-HOTrE	68676.791	142863.589	0.481	1.109	0.025	Down
6-Ketoestriol	15386.800	6470.896	2.378	0.378	0.038	
Anhydroretinol	27519.702	44678.996	0.616	0.527	0.044	
SCLAREOLIDE	1986.008	3589.731	0.553	0.159	0.045	

TABLE 5 Classification of the differential metabolites.

Metabolite	classA	classB	classC	Sig
13,14-Dihydro PGE1	Lipids and lipid-like molecules	Fatty Acyls	Eicosanoids	Down
9(S)-HOTrE	Lipids and lipid-like molecules	Fatty Acyls	Lineolic acids and derivatives	Down
Perilla alcohol	Lipids and lipid-like molecules	Prenol lipids	Monoterpenoids	Up
p-Mentha-1,3,8-triene	Lipids and lipid-like molecules	Prenol lipids	Monoterpenoids	Up
13(S)-HOTrE	Lipids and lipid-like molecules	Fatty Acyls	Lineolic acids and derivatives	Down

fetus and improvement in fetal growth needs (80). When VEGFA binds to VEGFR2, it blocks VEGF-induced AKT phosphorylation and inhibits endothelial cell migration, invasion, conduit formation, and angiogenesis (81) and activates STAT3 to achieve proliferative and antimodulatory cell signaling (82). Activation of the STAT3/VEGFA pathway enhances angiogenesis (83), and STAT3 is significantly upregulated in preeclamptic placenta (84). We previously demonstrated that EGFR-PI3K-AKT1 is a key regulatory pathway mediated by the main component of ALAEm. In the current study, we investigated the effect of ALAEm on this pathway in placental tissues and found that changes in the EGFR and VEGFR2 proteins induced the phosphorylation of their downstream pathway, the PI3K-AKT1 pathway, which have been shown to mediate inflammation (15), inhibit the infiltration and implantation of ectopic endometrium by decreasing the expression of Caspase-3 (85), and increase the expression of CCND1 (40). Previous studies on the mechanism of action of additives have shown that additives may improve sow reproduction through different mechanisms of action, e.g., supplementation of sows with *Artemisia annua* enhances inflammatory and innate immune responses by inhibiting TLR4/NF-κB and MAPK pathways, and ultimately attenuates oxidative and inflammatory responses associated with neonatal and early weaning. Our study have shown that flavonoid active ingredients (e.g., liquiritin), which are widely present in plant extracts such as *Glycyrrhiza uralensis* Fisch. and *Astragalus membranaceus* (Fisch.) Bge., can reduce inflammatory responses by inhibiting the activation of the extracellular signaling-associated kinase 1/2 (ERK1/2)/NF- κB pathway and promoting the activation of the Nrf2/Keap1 pathway (86), which are downstream of the PI3K-AKT1 signaling pathway. eNOS, which also belongs to the downstream factors of PI3K-AKT1, promotes the growth of uterine arteries and spiral arteries, which in turn induces VEGF to promote neointima formation (87). Therefore, it is inferred that ALAEm may promot placental growth and angiogenesis through the EGFR/VEGFR2-PI3K-AKT1-eNOS pathway and regulate placental function by regulating responses such as inflammation, apoptosis, and oxidation through EGFR/VEGFR2-PI3K-AKT1 pathway, in which the flavonoids in ALAEm play a key role.

Metabolomics analysis identified two upregulated and three downregulated differential metabolites. The downregulated metabolites included the oxidized lipids 13(S)-HOTrE, 9(S)-HOTrE, and 13,14-dihydro PGE1, which are associated with fatty acid metabolism and fatty acid transport into the placenta. These substances can be directly transported into the fetal circulation, where they meet the extensive metabolism of the placenta throughout gestation by oxidatively producing ATP (88, 89), a process that may induce oxidative stress in the placenta (90). Both 13(S)-HOTrE and 9(S)-HOTrE play an

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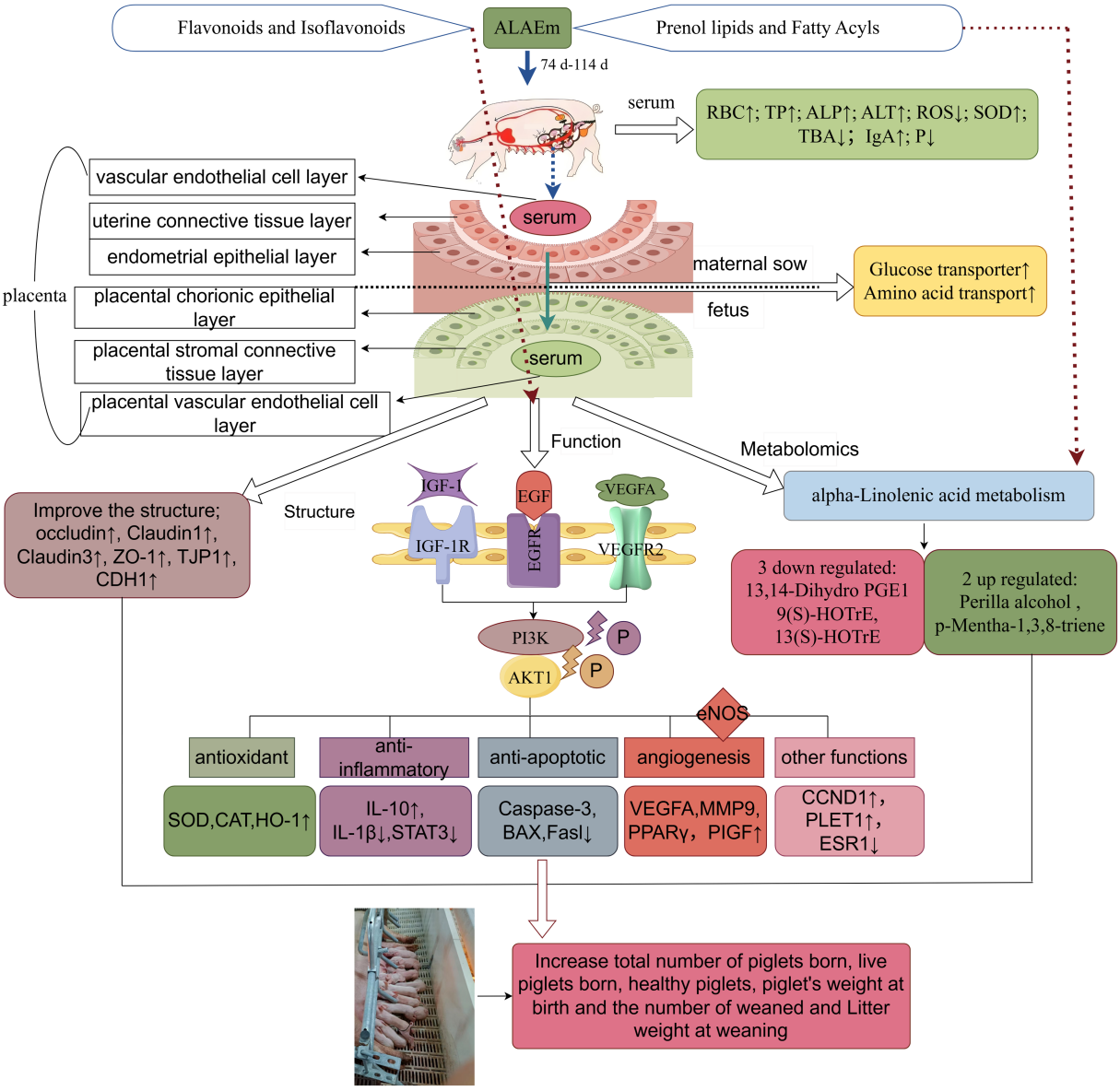

FIGURE 10
Schematic diagram of study overview and the mechanism of action of ALAEm in improving the reproductive performance of sows (by Figdraw).

important role in the inflammatory response (91). A previous study showed that 13(S)-HOTrE was upregulated in mares with endometritis compared with normal mares, and 13(S)-HOTrE was identified as a potential biomarker for the diagnosis of endometritis in mares (92). Additionally, 9(S)-HOTrE levels were elevated in plasma of postpartum dairy cows with the presence of uterine inflammation (93). 13,14-Dihydro PGE1 is an *in vivo* metabolite of PGE1. PGs are important regulators of pregnancy and are synthesized mainly from arachidonic acid and linoleic acid in the placenta; PGs reduce mitogenic activity induced by corticosteroid administration (94), improve lipid metabolism of the vascular wall (95), inhibit platelet aggregation, and constrict vasoconstriction (96). Metabolism of linoleic acid to PGs in the placenta will help ensures a positive pregnancy outcome (97, 98). p-Mentha-1,3,8-triene and perilla alcohol are upregulated in the ALAEm group and are generally found in essential oils with antimicrobial and antioxidant activities (94, 99), which reduce VEGF release in cancer cells, stimulate endothelial cell expression of angiopoietin 2 (Ang2), and inhibit tumor formation (100). These differential metabolites are mainly involved in the alpha-linolenic acid metabolism pathway; abnormalities in this pathway may lead to problems such as aberrant lipid metabolism in the body and decreased immunity. Severe abnormalities in lipid metabolism can increase the incidence of hypertension and preeclampsia during pregnancy. Some studies have shown that adding lipids to the lactation diet of sows improves milk fat secretion, which is beneficial to the development of lactating piglets and subsequent reproduction of sows (101). Increasing the intake of alpha-linolenic acid (ALA) during lactation increases litter size and pig weaning weights without affecting piglet survival or subsequent sow performance (102, 103). Fatty acids are ligands for the transcription factor PPAR, and reduced PPAR expression is associated with placental developmental disorders (e.g., delivery of animals with a low birth weight). ALA promotes the expression of PPARγ, which

regulates angiogenesis in the placenta (104). ALA was also detected in ALAEm components. ALA regulates genes related to oxidative stress and inflammation by affecting NF-κB (105); it inhibits pro-inflammatory factor formation such as TNF-α and IL-1β, which is consistent with our findings. The lipid active ingredient glycyrrhizic acid inhibits activation of the NF-κB signaling pathway, modulates immune cells at the maternal-fetal interface, attenuates endometrial pyroptosis and inflammation during pregnancy (106, 107), and significantly inhibits the development of endometriosis (108). Glabridin is involved in the regulation of genes related to lipid metabolism and has significant PPAR-γ binding activity (109). Therefore, we hypothesize that ALAEm-mediated alteration of metabolites related to the alpha-linolenic acid metabolism pathway in sow placental tissues may regulate sow reproduction. More experiments are needed to verify this possibility. A diagram summarizing the workflow and findings of this study is provided in Figure 10.

5 Conclusion

In this study, we showed that dietary addition of ALAEm in late gestation sows improved the levels of factors in the serum and cord blood of sows and improved placental structure. We inferred that ALAEm may attenuate the levels of inflammation, oxidation and apoptosis in placental tissues and promote placental growth, angiogenesis, and nutrient transport through the EGFR/VEGFR2-PI3K-AKT1 pathway. Additionally, lipid components of ALAEm may alter the levels of placental lipid metabolites by lipids through alpha-linolenic acid metabolism.

This study has several limitations. First, we did not conduct experimental examination of the potential regulatory effects of flavonoids or lipid-based active components on sow reproductive performance. Second, because there is no uniformly used positive drug used to regulate sow reproduction in late gestation in modern swine farms and because many factors affect sow reproduction, we were unable to include positive and model control groups. Third, further experiments are needed to investigate the effects of ALAEm on piglet growth performance, nutrients in umbilical cord blood, and the number of days to estrus after weaning of sows to more convincingly justify the results of this article exploring the mechanism of ALAEm in regulating sow postpartum performance and piglet growth. In future studies we will explore the role of ALAEm in combination with other additives and further investigate the mechanism of action of ALAEm through the use of inhibitors or gene silencing.

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 studies were approved by the Institutional Animal Care and Use Committee of Guangdong Ocean University. The studies were conducted in accordance with the local legislation and institutional requirements. Written informed consent was obtained from the owners for the participation of their animals in this study.

Author contributions

LZ: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Visualization, Writing – original draft. JZ: Data curation, Formal analysis, Validation, Writing – original draft. JH: Data curation, Formal analysis, Resources, Software, Validation, Writing – original draft. XM: Data curation, Writing – review & editing. ZY: Data curation, Resources, Writing – review & editing. YY: Data curation, Funding acquisition, Validation, Writing – review & editing. YL: Conceptualization, Supervision, Validation, Writing – review & editing. XJ: Funding acquisition, Resources, Supervision, Writing – review & editing. XL: Conceptualization, Funding acquisition, Investigation, Project administration, Supervision, Writing – review & editing, Writing – original draft.

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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.

Generative AI statement

The authors declare that no Gen AI was used in the creation of this manuscript.

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Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2025.1548263/full#supplementary-material

SUPPLEMENTARY FILE 1
A total of 803 active components in ALAEm.

SUPPLEMENTARY FILE 2
The top 30 components of ALAEm-secondary mass spectrometry.

SUPPLEMENTARY FILE 3
1532 metabolites.

SUPPLEMENTARY FILE 4
The box line diagrams of five differential metabolites.

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Glossary

ALAEm - A natural plant complex consisting of *Angelica sinensis* (Oliv.) Diels, *Glycyrrhiza uralensis* Fisch., *Astragalus membranaceus* (Fisch.) Bge., *Eucommia ulmoides* Oliv., *Atractylodes macrocephala* Koidz., *Populus tomentosa* Carr., *Perilla frutescens* (L.) Britt., Gynostemma pentaphyllum and *Leonurus japonicus* Houtt.

VEGFA - Vascular endothelial growth factor A

MMP9 - Matrix metalloproteinase 9

PPARγ - Peroxisome proliferator-activated receptor gamma

PIGF - Placental growth factor

VEGFR1 - Vascular endothelial growth factor receptor 1

VEGFR2 - Vascular endothelial growth factor receptor 2

EGF - Epidermal growth factor

IGF-1 - Insulin-like growth factor 1

EGFR - Epidermal growth factor receptor

IGF1R - Insulin-like growth factor 1 receptor

TGFβR1 - Transforming growth factor beta receptor 1

FGFR - Fibroblast growth factor receptor

CDH1 - Cadherin 1

CTNNB1 - Catenin beta 1

GLUT1 - Glucose transporter 1

SNAT1 - Sodium-coupled neutral amino acid transporter 1

SNAT2 - Sodium-coupled neutral amino acid transporter 2

PLET1 - Placenta-expressed transcript 1

ESR1 - Estrogen receptor 1

CCND1 - Cyclin D1

eNOS - Endothelial nitric oxide synthase

UPLC-MS/MS - Ultra-performance liquid chromatography coupled with tandem mass spectrometry

FC - Fold change

VIP - Variable importance on projection

IUGR - Intrauterine growth retardation

PGE1 - Prostaglandin E1

ALA - Alpha-linolenic acid

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Dietary supplementation with antibacterial peptide microcin J25 improved antioxidant capacity and intestinal health of pigeons

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Introduction: The present study aimed to evaluate the potential of antimicrobial peptide microcin J25 (MccJ25) as a feed additive for pigeons.

Methods: A total of 28-day-old pigeons were allocated to four groups and fed a basal diet (CON) or a basal diet supplemented with 100, 200, or 300 mg/kg MccJ25 (ABP100, ABP200, and ABP300) for 8 weeks.

Results: Dietary MccJ25 supplementation significantly improved survival rates in the ABP200 group compared to the control ($p < 0.05$). Serum analysis revealed that ABP200 and ABP300 groups exhibited increased levels of total protein (TP), globulin (GLB), immunoglobulin A (IgA), and immunoglobulin G (IgG), alongside reduced aspartate aminotransferase (AST), alanine aminotransferase (ALT), total cholesterol (TC), and triglyceride (TG) concentrations ($p < 0.05$). Furthermore, MccJ25 supplementation enhanced duodenal maltase and trypsin activity ($p < 0.05$) and upregulated intestinal and hepatic antioxidant capacity, as evidenced by elevated glutathione peroxidase (GSH-Px) and superoxide dismutase (SOD) activity ($p < 0.05$). Intestinal morphology improvements were observed in the jejunum and ileum, with increased villus height-to-crypt depth ratios (VH/CD) ($p < 0.05$). Additionally, ABP200 and ABP300 groups demonstrated significant upregulation of intestinal barrier-related genes occludin (*OCLN*), claudin 1 (*CLDN1*), zonula occluden protein 1 (*ZO1*), mucin 2 (*MUC2*), superoxide dismutase 1, 2 (*SOD1*, *2*), and catalase (*CAT*) in duodenum ($p < 0.05$).

Discussion: These findings indicate that MccJ25 supplementation improves systemic metabolism, enhances antioxidant defenses, strengthens intestinal barrier integrity, and ultimately promotes pigeon health and survival. This study supports the application of MccJ25 as a functional feed additive in poultry production.

KEYWORDS

antioxidant capacity, biochemical function, feed additive, microcin J25, pigeon

1 Introduction

Antibacterial peptides (ABPs), small molecular peptides that are important to innate immune defense system (1, 2), have emerged as promising alternatives to antibiotics in livestock production due to their broad-spectrum antimicrobial activity and low risk of inducing resistance (3, 4). However, the potential effect of ABPs on pigeon production and their possibility of being used as a feed additive product still need to be studied.

While studies in poultry (e.g., broilers) have demonstrated that AMPs enhance intestinal barrier function and modulate immune responses (1, 5), their application in pigeon production remains underexplored—a critical gap given the unique digestive physiology and commercial importance of pigeons in the global poultry industry.

Microcin J25 (MccJ25), a bacteriocin produced by Escherichia coli, has garnered attention for its unique stability and efficient antibacterial and anti-inflammatory properties (6). Recent advances highlight the interplay between AMPs and gut microbiota in mediating host health (7), and ABPs have been used as feed additives in livestock production (8, 9). Previous study showed that addition of ABP decreased the number of Escherichia coli in the ileum and cecum, which promoted intestinal health in broilers and then improved the production performance (10). Another study indicated that the addition of ABP to diets significantly reduced the number of coliforms in ileum and cecum and enhanced the humoral immune function, resulting in increased growth performance in weaned piglets (7, 11). It was also showed that dietary supplemented MccJ25 effectively improved performance, attenuated diarrhea and systematic inflammation, enhanced intestinal barrier function, and improved fecal microbiota composition of weaned pigs (12). In pigeons, however, the mechanisms by which AMPs influence gut microbiota composition, redox homeostasis, and systemic immunity remain unclear.

Pigeon meat industry has developed rapidly in recent years and has great market potential (13, 14). Pigeon squabs are first fed by crop milk secreted by their parents, and the digestive system of squabs is hypersensitive during the transition to self-feeding (15, 16). The weight of 28-day old King pigeons was 25.7 times that of the 1-day old, and then reaching their mature weight by day 28 (17). Our previous study found that ABP MccJ25 and improved the antioxidant capacity and production performance of pigeons, which is beneficial to the health of pigeon squabs (18).

This study aimed to evaluate the impact of MccJ25 supplementation on pigeon health and its potential as a feed additive. By integrating serum biochemistry, digestive enzyme profiling, and histopathological analyses, this work provides a comprehensive assessment of MccJ25ʹs potential as a sustainable feed additive, offering novel insights into AMP applications in niche poultry sectors.

2 Materials and methods

2.1 Ethics statement

This study was approved by the Laboratory Animal Welfare and Ethics Committee of Nanjing Agricultural University, China (Permit number: SYXK-2021-0014).

2.2 Animal experimental design, management and diet

A total of 360 28-day-old American Silver King pigeons (450 ± 10 g body weight) from a genetically homogeneous breeding line (Dongchen Pigeon Industry Co. Ltd., Nanjing, China) were randomly allocated into 4 dietary groups (n = 5 replicates/group). There were 18 pigeons for each replicate, which was divided into 6 cages with 3 pigeons per cage. Pigeons were raised in a ventilated three-layer ladder cage (45 × 40 × 40 cm^3) with free access to feed and water. Pigeons were raised in a semi-open dovecote which was ventilated both mechanically and naturally, with an average temperature of 25°C-32°C, relative humidity 55–70% and natural light. The control group (CON) received a basal diet, and the experimental groups (ABP100, ABP200, and ABP300) were fed a basal diet supplemented with 100, 200, or 300 mg/kg MccJ25 (Zhongnong Yingtai Linzhou Biological Co. Ltd., purity: 94.7%) for 8 weeks. The composition and nutrient content of the pigeon diet are shown in Table 1.

2.3 Survival rate

At the end of trial, the number of died pigeons in each replicate was counted and the survival rate was calculated. The formula for survival rate for each repetition is as follows:

$$\text{Survival Rate (\%)} = [(\text{Number of pigeons per repetition} - \textit{Number of dead pigeons per repetition}) / \textit{Number of pigeons per repetition}] \times 100\%$$

2.4 Sample collection

At 84 days of age, one pigeon from each replicate was randomly selected for left wing vein blood collection followed by cervical dislocation. Serum was separated by centrifugation of blood at 4,000 rpm for 15 min at 4°C and stored at −20°C. Duodenal and ileal contents were first collected aseptically, rapidly frozen and stored at −80°C. The intestines were re-moved, the intestinal contents were rinsed with phosphate buffer saline (PBS) and samples of duodenum, jejunum, ileum, jejunal mucosa, and liver were collected partly for intestinal morphology analysis and partly immediately cryopreserved at −80°C for further experiments.

2.5 Laboratory analysis

2.5.1 Serum biochemical parameters

The serum biochemical parameters, including total protein (TP), aspartate ami-notransferase (AST), glucose (GLU), albumin (ALB), alanine aminotransferase (ALT), globulin (GLB), total cholesterol (TC), alkaline phosphatase (ALP) and triglyceride (TG) were measured spectrophotometrically using an automated system (CLS880 analyzer, Nanjing, China). The contents of immunoglobulin A (IgA), immunoglobulin G (IgG), and immunoglobulin M (IgM) in pigeon serum were assayed using commercial chicken elisa kits (Nanjing Aoqing Biotechnology Co., Ltd., CAT, ANG-E32004C, ANG-E32009C, and ANG-E32005C, Nanjing, China).

TABLE 1 Basal diet nutrient levels and ingredients for pigeons.

Items	Content
Ingredient composition (%)	
Corn	42.55
Pea	25.53
Wheat	12.77
Sorghum	12.77
Green bean	6.38
Total	100.00
Calculated nutrients[a] (%)	
Metabolizable energy (MJ/kg)	12.31
Crude protein	13.50
Crude fat	2.67
Calcium	0.08
Total phosphorus	0.31
Analyzed nutrients (%)	
Crude protein	13.43
Crude fat	2.94
Calcium	0.18
Total phosphorus	0.39
Ingredients of grit meal[b] (%)	
Limestone	52.93
Shell meal	28.10
Yellow mud	14.05
Salt	1.41
Ferrous sulfate (monohydrate)	0.23
Premix[c]	3.28
Total	100.00

[a]Nutrition values and metabolizable energy values determined in pigeons were calculated from table of feed composition and nutritive values in China (31st edition, 2020).
[b]The grit meal was offered to pigeons consistently.
[c]The per kilogram of diet provided by the premix is as follows: vitamin A 5,000 IU, vitamin D_3 2,000 IU, vitamin E 500 IU; C_U (as copper sulfate) 15 mg, Fe (as ferrous sulfate) 100 mg, Mn (as manganese sulfate) 45 mg, Zn (as zinc sulfate) 90 mg.

2.5.2 Digestive enzyme activity

The contents of duodenum in pigeons were collected and were homogenized with ice-cold sodium chloride solution by using an Automatic Sample Rapid Grinding Machine. Then, the homogenization buffer was centrifuged at 3,500 rpm for 10 min at 4°C. The supernatant was used for assaying the enzyme activity of lipase (LPS), amylase (AMS), maltase, trypsin, and chymotrypsin in the duodenum contents of pigeons by using commercial kits (Nanjing Aoqing Biotechnology Co. Ltd., CAT, ZS3151, MZ4302, MZ4638, ZM4288, and ZM4292, respectively, Nanjing, China). The absorbance of each sample was spectrophotometrically measured using a spectrometer against a blank and then the activity was calculated according to the instructions provided by the manufacturer.

2.5.3 Intestinal morphology

Approximately 1-cm intestinal segments (duodenum, jejunum and ileum) were collected and fixed in 4% paraformaldehyde for 24 h. Each sample was dehydrated, cleared and embedded in paraffin and cut at the thickness of 5 μm by using a microtome (LeicaBiosystems, Wetzlar, Germany). Then sections were stained with hematoxylin and eosin (H&E). Five fields of each section were randomly selected for image acquisition under a microscope (OlympusBX50, Tokyo, Japan). The villus height (VH), crypt depth (CD), and the ratio of villus height to crypt depth (V/C) were measured.

2.5.4 Antioxidant indicators

The liver and serum samples were collected from pigeons on day 84 to test the activity of catalase (CAT), glutathione peroxidase (GSH-Px), superoxide dismutase (SOD), total antioxidant capacity (T-AOC), and the content of malonaldehyde (MDA). The level of SOD1 (Cu/Zn-SOD) and SOD2 (Mn-SOD) were measured respectively. Liver samples of approximately 1 g were homogenized with ice-cold sodium chloride solution according to the ratio of mass (g): volume (mL) = 1:9. Then, the homogenization buffer was centrifuged at 3,000 rpm for 10 min at 4°C. The supernatant was used for assaying the antioxidant indices by using commercial kits (Nanjing Aoqing Biotechnology Co. Ltd., CAT, YH1206, YH1267, YH1201, YH1248, and YH1218, respectively, Nanjing, China).

2.5.5 Genes expression

Total RNA was extracted from intestinal, jejunal mucosa, and liver samples with Trizol, and cDNA was synthesized from the RNA using a cDNA synthesis kit (ABM, Richmond, CAN). The expression of target genes was measured using a BlasTaq 2 X qPCR MasterMix (ABM, Richmond, CAN) and a CFX Connect PCR Detection System (Bio-Rad, Hercules, CA, USA) using a reaction program of initial denaturation at 95°C for 3 min and then continued with 40 cycles of 95°C for 15 s and 60°C for 1 min. The primer sequence information of the genes is shown in Table 2. The relative mRNA expression of the target genes were calculated using the $2^{-\Delta\Delta CT}$ method with β-actin as the control.

2.6 Statistical analysis

Normality of data was confirmed via the Shapiro-Wilk test by using SPSS 20.0 software (SPSS Inc., Chicago, IL, USA). One-way ANOVA with Tukey multiple range test was applied for group comparisons. Survival rate was measured in replicates as the experimental unit, and the experimental unit for the other parameters was the individual bird. The statistical results were expressed as mean ± SEM, with significance set at $p < 0.05$. The graphs were drawn by using GraphPad Prism 6.0 software (San Diego, CA).

TABLE 2 Specific primers used for quantitative real-time PCR.

Gene	Accession number	Primer sequences (5′ → 3′)	Product length
OCLN	XM_005509325.2	F: GCCTCATCTGCTTCTTCGCTCAC R: GTCCACCACATTCTTCACCCACTC	134
CLDN1	XM_005513213.2	F: ACATCATGGTATGGCAACAGAGTGG R: ACAGGAGCAGCAGAGGAAGGC	150
ZO-1	XM_021299312.1	F: TGGGTGAGAAACGCTATGAG R: GCTTGTGATGTGCTGGGAGA	123
MUC2	XM_021296699.1	F: TGGCTCCACAGACAGGCAGAC R: TGGCTGACACATGAGGCACATTC	137
SOD1	XM_013370038.1	F: GCAGGGCATCATCCACTTCCAG R: ATCTCCGTCAGCCAAGCCATTG	82
SOD2	XM_013368727.2	F: AATGGAGGAGGAGAGCCTAAAGGAG R: AGCCTGATCCTTGAACACCAACTG	118
CAT	XM_005511042.2	F: CTGGAGAATCTGGCTCTGCTGATAC R: TGGATGAAGGACGGAAACAACAGTG	148
ACTB	XM_005504502.2	F: CCAGCCATGTATGTAGCCATCCAG R: AACACCATCACCAGAGTCCATCAC	90

OCLN, Occludin; CLDN1, Claudin 1; ZO-1, Zonula occluden protein 1; MUC2, Mucin 2; SOD1, Superoxide dismutase 1; SOD2, Superoxide dismutase 2; CAT, Catalase; ACTB, Actin beta.

TABLE 3 Effects of ABP MccJ25 on survival rate in pigeons from 28 to 84 days of age.

Item	Treatment[1]				*P*-value[2]
	CON	ABP100	ABP200	ABP300	
Survival rate (%)	88.89±1.76^{b}	92.22±1.36^{ab}	95.55±1.11^{a}	93.33±2.08^{ab}	0.045

[1]CON: Basal diet; ABP100, ABP200, ABP300: Basal diet supplemented with 100 mg/kg 200 mg/kg, and 300 mg/kg antibacterial peptide MccJ25, respectively.
[2]p value was obtained by ANOVA; ^{a,b} The values in a row with different letters show significant differences ($p < 0.05$). Data were presented as mean ± SEM (n = 90).

3 Results

3.1 Effect of ABP MccJ25 on survival rate in pigeons

Dietary MccJ25 supplementation significantly improved survival rates (p = 0.045; Table 3). From 28 to 84 days, the survival rate of all ABP groups was obviously increased compared with the control group, and ABP200 group showed the highest survival rate (Table 3).

3.2 Effect of ABP MccJ25 on biochemical and immunological parameters in pigeon serum

The impact of ABP treatment on the serum biochemical parameter of pigeons is presented in Table 4. Compared with the control group, ABP200 and ABP300 groups significantly increased the level of TP and GLB in pigeon serum (Table 4). ABP groups significantly reduced the level of AST and TG compared with the control group (Table 4), and the level of ALT and total TC were remarkably lower in the ABP200 and ABP300 groups than that of the control group (Table 4). But ALP and GLU showed no remarkable difference after the supplement of ABP (Table 4). The level of IgA and IgG in pigeon serum was significantly higher in all ABP groups than in the control group (Table 4). In addition, the content of IgM in pigeon serum was also increased in ABP groups, and it was significantly higher in ABP200 group than that of the control group (Table 4).

3.3 Effect of ABP MccJ25 on the digestive enzyme activity in pigeon duodenum

Compared with the control group, the activity of maltase and trypsin in pigeon duodenal contents was significantly increased in ABP groups (Table 5), and chymotrypsin activity was also significantly increased in ABP200 and ABP300 groups (Table 5). In addition, AMS activity in the ABP200 group and LPS activity in the ABP300 group were significantly higher than control group (Table 5).

3.4 Effect of ABP MccJ25 on the intestinal morphology and barrier function in pigeons

The intestinal villi of pigeons in ABP groups showed complete structure in morphology and high density compared with the control group (Figure 1). Villus height (VH) significantly increased by 15.4% ($p < 0.001$), V/C ratio by 12.7% in duodenum; VH (+10.8%) and V/C ratio (+11.3%) ($p < 0.01$) in jejunum; VH (+8.2%) and V/C ratio (+11.1%) ($p < 0.05$) in ileum by diet supplement with ABP200 (Table 6).

ABP groups significantly up-regulated the mRNA expression level of the OCLN, CLDN1, ZO-1 and MUC2 in pigeon duodenum

TABLE 4 Serum biochemical and immunological parameters of 84-day-old pigeons.

Items	Treatment[1]				*p* value[2]
	CON	ABP100	ABP200	ABP300	
TP (g/L)	26.84 ± 1.17^{b}	27.20 ± 0.74^{b}	32.34 ± 1.30^{a}	33.60 ± 0.80^{a}	<0.001
ALB (g/L)	11.74 ± 0.31^{b}	12.92 ± 0.52^{ab}	13.12 ± 0.48^{ab}	13.78 ± 0.65^{a}	0.043
GLB (g/L)	15.10 ± 0.73^{b}	14.48 ± 0.31^{b}	19.22 ± 1.26^{a}	19.82 ± 0.85^{a}	<0.001
AST (U/L)	124.26 ± 4.01^{a}	112.22 ± 4.36^{b}	113.30 ± 3.18^{b}	107.86 ± 2.78^{b}	0.035
ALT (U/L)	15.56 ± 0.48^{a}	15.36 ± 0.69^{ab}	13.66 ± 0.57^{c}	13.92 ± 0.24^{bc}	0.040
ALP (U/L)	446.76 ± 13.21	439.98 ± 20.2	438.72 ± 11.06	440.24 ± 8.85	0.976
GLU (mmol/L)	23.69 ± 0.73	24.72 ± 0.40	24.12 ± 0.48	26.65 ± 0.79	0.173
TC (mmol/L)	7.72 ± 0.48^{a}	6.98 ± 0.24^{ab}	6.62 ± 0.25^{b}	6.71 ± 0.19^{b}	0.034
TG (mmol/L)	2.08 ± 0.06^{a}	1.82 ± 0.06^{b}	1.68 ± 0.04^{b}	1.66 ± 0.05^{b}	<0.001
IgA (ng/mL)	170.83 ± 6.07^{c}	192.68 ± 7.84^{b}	200.32 ± 4.37^{b}	234.37 ± 5.66^{a}	<0.001
IgG (μg/mL)	190.86 ± 5.41^{d}	243.09 ± 7.37^{c}	275.88 ± 4.49^{b}	298.81 ± 6.08^{a}	<0.001
IgM (μg/mL)	2.25 ± 0.05^{b}	2.26 ± 0.08^{b}	2.89 ± 0.11^{a}	2.48 ± 0.08^{b}	<0.001

[1]CON: Basal diet; ABP100, ABP200, ABP300: Basal diet supplemented with 100 mg/kg 200 mg/kg, and 300 mg/kg antibacterial peptide MccJ25, respectively.
[2]P value was obtained by ANOVA; $^{a-d}$ The values in a row with different letters show significant differences ($p < 0.05$). Data were presented as mean ± SEM (n = 5).
TP, total protein; ALB, albumin; GLB, globulin; AST, aspartate aminotransferase; ALT, alanine aminotransferase; ALP, alkaline phosphatase; GLU, glucose; TC, total cholesterol; TG, triglyceride; IgA, immunoglobulin A; IgG, immunoglobulin G; IgM, immunoglobulin M.

TABLE 5 Digestive enzyme activity in the duodenum of 84-day-old pigeons.

Items	Treatment[1]				*p* value[2]
	CON	ABP100	ABP200	ABP300	
LPS (U/g)	13.68 ± 0.49^{b}	14.67 ± 0.64^{b}	15.37 ± 0.52^{b}	18.71 ± 0.64^{a}	<0.001
AMS (mg/min/mg)	0.66 ± 0.05^{b}	0.72 ± 0.03^{b}	1.09 ± 0.11^{a}	0.75 ± 0.05^{b}	0.001
Maltase (U/mg)	13.23 ± 0.58^{c}	16.18 ± 0.53^{b}	17.05 ± 0.39^{b}	19.86 ± 1.16^{a}	<0.001
Trypsin (U/mg)	3.74 ± 0.15^{c}	4.82 ± 0.29^{b}	4.85 ± 0.32^{b}	5.98 ± 0.40^{a}	0.001
Chymotrypsin (U/mg)	6.11 ± 0.40^{c}	6.76 ± 0.41^{bc}	7.73 ± 0.29^{ab}	8.57 ± 0.62^{a}	0.007

[1]CON: Basal diet; ABP100, ABP200, ABP300: Basal diet supplemented with 100 mg/kg 200 mg/kg, and 300 mg/kg antibacterial peptide MccJ25, respectively.
[2]p value was obtained by ANOVA; $^{a-c}$ The values in a row with different letters show significant differences ($p < 0.05$). Data were presented as mean ± SEM (n = 5).
LPS, lipase; AMS, amylase.

compared with control group (Figure 2). In pigeon jejunum, ABP groups obviously up-regulated the mRNA expression level of the ZO-1 and MUC2 compared with control group, and ABP200 and ABP300 groups significantly increased the mRNA expression of OCLN (Figure 2). In pigeon ileum, compared with control group, the mRNA expression of the OCLN, CLDN1 and ZO-1 was significantly increased in the ABP200 and ABP300 groups (Figure 2). In pigeon jejunal mucosa, compared with the control group, ABP200 and ABP300 groups significantly up-regulated the mRNA expression level of the CLDN1, ZO-1 and MUC2 (Figure 2).

3.5 Effect of ABP MccJ25 on the antioxidant capacity in pigeons

In pigeon serum, the activity of T-AOC and GSH-PX was remarkably increased and MDA content was dramatically decreased in ABP200 and ABP300 groups compared with the control group (Table 7). The activity of SOD in ABP200 group was significantly higher than control group (Table 7). In pigeon liver, ABP200 and ABP300 groups notably enhanced the activity of GSH-PX, SOD and CAT in pigeons compared with the control group, but the content of MDA in ABP200 group was significantly lower than the control group (Table 7).

Compared with the control group, the ABP200 and ABP300 groups significantly up-regulated the mRNA expression level of the SOD1, SOD2 and CAT in pigeon duo-denum, jejunum and liver (Figure 3). The mRNA expression of the SOD1 and CAT was significantly increased in the ABP200 and ABP300 groups in pigeon ileum compared with the control group (Figure 3). In pigeon jejunal mucosa, ABP200 and ABP300 groups significantly increased the mRNA expression of SOD1 and SOD2 compared with the control group, and the mRNA expression of CAT was also significantly increased in ABP200 group (Figure 3).

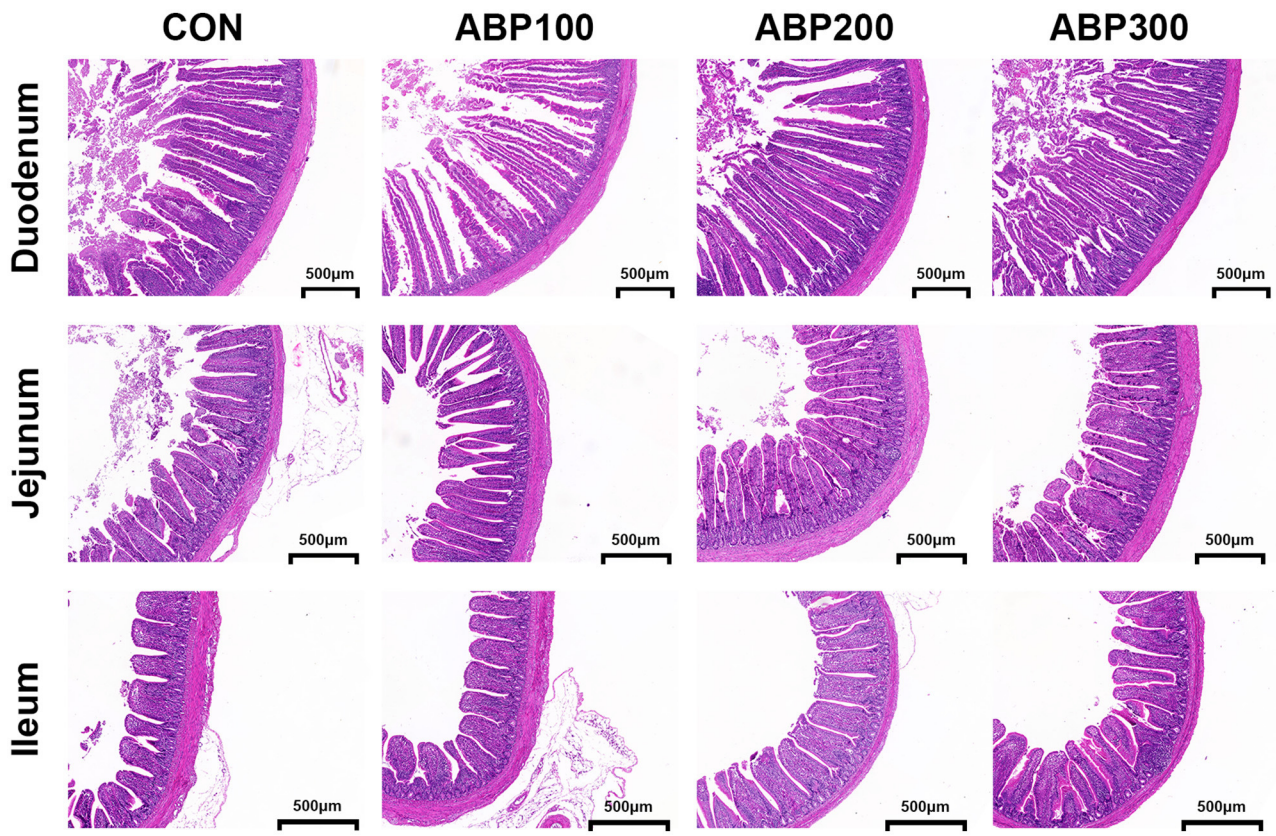

FIGURE 1
Intestinal morphology of 84-day-old pigeons. The morphology of intestinal villi was observed by hematoxylin and eosin (H&E) staining. Scale bar, 500 μm.

TABLE 6 Intestinal morphology of 84-day-old pigeons.

Items	Treatment[1]				*p* value[2]
	CON	ABP100	ABP200	ABP300	
Duodenum					
Villus height (μm)	1,308.80 ± 13.12^{b}	1,336.02 ± 11.57^{b}	1,510.77 ± 17.86^{a}	1,487.35 ± 14.92^{a}	<0.001
Crypt depth (μm)	95.88 ± 0.72	98.17 ± 1.08	98.18 ± 1.08	97.62 ± 1.36	0.413
Villus height/Crypt depth	13.66 ± 0.20^{b}	13.61 ± 0.12^{b}	15.39 ± 0.12^{a}	15.24 ± 0.14^{a}	<0.001
Jejunum					
Villus height (μm)	661.98 ± 11.67^{b}	701.40 ± 12.26^{a}	733.58 ± 11.54^{a}	715.78 ± 13.83^{a}	0.004
Crypt depth (μm)	94.92 ± 1.90	95.55 ± 2.21	94.48 ± 1.86	94.88 ± 1.70	0.815
Villus height/Crypt depth	6.97 ± 0.08^{b}	7.34 ± 0.11^{a}	7.76 ± 0.11^{a}	7.54 ± 0.12^{ab}	<0.001
Ileum					
Villus height (μm)	349.82 ± 5.21^{b}	362.27 ± 6.94^{ab}	378.37 ± 7.18^{a}	377.92 ± 7.39^{a}	0.019
Crypt depth (μm)	80.65 ± 1.26^{ab}	80.27 ± 1.27^{ab}	78.50 ± 0.68^{b}	82.17 ± 0.60^{a}	0.038
Villus height/Crypt depth	4.34 ± 0.08^{c}	4.51 ± 0.12^{b}	4.82 ± 0.14^{a}	4.60 ± 0.17^{b}	<0.001

[1]CON: Basal diet; ABP100, ABP200, ABP300: Basal diet supplemented with 100 mg/kg 200 mg/kg, and 300 mg/kg antibacterial peptide MccJ25, respectively.
[2]p value was obtained by ANOVA; $^{a-c}$ The values in a row with different letters show significant differences ($p < 0.05$). Data were presented as mean ± SEM (n = 5).

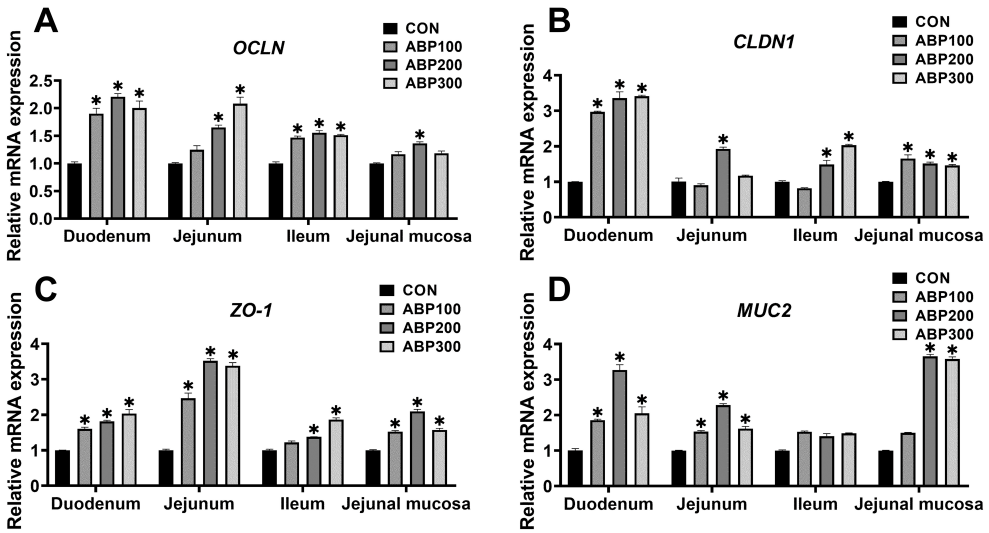

FIGURE 2
Relative mRNA expression of intestinal barrier-related genes in 84-day-old pigeons. The mRNA expression of tight junction genes *OCLN* **(A)**, *CLDN1* **(B)**, *ZO-1* **(C)** and *MUC2* **(D)** was measurted by qRT-PCR. *OCLN,* Occludin; *CLDN1,* Claudin 1; *ZO-1,* Zonula occluden protein 1; *MUC2,* Mucin 2. Data are presented as mean ± SEM. ns, $p > 0.05$; *$p < 0.05$ vs. CON.

TABLE 7 Antioxidative parameters in the serum and liver of 84-day-old pigeons.

Items	Treatment[1]				*p* value[2]
	CON	ABP100	ABP200	ABP300	
Serum					
T-AOC (U/mL)	49.96 ± 2.05^{b}	49.75 ± 2.55^{b}	58.43 ± 2.00^{a}	57.70 ± 1.33^{a}	0.009
MDA (nmol/mL)	0.90 ± 0.03^{a}	0.88 ± 0.04^{a}	0.77 ± 0.01^{b}	0.78 ± 0.03^{b}	0.015
GSH-Px (nmol/min/mL)	46.85 ± 2.35^{c}	51.33 ± 2.49^{bc}	60.89 ± 2.5^{a}	55.10 ± 1.81^{ab}	0.004
SOD (U/mL)	12.17 ± 1.14^{b}	12.11 ± 1.00^{b}	17.03 ± 1.10^{a}	15.01 ± 0.89^{ab}	0.010
Liver					
T-AOC (U/mL)	17.79 ± 0.41^{b}	18.49 ± 1.17^{b}	19.30 ± 1.23^{b}	25.11 ± 0.78^{a}	< 0.001
MDA (nmol/mL)	1.28 ± 0.01^{a}	1.17 ± 0.05^{ab}	1.09 ± 0.03^{b}	1.16 ± 0.06^{ab}	0.049
GSH-Px (nmol/min/mL)	21.41 ± 1.49^{c}	21.01 ± 1.33^{c}	26.39 ± 1.61^{b}	33.24 ± 2.06^{a}	< 0.001
SOD (U/mL)	134.23 ± 6.79^{c}	148.16 ± 9.73^{bc}	169.42 ± 6.15^{b}	230.20 ± 7.31^{a}	< 0.001
CAT(μmol/min/mL)	16.08 ± 0.55^{c}	16.16 ± 0.45^{c}	22.11 ± 0.81^{b}	24.55 ± 0.74^{a}	< 0.001

[1]CON: Basal diet; ABP100, ABP200, ABP300: Basal diet supplemented with 100 mg/kg 200 mg/kg, and 300 mg/kg antibacterial peptide MccJ25, respectively.
[2]p value was obtained by ANOVA; $^{a-c}$ The values in a row with different letters show significant differences ($p < 0.05$). Data were presented as mean ± SEM (n = 5).
T-AOC, total antioxidant capacity; MDA, malonaldehyde; GSH-Px, glutathione peroxidase; SOD, superoxide dismutase; CAT, catalase.

4 Discussion

Antimicrobial peptides (AMPs) represent a promising alternative to antibiotics in livestock production, with demonstrated efficacy in enhancing gut health, immune function, and oxidative homeostasis across species (19–21). Pigeons are susceptible to bacterial infections in the early stages of growth, leading to a low survival rate, therefore, this study aimed to investigate the potential application of ABP MccJ25 in young pigeons. The percent study revealed that MccJ25 supplementation at 200 mg/kg exerted significant effects on intestinal barrier reinforcement, redox balance modulation, and humoral immunity enhancement, then improved survival rates in pigeons.

The improved intestinal morphology (villus height, V/C ratio) and upregulated tight junction proteins (OCLN, CLDN1, ZO-1) observed here are consistent with AMP-mediated gut protection mechanisms reported in poultry (10, 22, 23). It has been shown that the addition of ABPs in the diet remarkably increased the VH of jejunum, improved the V/C of duodenum and ileum, and significantly increased the expression of the intestinal tight junction protein genes (*ZO-1*, *CLDN3*, and *MUC2*) in broilers (24). Notably, the enhanced MUC2 expression suggests MccJ25 promotes mucin

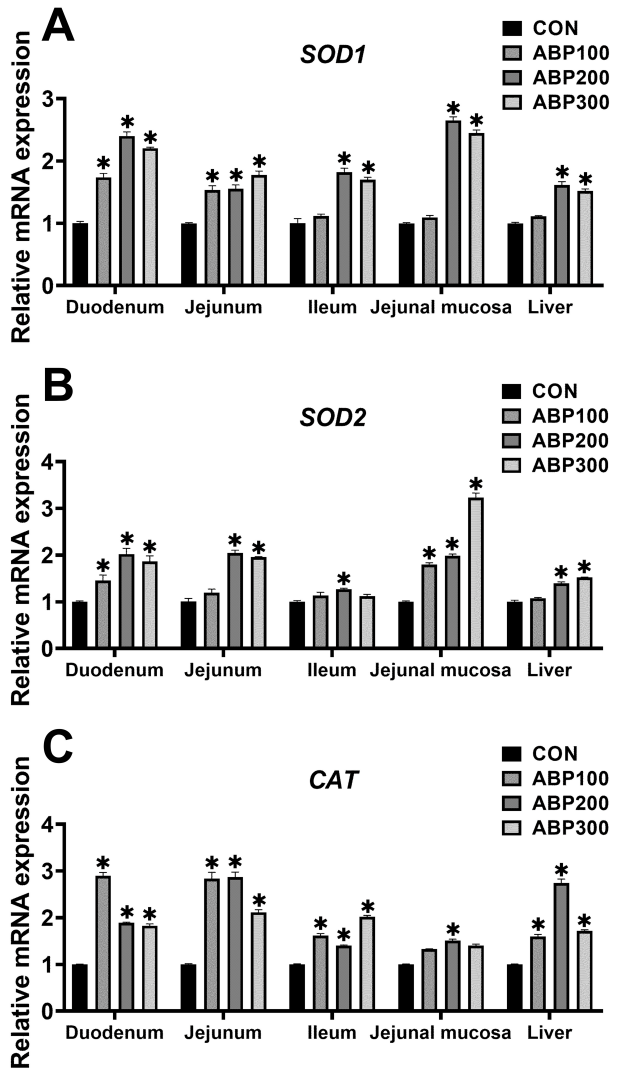

FIGURE 3
Relative mRNA expression of antioxidant relative genes in intestinal and liver in 84-day-old pigeons. The mRNA expression of the antioxidant genes *SOD1* **(A)**, *SOD2* **(B)**, and *CAT* **(C)**. *SOD1*, Superoxide dismutase 1; *SOD2*, Superoxide dismutase 2; *CAT*, Catalase. Data are presented as mean ± SEM ($n = 5$). ns, $p > 0.05$; *$p < 0.05$ vs.CON.

secretion, a critical component of the mucosal barrier against pathogens (25). The improved intestinal morphology and barrier function may be related to the intestinal flora, and ABP Plectasin significantly reduced the number of *Escherichia coli* (*E. coli*) in the ileum of yellow-feathered chickens, improved intestinal morphology, and significantly increased the expression of barrier genes *ZO-1* and *CLDN3* in the ileum (26). Similarly, this study found that ABP MccJ25 significantly increased the VH, decreased the V/C of small intestine, improved the intestinal morphology in pigeons and was conducive to intestinal absorption of nutrients, which may be the reason for the increase of its duodenal digestive enzyme activity. Recent studies highlight that polysaccharide-induced microbiota modulation alleviates oxidative organ damage by restoring microbial balance (27), while a study identified host-microbe interactions as key determinants of intestinal barrier integrity in swine (28). Although our study did not directly profile gut microbiota, the improved digestive enzyme activity (maltase, trypsin) and reduced systemic inflammation (lower AST/ALT) imply MccJ25 may exert prebiotic-like effects by suppressing pathogenic bacteria (e.g., E. coli) while favoring commensal species—a mechanism previously observed with plectasin in poultry (26). Future metagenomic analyses, as proposed by Huang et al. (28), could clarify MccJ25′s microbiota-specific impacts in pigeons.

Serum biochemical indicators indicate the nutritional metabolism and health status of the animal (29–31). Previous study showed that serum biochemical indicators were improved by the addition of ABPs in broilers (32). Similar results was obtained in this study that the addition ABP MccJ25 significantly increased the level of TP, ALB and GLB in pigeon serum, indicating that protein metabolism was vigorous and the degree of protein digestion and utilization was improved. It has been shown that ABPs significantly reduced TC content in the serum of weaned piglets (33), which potentially via AMP-mediated lipid regulation (34). Our observation of reduced serum TC/TG levels in MccJ25-supplemented pigeons further supports this result. It has been shown that ABPs elevated the activity of intestinal AMS, LPS and trypsin in broilers (10). This study showed that ABP MccJ25 increased the activity of duodenal AMS, maltase and LPS in pigeons and promoted the absorption and utilization of nutrients in the duodenum.

Serum AST and ALT reflect liver function or liver damage (35). In our study, ABP MccJ25 showed a significant decrease in the level of AST and ALT in pigeon serum, suggesting a protective effect of ABP MccJ25 on the liver. Immunoglobulins (IgG, IgA, IgM) are important components of the humoral immune system (10, 36). Previous studies found that ABP Plectasin significantly increased the levels of IgG and IgM in serum of broilers and weaned piglets (11). The use of ABP pratt and full-tide promotes the development of immune organs such as the bursa and thymus in broilers, and has the effect of preventing diseases and reducing mortality in broilers (24). Additionally, the elevated immunoglobulins (IgA, IgG, IgM) corroborate findings by Chen et al. (37). The present results were similar to the previous results, where ABP MccJ25 significantly increased the levels of IgA, IgG and IgM in pigeon serum, enhanced their humoral immune function, and increased the survival rate of the pigeons. This result corroborated the immune function of ABPs (2, 4, 20).

When the oxidative balance of the body is broken, it will cause oxidative stress in the organism, resulting in cell and tissue damage, which leads to a variety of diseases (38). SOD, GSH-PX, and CAT synergistically eliminate free radicals and peroxides to reduce oxidative damage (13). MDA is lipid peroxide produced by the metabolism of free radicals in the body (39), and its content is inversely proportional to the health status of the body. Consistent with previous studies, the results showed that ABP groups led to significant increases in T-AOC, GSH-Px, SOD, and CAT levels, while reducing MDA content in both pigeon serum and liver, and enhanced the oxidative defense system

and scavenging capacity of the body against oxidative factors. It has been shown that ABPs improved the intestinal flora and showed resistance to bacterial infections, and also significantly increased the expression of intestinal antioxidant genes *SOD1* and *CAT* to enhance the antioxidant capacity of broiler chickens (20). It was also found in this study, suggesting the enhanced antioxidant capacity of ABPs in pigeons. While the potential influence of confounding factors such as genetic variability and environmental conditions on experimental outcomes was existed, future studies will delineate microbiome differences or epigenetic modifications induced by these factors. Despite these limitations, our study provided robust support for the causality inferred between MccJ25 supplementation and the observed phenotypic improvements.

The survival rate improvement (6.7% increase in ABP200 vs. CON) holds significant economic potential for pigeon farming. ABP MccJ25 indirect health promotion via gut-liver axis modulation. Notably, the absence of adverse effects at 300 mg/kg contrasts with reports of high-dose AMP toxicity in mice (40, 41), suggesting pigeons may tolerate higher doses for therapeutic applications. However, pigeon-specific microbiota interactions, long-term safety, and comparative efficacy with structurally distinct AMPs (e.g., cLFchimera) warrant further investigation. Future studies should integrate multi-omics approaches to decode host-microbe crosstalk and clarify structure-function relationships in avian species.

5 Conclusion

The present study demonstrated that dietary supplementation with ABP MccJ25 improved serum biochemical indices, elevated intestinal digestive enzyme activity and strengthened systemic antioxidant capacity and ameliorated intestinal barrier integrity, thereby improved survival rates in pigeons. These results collectively highlight the roles of ABP MccJ25 in promoting intestinal health. Based on its dose-dependent efficacy, the inclusion of 200 mg/kg MccJ25 in diets is recommended as a sustainable strategy for improving productivity in pigeons. This investigation provides both theoretical and practical foundations for adopting ABP MccJ25 additives as alternatives in the pigeon industry.

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 studies were approved by the Laboratory Animal Welfare and Ethics Committee of Nanjing Agricultural University, China. The studies were conducted in accordance with the local legislation and institutional requirements. Written informed consent was obtained from the owners for the participation of their animals in this study.

Author contributions

HC: Conceptualization, Investigation, Writing – original draft. YL: Resources, Writing – review & editing. XZ: Investigation, Writing – review & editing. FL: Formal analysis, Writing – review & editing. ML: Methodology, Writing – review & editing. JZ: Software, Writing – review & editing. HH: Formal analysis, Writing – review & editing. QM: Software, Writing – review & editing. MY: Conceptualization, Methodology, Writing – review & editing.

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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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The author(s) declare that no Gen AI was used in the creation of this manuscript.

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Enhancing fermentation quality and fiber decomposition of *Phragmites australis* silage by introducing *Bacillus subtilis* and lactic acid bacteria consortia

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Introduction: As a low-cost, high-fibre biomass resource, *Phragmites australis* (reed) has significant potential for feed applications, particularly as a partial replacement for conventional roughage in ruminant diets.

Methods: This study investigated the effects of integrating *Bacillus subtilis* (*B. subtilis* BNCC109047) with homofermentative/ heterofermentative lactic acid bacteria (LAB) consortia on the fermentation and nutritional quality of *Phragmites australis* (reed) silage. Five treatments were evaluated: a Control (CK, without inoculum) and four inoculants—LAB (1.5 × 108 CFU/kg LAB, 1:4 homofermentative (*Lentilactobacillus plantarum* BNCC 336421 and *Pediococcus pentosaceus* BNCC 135034 in a ratio of 1:1): heterofermentative (*L. buchneri* BNCC 187961) ratio), LAB-BS2.5 (LAB plus 2.5 × 10^7 CFU/kg *B. subtilis*), LAB-BS5.0 (LAB plus 5.0 × 10^7 CFU/kg *B. subtilis*), and LAB-BS10.0 (LAB plus 1.0 × 10^8 CFU/kg *B. subtilis*)—with triplicate samples per group. Silage fermentation was conducted for 90 days.

Results: LAB-BS10.0 demonstrated superior fermentation outcomes, achieving the highest lactic acid-to-total acid ratio (62.3%, $p < 0.05$) and the lowest ammonia nitrogen (NH_3-N) content (0.60 ± 0.09 g/kg, $p < 0.05$). Acetic and butyric acid concentrations were significantly reduced ($p < 0.05$), while neutral detergent fiber (NDF) decreased by 5.9% compared to the Control. Ether extract (EE) increased to 4.76% ($p < 0.01$), highlighting enhanced lipid preservation.

Conclusion: These results emphasize the synergistic potential of *B. subtilis* and LAB to optimize *P. australis* silage, providing a sustainable strategy to enhance forage quality and tackle global feed shortages.

KEYWORDS

silage, microbial inoculant, homofermentative, heterofermentative, *Lactobacillus*, reed, silage quality

Introduction

Phragmites australis (reed) is a perennial aquatic grass with global distribution, recognized for its high biomass yield and adaptability to wetland ecosystems (1). Its structural components—roots, stems, and leaves—have been utilized in diverse applications, ranging from construction materials to phytoremediation (2, 3). Recently, *P. australis* has gained attention as a high-biomass forage candidate, offering a sustainable alternative to conventional fodder crops (4, 5). As a promising unconventional silage material, reed shows considerable potential for forage applications. However, similar to other non-traditional feedstocks such as sorghum, barley, and oats, its use in silage systems remains poorly understood, particularly in optimizing fermentation efficiency and nutrient retention (6). The plant's high lignocellulosic fiber content promotes undesirable microbial activity during ensiling, leading to excessive butyric acid production, pH instability, and nutrient loss (7–9). These factors compromise silage quality, limiting its adoption in livestock feed systems (10, 11).

Recent advances in silage microbiology highlight the potential of microbial inoculants to enhance fermentation quality (12, 13). Homofermentative lactic acid bacteria (LAB), such as *Lentilactobacillus plantarum* (14), excel in lactic acid (LA) production, effectively acidifying various lignocellulosic substrates, including sweet sorghum bagasse, barley, *P. australis*, corn, and rice (15). This rapid acidification process significantly suppresses spoilage microorganisms (16–18). Heterofermentative LAB, including *Lactobacillus buchneri*, play a complementary role in the silage fermentation of various forage crops, including lucerne, maize, and Napier grass. These bacteria convert residual carbohydrates to acetic acid through their unique metabolic pathway, providing an additional antimicrobial barrier against spoilage microorganisms such as molds and yeasts (19–22). However, the limited fiber-degrading capacity of LAB often hinders the utilization of lignocellulose-rich forages like *P. australis*.

To address this limitation, *Bacillus subtilis* has emerged as a promising adjunct. This bacterium produces bacteriocins that inhibit undesirable microbes while secreting cellulases and xylanases to break down recalcitrant fibers (23). Synergistically, *B. subtilis* enhances nutrient availability, elevates flavor compounds, and improves silage palatability (24–27). Despite these benefits, research on *B. subtilis*-LAB consortia in unconventional forage silage, particularly *P. australis*, remains scarce.

Current research on optimizing *P. australis* silage through microbial interventions remains sparse. While LAB is widely used to enhance silage fermentation, its synergy with fiber-degrading bacteria like *Bacillus subtilis* (*B. subtilis*) remains a mystery. This study investigates the novel combination of homofermentative/heterofermentative LAB consortia (*L. plantarum*, *Pediococcus pentosaceus*, and *L. buchneri*) with incremental doses of *B. subtilis*. We aimed to (1) evaluate the effects on fermentation parameters (e.g., LA, NH_3-N), (2) assess fiber degradation (e.g., NDF, ADF), and (3) establish an optimal inoculant ratio for enhancing the nutritional quality and digestibility of *P. australis* silage, offering a sustainable solution to mitigate the growing feed supply–demand imbalance.

Abbreviations: AA, acetic acid; ADF, acid detergent fiber; BA, butyric acid; BS, Bacillus subtilis; CP, crude protein; DM, dry matter; EE, ether extract; LA, lactic acid; LAB, lactic acid bacteria; LAB-BS, lactic acid bacteria combined with B. subtilis; NDF, neutral detergent fiber; NH3-N, ammonia nitrogen; PA, propionic acid; WSC, soluble carbohydrates; TN, total nitrogen.

Materials and methods

Plant materials preparation

Phragmites australis (reed) was harvested at the vegetative growth stage from Dongting Lake District, Yueyang City, Hunan Province, China, in 2021. The biomass and protein content simulation of *P. australis* plant (containing stems and leaves) at the early vegetative growth stage is presented in Figure 1. The plants were chopped into 2–3 cm segments, and surface moisture was air-dried. Baseline nutrient composition of raw material was analyzed (Table 1), including dry matter (DM), crude protein (CP), water-soluble carbohydrates (WSC), neutral detergent fiber (NDF), acid detergent fiber (ADF), ether extract (EE), and gross energy (GE).

Microbial inoculants and experimental design

Microbial strains

Homogenenous LAB: *L. plantarum* (3.0×10^9 CFU/g, BNCC 336421), *P. pentosaceus* (1.0×10^{10} CFU/g, NBCC 135034).

TABLE 1 Primary nutritional composition of *P. australis* (%, DM basis).

Items	Content
DM, g/kg	283.76
CP, g/kg	87.18
WSC, g/kg	21.99
NDF, g/kg	786.05
ADF, g/kg	461.87
EE, g/kg	62.26
GE, MJ/kg	16.41

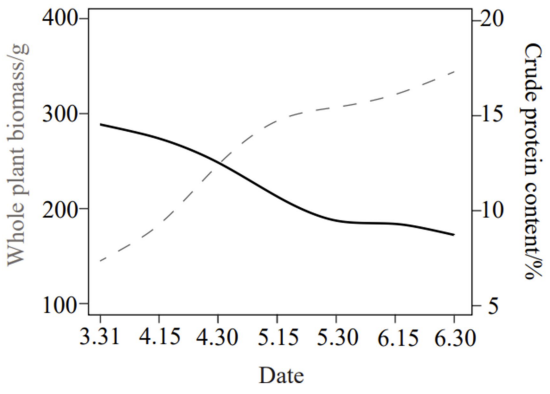

FIGURE 1
The biomass and protein content simulation among the life cycle of *P. australis*.

Heterogeneous LAB: *L. buchneri* (1.0×10^{10} CFU/g, BNCC 187961). Functional bacteria: *B. subtilis* (5.0×10^{10} CFU/g, BNCC 109047). All strains were procured from the BeNa Culture Collection (Beijing, China).

Silage treatments

Five groups with three replicates each were established, including the control group (CK), inoculum LAB, inoculum LAB-BS2.5, inoculum LAB-BS5.0, and inoculum LAB-BS10.0 groups. The control group (CK) was *P. australis* silage without inoculants. The inoculum LAB group was the *P. australis* with homogenous LAB (*L. plantarum* + *L. pentosaceus* at a 1:1 ratio) combined with heterogeneous LAB (*L. buchneri*) at a 1:4 ratio (total LAB: 1.5×10^8 CFU/kg fresh silage). The inoculum LAB-BS2.5, LAB-BS5.0, and LAB-BS10.0 were LAB consortium plus *B. subtilis* with 2.5×10^7, 5.0×10^7, and 1.0×10^8 CFU/kg, respectively. The details are presented in Table 2. The fermentation process and the determination index of silage are shown in Figure 2. The *B. subtilis* gradient (2.5×10^7 to 1.0×10^8 CFU/kg) was selected to evaluate dose-dependent effects on fiber degradation and fermentation efficiency.

Silage preparation and fermentation

Inoculation

Microbial suspensions were prepared by dissolving strains in distilled water (10 mL/kg fresh weight), activated at 30°C for 2 h, and uniformly sprayed onto chopped *P. australis*.

Packing and storage

reated material was packed into 40 cm × 60 cm polyethylene bags (1 kg/bag), vacuum-sealed, and stored at 25–30°C for 90 days (18). This duration was based on preliminary trials confirming pH stabilization and organic acid equilibrium.

Silage quality assessment

Silage fermentation quality

After homogenizing *P. australis* silage, 50 g of the sample was combined with 450 mL of distilled water in a sealed 500 mL Erlenmeyer flask. The mixture was refrigerated at 4°C for 24 h, filtered through polyester cloth, and pressed to extract the residual liquid. The filtrate was further purified using qualitative filter paper and stored in 15 mL centrifuge tubes for subsequent analyses.

TABLE 2 Microbial inoculant composition (CFU/kg fresh silage material) for silage.

Treatments	LAB Consortium (*L. plantarum*: *P. pentosaceus*: *L. buchneri*, in ratio of 1.0: 1.0: 8.0)	*B. subtilis* (BS)
Control (CK)	–	–
LAB	1.5×10^8	–
LAB-BS2.5	1.5×10^8	2.5×10^7
LAB-BS5.0	1.5×10^8	5.0×10^7
LAB-BS10.0	1.5×10^8	1.0×10^8

Control (CK, without inoculum), LAB (consisting of 1.5×10^8 CFU/kg of homogeneous [*L. plantarum* BNCC 336421 and *P. pentosaceus* BNCC 135034 in a ratio of 1:1], to heterogenous LAB species [*L. buchneri* BNCC 187961] in a ratio of 1:4), LAB-BS2.5 (LAB plus 2.5×10^7 CFU/kg *B. subtilis*), LAB-BS5.0 (LAB plus 5.0×10^7 CFU/kg *B. subtilis*), and LAB-BS10.0 (LAB plus 1.0×10^8 CFU/kg *B. subtilis*).

pH and organic acid analysis

The pH value was measured using a calibrated pH meter (HI2211, Hanna Instruments). Lactic acid (LA) was quantified using high-performance liquid chromatography (HPLC; Agilent 1,290, USA). Volatile fatty acids, such as acetic acid (AA), butyric acid (BA), and propionic acid (PA), were analyzed using gas chromatography (GC; Agilent 7890A, USA) equipped with a flame ionization detector (FID) and an FFAP capillary column (15 m × 0.32 mm × 0.25 μm). Operational parameters included helium carrier gas (19.991 kPa), injector temperature (250°C), and a 2 μL injection volume.

Ammonia nitrogen (NH_3-N) determination

NH_3-N content was assessed using phenol-sodium hypochlorite colorimetry. Specifically, a 1.5 mL aliquot of extract was mixed with 0.15 mL of 25% metaphosphoric acid (10,1, v/v), stabilized for 30 min, and centrifuged (1,500 rpm, 4°C, 15 min). The supernatant was filtered through a 0.45 μm filter membrane prior to spectrophotometric analysis.

Nutritional component determination

Silage samples (200 g) were oven-dried at 65°C to constant weight, ground through a 40 mesh sieve, and stored in airtight containers. DM was determined by further drying at 105°C. Nutritional parameters were analyzed as follows: CP: Kjeldahl nitrogen method; WSC: anthraquinone-sulfuric acid colorimetry; NDF and ADF: sequential detergent filtration (Fan's method); EE: Sohren's extraction with petroleum ether; Crude ash: high-temperature incineration (550°C, 6 h) (24, 28).

Silage evaluation standards

Silage sensory quality was graded per China's *Standard for silage quality evaluation* (Table 3), assessing: color (turquoise to dark brown, score 0–20), odor (aromatic sour to moldy, score 0–25), texture (loose to sticky, score 0–10), moisture (compacted to watery, score 0–20), and pH values (3.4–4.8, score 0–25).

Statistical analysis

All experimental data were reported as mean ± standard error (SE), indicating variability around the mean estimate. Data normality was verified using the Shapiro–Wilk test (29). One-way ANOVA with Duncan's post-hoc test compared treatment means was conducted in SPSS (v22.0), with significance thresholds at $p < 0.05$ (significant) and $p < 0.01$ (highly significant). Spearman's correlation coefficients were calculated using the R package psych (v 2.4.1), and results were visualized with corrplot (v 0.92).

Results

Sensory assessment and pH score of *P. australis* silage

All silage treatments exhibited favorable sensory profiles, with no signs of mold or spoilage (Table 4). The silage color ranged from

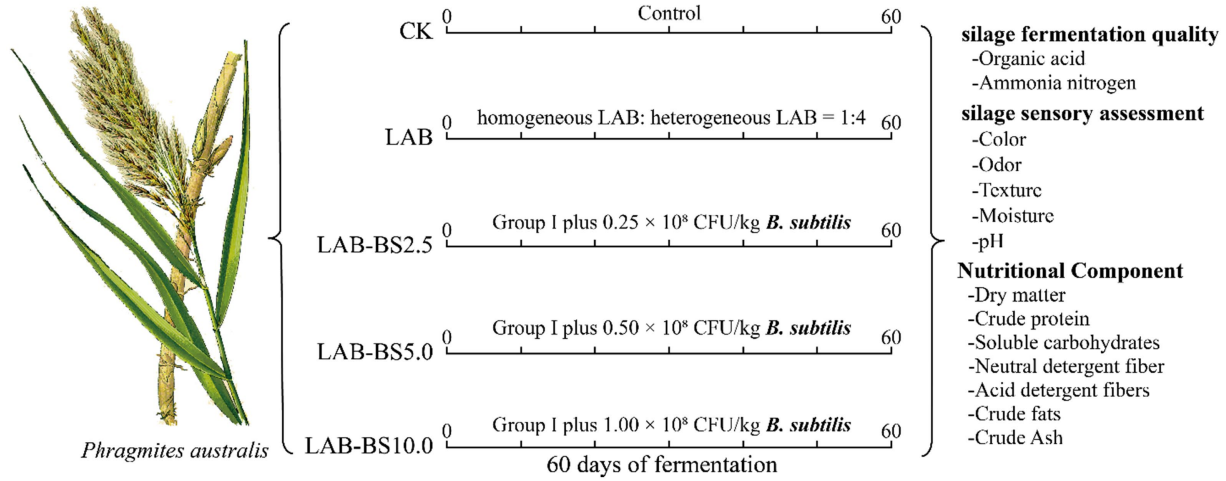

FIGURE 2
Schematic diagram of experimental design and silage processes.

TABLE 3 Silage quality evaluation standards.

Parameter	Total Score	Excellent	Good	Generally	Poor
Color	20	Turquoise/chartreuse (14~20)	Yellow-green (8~13)	Brownish-yellow (1~7)	Dark brown (0)
Odor	25	Aromatic sour (18~25)	Light sour (9~17)	Pungent sour (1~8)	Rotten/moldy (0)
Texture	10	Loose, non-sticky (8~10)	Soft, cohesionless (4~7)	Slightly viscous (1~3)	Sticky (0)
Moisture (%)	20	Moist, no droplets (14~20)	Moist, droplets (8-13)	Watery (1~7)	Drying/waterlog ged (0)
pH	25	3.4 ~ 3.8 (18 ~ 25)	3.9–4.1 (10–17)	4.2 ~ 4.7 (1 ~ 8)	> 4.8 (0)
Total Score	100	76 ~ 100	51 ~ 75	26 ~ 50	0 ~ 25
Grade		(Excellent)	(Good)	(Generally)	(Poor quality)

yellow-green to brownish-yellow, displaying a soft, non-sticky texture and optimal moisture content (14–17/20) without water droplet formation. A pronounced wine-like aroma was observed across treatments, particularly in LAB-BS5.0 and the Control (CK), which scored highest in odor (12/25). pH values ranged between 3.9 and 4.1, with LAB-BS5.0 (3.98) and LAB-BS10.0 (3.94) showing lower pH than CK (3.99), while LAB-BS2.5 (4.12) and LAB-BS2.5 (4.12) and LAB (4.02) had slightly higher values. Total sensory scores classified all treatments as "Good" (60–70/100), with LAB-BS 5.0 and CK achieving the highest scores (70/100).

Effects on organic acid profiles and ammonia nitrogen in *P. australis* silage by introducing *B. subtilis* and LAB consortia

The addition of *B. subtilis* significantly influenced organic acid profiles and NH_3-N content (Table 5). While LAB increased LA content (1.86 ± 0.04 g/kg) compared to CK (1.76 ± 0.03 g/kg), the difference was non-significant. However, LAB-BS2.5 (2.00 ± 0.11 g/kg) and LAB-BS2.5 (1.93 ± 0.06 g/kg) showed significantly higher LA than CK ($p < 0.05$). BA and PA concentrations increased significantly in LAB (1.21 ± 0.05 g/kg and 0.43 ± 0.02 g/kg, respectively) compared to CK ($p < 0.01$). NH_3-N decreased progressively with higher *B. subtilis* doses, reaching the lowest value in LAB-BS10.0 (0.60 ± 0.09 g/kg vs. CK: 0.89 ± 0.03 g/kg; $p < 0.05$). All treatments reduced acetic acid (AA) and PA compared to CK ($p < 0.01$), with LAB-BS10.0 showing the most pronounced reduction (AA: 2.01 ± 0.01 g/kg; PA: 0.17 ± 0.00 g/kg).

In summary, LAB-BS10.0 achieved the highest LA-to-total acid ratio (62.3%, $p < 0.01$) and the lowest NH_3-N content, indicating superior fermentation efficiency. LAB-BS2.5 and LAB-BS5.0 demonstrated a dose-dependent enhancement in LA production, highlighting *B. subtilis*'s role in acidification.

Effect on the nutrient content of *P. australis* silage

The inclusion of *B. subtilis* significantly influenced the nutritional profile of *P. australis* silage (Table 6). DM content decreased ($p < 0.01$) in all inoculated groups compared to CK, with the lowest values observed in LAB-BS2.5 (24.92%) and LAB-BS5.0 (24.99%). DM followed a descending order: LAB-BS10.0 (26.43%) > LAB-BS5.0 > LAB-BS2.5.

CP content increased incrementally with higher *B. subtilis* doses, peaking in LAB-BS10.0 (6.76%), though differences from CK (5.42%) were non-significant ($p = 0.09$). LAB-BS2.5 exhibited the highest WSC

TABLE 4 Sensory evaluation scores of *P. australis* silage by introducing *B. subtilis* and LAB consortia.

Treatments	Index scores					Total	Grade
	Color	Smell	Structure	Moisture	pH score/ values		
Control (CK)	16	12	10	17	15/3.99	70	Good
LAB	17	9	10	17	14/4.02	67	Good
LAB-BS2.5	16	10	9	16	9/4.12	60	Good
LAB-BS5.0	17	12	10	16	15/3.98	70	Good
LAB-BS10.0	15	10	10	16	16/3.94	67	Good

Control (CK, without inoculum), LAB (consisting of 1.5×10^8 CFU/kg of homogeneous (*L. plantarum* BNCC 336421 and *P. pentosaceus* BNCC 135034 in a ratio of 1:1), to heterogenous LAB species (*L. buchneri* BNCC 187961) in a ratio of 1:4), LAB-BS2.5 (LAB plus 2.5×10^7 CFU/kg *B. subtilis*), LAB-BS5.0 (LAB plus 5.0×10^7 CFU/kg *B. subtilis*), and LAB-BS10.0 (LAB plus 1.0×10^8 CFU/kg *B. subtilis*).

TABLE 5 Silage fermentation parameters for *P. australis* silage by introducing *B. subtilis* and LAB consortia.

Treatments	LA	AA	BA	PA	NH_3-N
Control (CK)	1.76 ± 0.03^{bc}	3.35 ± 0.01^{a}	1.09 ± 0.01^{b}	0.32 ± 0.00^{c}	0.89 ± 0.03^{a}
LAB	1.86 ± 0.04^{ab}	3.41 ± 0.14^{a}	1.21 ± 0.05^{a}	0.43 ± 0.02^{a}	0.84 ± 0.07^{a}
LAB-BS2.5	2.00 ± 0.11^{a}	2.89 ± 0.15^{b}	1.15 ± 0.06^{ab}	0.37 ± 0.02^{b}	0.79 ± 0.01^{a}
LAB-BS5.0	1.93 ± 0.06^{a}	3.33 ± 0.03^{a}	1.14 ± 0.01^{ab}	0.36 ± 0.00^{bc}	0.78 ± 0.01^{a}
LAB-BS10.0	1.68 ± 0.03^{c}	2.01 ± 0.01^{c}	0.65 ± 0.00^{c}	0.17 ± 0.00^{d}	0.60 ± 0.09^{b}
SEM	0.04	0.18	0.07	0.03	0.03
p-value	0.02	<0.01	<0.01	<0.01	0.02

Different lowercase letters within the same column indicate significant differences between the groups at a *p*-value of 0.05. LA, lactic acid; AA, acetic acid; BA, butyric acid; PA, propanoic acid; NH_3-N, ammonia nitrogen. Control (CK, without inoculum), LAB (consisting of 1.5×10^8 CFU/kg of homogeneous (*L. plantarum* BNCC 336421 and *P. pentosaceus* BNCC 135034 in a ratio of 1:1), to heterogenous LAB species (*L. buchneri* BNCC 187961) in a ratio of 1:4), LAB-BS2.5 (LAB plus 2.5×10^7 CFU/kg *B. subtilis*), LAB-BS5.0 (LAB plus 5.0×10^7 CFU/kg *B. subtilis*), and LAB-BS10.0 (LAB plus 1.0×10^8 CFU/kg *B. subtilis*). The units (g/kg fresh silage for LA, AA, BA, PA, and NH_3-N) represent the mass weight (g) of each parameter per kilogram of fresh silage material.

(0.87%, $p < 0.05$), a 40.3% increase over CK. NDF decreased significantly in LAB-BS2.5 (64.19%, $p < 0.05$) and LAB-BS10.0 (67.80%, $p < 0.05$), representing reductions of 9.51 and 5.9%, respectively. ADF was lowest in LAB-BS10.0 (22.51%), though differences from CK (26.00%) were non-significant. Ash content decreased ($p < 0.01$) in LAB (9.93%) and LAB-BS10.0 (8.96%). EE increased progressively with *B. subtilis* dosage, reaching 4.76% in LAB-BS10.0 ($p < 0.01$). LAB-BS10.0 optimized fiber degradation (NDF: 67.80%) while enhancing CP (6.76%) and EE (4.76%). LAB-BA2.5 maximized WSC accumulation (0.87%), critical for microbial activity during fermentation.

Correlation analysis between nutrients and fermentation parameters by introducing *B. subtilis*

Key correlations between nutrients and fermentation parameters of *P. australis* silage were identified (Figure 3). Positive correlations between NDF/ADF and AA/TA ($r = 0.82$), BA ($r = 0.76$), and NH_3-N ($r = 0.68$) suggest that fiber-rich substrates favor acetic/butyric acid production. Positive association of *B. subtilis* with CP ($r = 0.71$) and negative correlation with NH_3-N ($r = -0.65$) indicate its role in converting ammonia to microbial protein. *B. subtilis* correlated positively with LA/TA ($r = 0.89$) and negatively with pH ($r = -0.78$), highlighting its dual role in acid production and fiber breakdown.

B. subtilis enhances lactic acid dominance (LA/TA > 60%) while reducing NH_3-N, critical for high-quality silage. Fiber degradation by *B. subtilis* improves digestibility, supporting *P. australis* as a viable unconventional forage.

Discussion

Effect on fermentation quality of *P. australis* silage

NH_3-N, LA, and volatile fatty acids (VFAs) are critical indicators of silage fermentation quality (30). In this study, NH_3-N content decreased progressively with increasing *B. subtilis* inoculation, reaching the lowest level in LAB-BS10.0 (0.60 ± 0.09 g/kg vs. Control: 0.89 ± 0.03 g/kg; $p < 0.05$). This aligns with prior findings that *B. subtilis* produces bacteriocin-like metabolites, suppressing yeasts and molds while enhancing aerobic stability and reducing NH_3-N through proteolysis (27, 31–33). Additionally, *B. subtilis*-mediated acetolactate synthase activity likely catalyzed pyruvate conversion to acetolactic acid, improving both nutritional value and palatability (27, 32). Another factor affecting silage quality was the silage fermentation time, which ranged from 60 to 120 days, particularly 60 to 90 days (15, 18, 34).

LABs are well-documented for their role in rapid acidification, which preserves silage by inhibiting spoilage microorganisms (35–37). Homogeneous LAB (e.g., *Lentilactobacillus plantarum*) excel in LA production but offer limited inhibition of harmful bacteria (38, 39). Conversely, heterogeneous LAB (e.g., *L. buchneri*) metabolize residual

TABLE 6 Nutrient compositions of *P. australis* silage (%, DM basis) by introducing *B. subtilis* and LAB consortia.

Treatments	DM	CP	WSC	NDF	ADF	Ash	EE
Control (CK)	27.36 ± 0.01^{a}	5.42 ± 0.59	0.62 ± 0.04^{b}	73.70 ± 0.96^{a}	26.00 ± 1.02	10.97 ± 0.02^{a}	2.57 ± 0.00^{c}
LAB	25.82 ± 0.06^{c}	5.19 ± 0.05	0.69 ± 0.02^{b}	72.42 ± 3.73^{ab}	24.01 ± 1.98	9.93 ± 0.19^{b}	3.10 ± 0.09^{c}
LAB-BS2.5	24.92 ± 0.04^{d}	5.98 ± 0.32	0.87 ± 0.02^{a}	64.19 ± 2.21^{c}	23.34 ± 0.65	10.40 ± 0.09^{ab}	3.88 ± 0.01^{b}
LAB-BS5.0	24.99 ± 0.05^{d}	6.49±0.87	0.68 ± 0.05^{b}	71.06 ± 1.28^{ab}	24.69 ± 2.06	10.58 ± 0.55^{ab}	4.62 ± 0.49^{ab}
LAB-BS10.0	26.43 ± 0.02^{b}	6.76 ± 0.17	0.73 ± 0.07^{b}	67.80 ± 1.69^{bc}	22.51 ± 1.72	8.96 ± 0.09^{c}	4.76 ± 0.46^{a}
SEM	0.31	0.23	0.03	1.26	0.54	0.24	0.29
p-value	<0.01	0.09	0.02	0.04	0.35	<0.01	<0.01

DM, dry matter; CP, crude protein; WSC, water-soluble carbohydrates; ADF, acid detergent fiber; NDF, neutral detergent fiber; Ash, crude Ash; EE, ether extract. Control (CK, without inoculum), LAB (consisting of 1.5× 10^{8} CFU/kg of homogeneous (*L. plantarum* BNCC 336421 and *P. pentosaceus* BNCC 135034 in a ratio of 1:1), to heterogenous LAB species (*L. buchneri* BNCC 187961) in a ratio of 1:4), LAB-BS2.5 (LAB plus 2.5 × 10^{7} CFU/kg *B. subtilis*), LAB-BS5.0 (LAB plus 5.0 × 10^{7} CFU/kg *B. subtilis*), and LAB-BS10.0 (LAB plus 1.0 × 10^{8} CFU/kg *B. subtilis*).

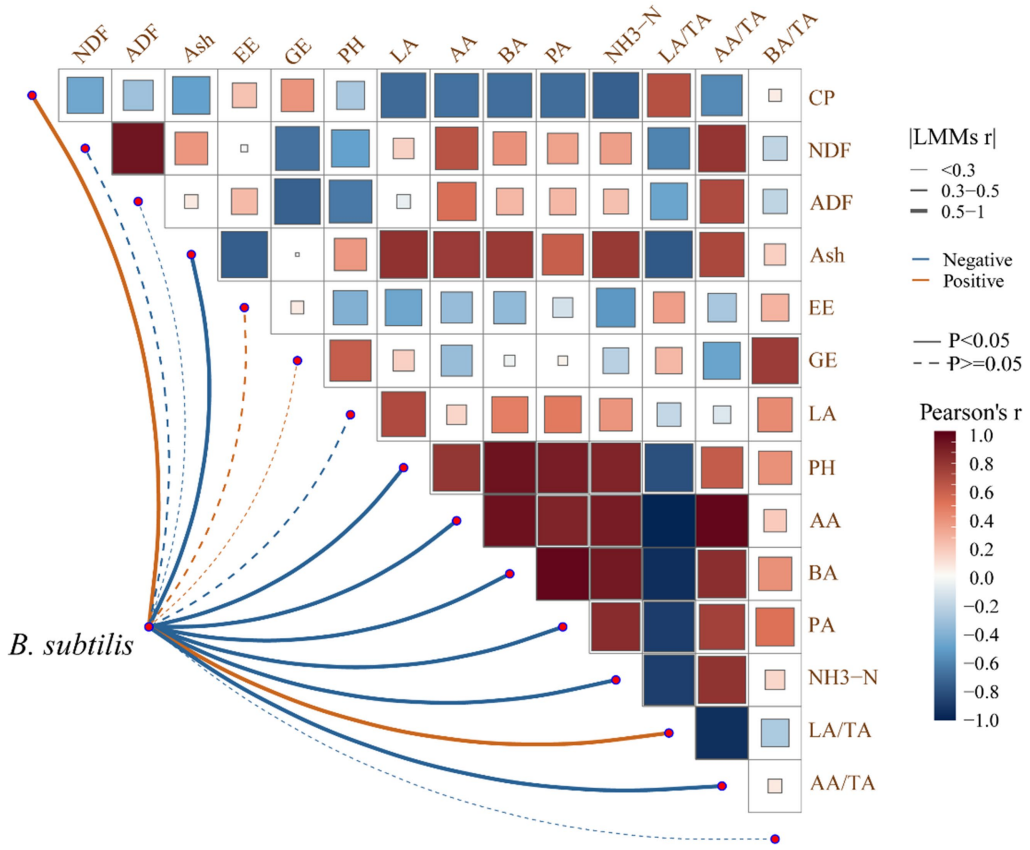

FIGURE 3
Correlation analysis between nutrients and fermentation parameters of *P. australis* by introducing *B. subtilis* in the LAB fermentation system.

sugars into AA, effectively suppressing molds (12). This study's combination of homofermentative and heterofermentative LAB at a 1:4 ratio synergistically enhanced LA yield while maintaining AA-driven mold inhibition (Table 5).

Notably, LAB-BS10.0 achieved the highest LA-to-total acid ratio (62.3%, $p < 0.01$), despite a slight reduction in absolute LA content compared to LAB-BS2.5 (2.00 ± 0.11 g/kg). This paradox highlights *B. subtilis*'s dual role: (1) promoting fiber degradation to release fermentable substrates for LA synthesis and (2) redirecting metabolic pathways to prioritize LA over VFAs like BA and PA (40, 41). The progressive decline in AA, BA, and PA with increasing *B. subtilis* doses ($p < 0.01$) further underscores its ability to refine fermentation profiles, favoring LA dominance.

Effect on the nutritional value of *P. australis* silage

DM content is a critical indicator of silage preservation efficiency (42). In this study, DM decreased significantly ($p < 0.01$) in all inoculated groups compared to the Control (CK: 27.36%), likely due to microbial utilization of soluble carbohydrates during fermentation

(Table 6). Notably, LAB-BS20.0 retained higher DM (26.43%) than other inoculated groups, suggesting *B. subtilis* moderates substrate consumption while enhancing fiber degradation.

CP content, a key nutritional metric (43), showed no significant differences between treatments ($p = 0.09$), though LAB-BS10.0 achieved the highest CP (6.76%). This aligns with Bai et al. (44), where *B. subtilis* improved protein retention in corn silage. Conversely, Bonaldi et al. (32) observed no CP enhancement with *B. subtilis*, possibly due to differences in substrate composition.

WSC peaked in LAB-BS2.5 (0.87%, $p < 0.05$), reflecting *B. subtilis*'s role in hydrolyzing structural carbohydrates. However, higher *B. subtilis* doses reduced WSC, likely due to accelerated microbial metabolism. *B. subtilis*'s cellulase activity (45) likely contributed to NDF reduction in LAB-BS2.5 (64.19%, $p < 0.05$) and LAB-BS10.0 (67.80%, $p < 0.05$), contrasting with Guo et al. (41), who reported minimal fiber impact.

Ash content decreased significantly ($p < 0.01$) in LAB (9.93%) and LAB+BS10.0 (8.96%), likely due to BS-driven mineral solubilization. Ether extract (EE) increased progressively with BS dosage, peaking at 4.76% in LAB+BS10.0 ($p < 0.01$), underscoring *B. subtilis*'s role in lipid preservation. *B. subtilis* synergizes with LAB to enhance fiber degradation (decrease NDF) and lipid retention (increase EE), though its dose-dependent effects on WSC and ash warrant further mechanistic exploration.

Conclusion

The integration of homogeneous LAB consortia with graded doses of *B. subtilis* significantly enhanced the fermentation and nutritional quality of *P. australis* silage. The optimal treatment, LAB-BS10.0 (1×10^8 CFU·kg-1 *B. subtilis*), demonstrated the highest lactic acid-to-total acid ratio (62.3%) alongside marked reductions in NH_3-N (0.60 ± 0.09 g/kg) and NDF (67.80%). Concurrently, EE increased to 4.76%, emphasizing *B. subtilis*'s role in lipid preservation and fiber degradation. These findings validate the synergistic potential of LAB-BS consortia to improve *P. australis* silage quality, offering actionable insights for scaling its silage production as a sustainable feed resource. This study provides a technical framework for optimizing microbial inoculants in *P. australis* silage system, addressing local forage shortages through innovative biomass valorization.

Data availability statement

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

Author contributions

YL: Conceptualization, Funding acquisition, Investigation, Writing – original draft. SZ: Formal analysis, Methodology, Writing – original draft. JL: Project administration, Software, Validation, Writing – original draft. NK: Data curation, Formal analysis, Resources, Writing – original draft. ST: Funding acquisition, Investigation, Resources, Writing – original draft. CZ: Project administration, Writing – original draft. ZT: Conceptualization, Data curation, Writing – original draft. AE: Conceptualization, Writing – original draft. IR: Funding acquisition, Writing – original draft. FZ: Funding acquisition, Writing – original draft. AS: Validation, Writing – original draft, Writing – review & editing.

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Conflict of interest

SZ was employed by Liuyang Liu'an Agricultural Technology Comprehensive Development 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.

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Heyndrickxia coagulans SANK70258 supplementation improves growth performance, gut health, and liver function in growing pigs

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Heyndrickxia coagulans is a Gram-positive, facultatively anaerobic, and spore forming bacterium. This species is often used as probiotics, therefore it is known for its health-conferring effects on livestock and humans. Previously, we showed that administering *H. coagulans* SANK70258 (SANK70258) to coccidiosis-infested broilers improved their growth performance by inducing anti-inflammation. Nonetheless, a few studies have observed the effects not only of *H. coagulans* spp. but also of SANK70258 in pigs. Here, we evaluated if SANK70258 could help improve the growth of pigs from weaning (days 0–42), growing (days 42–84) to fattening (days 84–126) periods. Twenty-four weaned crossbred (Duroc × Large White × Landrace) piglets were divided into control (CC; 4 replicants, n = 8) and 0.01% SANK70258 supplementation (P; 8 replicants, n = 16) groups. Diets and water were given *ad libitum*. After the weaning period (day 42), the pigs in group P were further sub-divided into pens with (PP; 4 replicants, n = 8) or without SANK70258 administration (PC; 4 replicants, n = 8). SANK70258 improved growth performance during the growing period [statistical differences were observed on days 42–56 (PP) and 70–84 (PP and PC)]. During weaning, a period well recognized as a frequent pathogen infection stage, due to the portal blood of pigs with leaky guts flows into the liver carrying gut microbes and their products such as endotoxin and bacterial DNA. P pigs experienced an improved liver function evidenced by the plasma alanine aminotransferase reduction and hence, a putative improved leaky gut condition evidenced by the plasma lactulose/mannitol ratio reduction. Nonetheless, during the fattening period, the positive effects became confounding with a pathogenic infection expressing a drastic increase in plasma aspartate aminotransferase on day 126. Nonetheless, stearate composition in meat of PP pigs were observed on day 126. Our results suggested that the SANK70258 administration may be a good natural product to improve the growth performance of pigs during production stages with a lower probability of infection like the growing period.

KEYWORDS

growing pig, growth performance, leaky gut, liver function, probiotics, *Heyndrickxia coagulans* SANK70258

1 Introduction

Pork is one of the most consumed meat globally (1). Indeed, in 2022 alone the consumption of pork in the world reached 113 million tons (2). Thus, growth performance is one of the most important productive traits in pig production (3). However, during weaning, piglets are vulnerable to several conditions that decrease their growth performance. For example, changes in the environment, diet, management and separation from dams exert negative effects on the growth performance of piglets (4). These stressors lead to negative changes in the intestinal health of piglets, which in turn cause diarrhea, slow growth and a decreased ability to withstand diseases (4).

The health of the intestine of the pig is very important to achieve optimal growth, as it is the place where digestion and absorption of nutrients take place (5). In addition, a healthy intestinal barrier protects the piglet from feed-derived antigens, pathogenic microorganisms and other harmful metabolites (5). The intestine of the pig is populated by a diverse microorganism community (6). Indeed, bacterial colonization of the pig's colon is known to be about 10^{10}–10^{11} per g or mL of content (7). The condition of the gut microbiota is linked to the health and growth performance of pigs (8). Apart from the microbial barrier, the intestine of the pig is protected by the mucosal and the immunological barriers (9). A leaky gut occurs when the intestinal barrier is compromised, and intestinal inflammation takes place (10). The portal blood of pigs with leaky guts flows into the liver carrying gut microbes and their products such as endotoxin and bacterial DNA (11). This condition contributes to persistent chronic inflammation and progressive liver damage (12, 13). Probiotics help restore the gut barrier integrity and modulate the microbiota, thus reducing lipopolysaccharide translocation and systemic inflammation (14). We have also described the cell preparation of *Enterococcus faecalis* EC-12, which stimulates the hepatic superoxide disumutase activity (15). Consecutive administration of this strain improves the liver damage such as fatty degradation in murine aged model (16). Thus, probiotics offer a therapeutic avenue for improving liver health. Furthermore, high levels of aspartate aminotransferase (AST) and/or alanine aminotransferase (ALT) in the blood indicate damage of the liver function (17), but a probiotic administration often suppresses these parameters (18).

Probiotics is a generic term to refer to those microorganisms that confer beneficial effects to the host, following ingestion. *Heyndrickxia coagulans*, also known as *Lactobacillus sporogenes* (19) or *Bacillus coagulans* (20) has been shown to modulate the microbiotas and immunostimulate the microbiotas of broilers (21). It is known that some strains of *H. coagulans* have antimicrobial activity (22), and that *H. coagulans* SANK70258 stimulates antimicrobial peptide secretion in the mucosa (23). Gut microbiota modulation by *H. coagulans* SANK70258 administration was at least partly caused by these functions. In addition, *H. coagulans* administration has been shown to be beneficial to patients with alcoholic fatty liver disease (24). An immune modulation effect of *H. coagulans* was also known as the common function (24); some strains of *H. coagulans* such as MTCC5856 (25) and BC198 (26) suppressed the inflammation and/or anti-inflammatory cytokine stimulation. Also, *H. coagulans* SANK70258 can modulate the immune function with suppression of inflammation, as described elsewhere (27). In pigs, several studies have reported the benefits of probiotics to weaning pigs (28, 29). Benefits of these probiotic administration were expected in the form of prevention of pathogen infection (30, 31), because pigs after weaning can be easily infected by many pathogens (32, 33). On the other hand, after the weaning period, the enhancement of growth performance became unclear (34), because pigs no longer need enhanced supplementation to prevent pathogen infections (33). Nonetheless, chronic inflammation, caused by stress and inflammation-inducing ingredients in feed, is always found in intensive livestock production systems; thus anti-inflammation inducing-materials are beneficial to promote growth performance (35). To the best in our knowledge, only one study has described the use of *H. coagulans* for pigs in the growing and fattening periods (36), but the reported growth performance was not improved remarkably. Based on this background, we hypothesized that *H. coagulans* SANK70258, known as an anti-inflammation-inducing probiotic, can enhance the growth performance particularly in pigs during the growing and fattening periods.

The aim of the present study was to assess if the administration of probiotic *H. coagulans* SANK70258 could help improve the growth performance of pigs from the weaning to the fattening periods. Because *H. coagulans* SANK70258 stimulated gene expressions of tight junction protein, suppressed gene expressions of inflammatory cytokines, and modulated the microbiota composition in the gut of broilers (27), we designed three analyses to determine the improvement mechanisms. (1) Plasma AST and ALT concentrations were measured every 2 weeks to evaluate the liver condition. (2) Lactulose/mannitol (L/M) test were observed at the weaning period (day 28) to evaluate the leaky gut condition. (3) mRNA expressions associated with antimicrobial peptide, tight junction proteins, IgA secretion and cytokines in jejunal, ileal, and cecal mucosae were observed. Furthermore, the organic acid concentration in the cecal content, and plasma in the ileal and cecal veins, the cecal microbiome and the fatty acid composition in muscle were also determined.

2 Materials and methods

2.1 Probiotics

Probiotic *H. coagulans* SANK70258 (SANK70258) manufactured by Mitsubishi Chemical Cooperation (Tokyo, Japan) containing 1×10^9 CFU per g was used. Supplementation was given to groups at 0.01% (w/w), so that diets contained 1×10^5 CFU per g of feed (0.1 g/kg of feed). Based on results from a previous chicken study (21), we expected that this concentration was enough to enhance the growth performance of pigs.

2.2 Animals and diet

The experimental design of this study is shown in Figure 1. Twenty-four weaned piglets (Duroc × Large White × Landrace; 30-day-old; 12 castrated male and 12 female) were purchased from a commercial supplier (Oita, Japan) and kept in an open shed at the KYODOKEN Institute (Kyoto, Japan). They were kept in 12 individual pens (W: 1.7 × D: 4.0 m; one castrated male and one female) and acclimatized for 3 days. After acclimatization, pigs in the pens were divided into treatment-free control (CC, 4 pens) and 0.01%

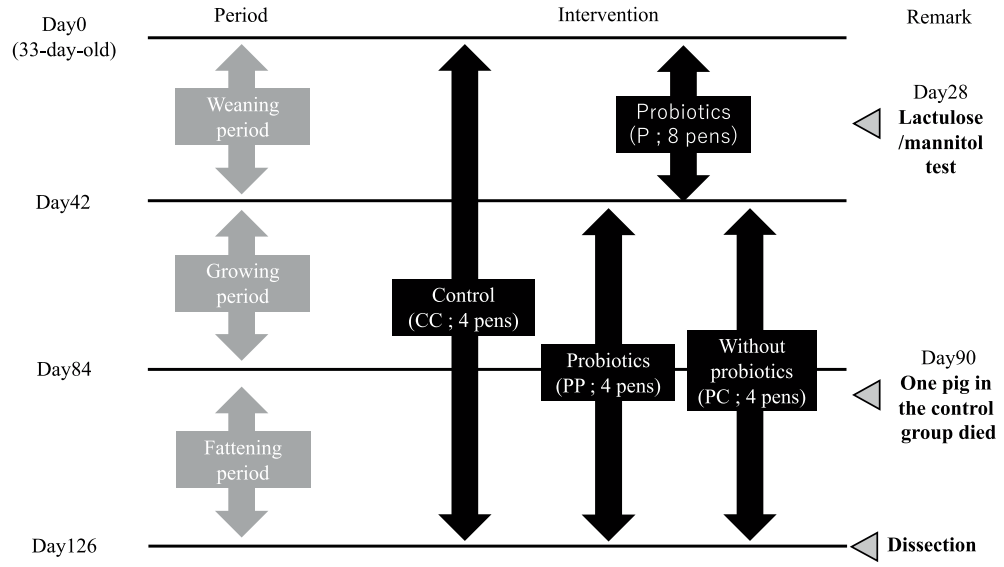

FIGURE 1
Schematic design of this study. Each pen housed two pigs. However, one pig in C group died on day 90. On day 28, a Lactulose/Mannitol test was performed, and blood was collected from the jugular vein. At dissection on day 126, muscle, blood, cecal contents, and intestinal tissues were sampled. *H. coagulans* SANK 70258 was supplemented to the diet at 1×10^5 CFU/g feed. For statistical analysis, a two-group comparison was conducted during the weaning period, while a multiple comparison test was performed among three groups during the growing and fattening periods.

SANK70258 supplementation (P, 8 pens) groups. The mean body weight of the pigs in groups was the same (8.8 ± 0.8 kg).

Piglets in each group were housed in a floored-pen with sawdust bedding and equipped with a gas brooder (Nakajima Seisakusyo, Nagano, Japan) to maintain an adequate, stable environment. Feed and drinking water were supplied *ad libitum*. The basal diet was formulated by Marubeni Nisshin Feed Co. Ltd. (Tochigi, Japan). The composition and the nutrient content (%) of the diets are shown in the Supplementary Table S1. The diets were given to the piglets throughout the present study (18 weeks). As the piglets developed, the diets were changed from the weaning period to the growing period on day 42, and then from the growing period to the fattening period on day 84. After the weaning period (day 42), the pens of group P were further sub-divided into SANK70258 administration (PP; four pens) and without SANK70258 administration (PC; four pens) groups. The mean body weight of each group was similar (PP 39.1 ± 1.5 kg vs. PC 37.3 ± 1.9 kg).

2.3 Body weight, feed intake measurements and clinical observation

Clinical symptoms such as vitality, appetite, respiratory difficulties and diarrhea were checked individually and the worse score between the two pigs were recorded as the daily score in each pen. The scoring criteria of clinical symptoms were described elsewhere (37). A female pig in CC group had a severe clinical sign of respiratory difficulties in the morning of day 90. A veterinarian checked the symptoms of the animal and injected her Marbofloxacin (Marbocyl 10%, Meiji Animal Health, Tokyo, Japan) and Flunixin meglumine (Forvet 50, MSD Animal Health, Tokyo, Japan). Unfortunately, this pig died in the evening of day 90. The pig was dissected to assess the cause of death. Typical adhesion was observed between the pleura and the lungs. Therefore, a veterinarian diagnosed that the pig died of acute pleuropneumonia. Afterwards, the growth performance of this pen was calculated by the parameters of the remaining castrated male. No other pigs used in the present study showed clinical signs of respiratory difficulties throughout this study. Therefore, the remaining pigs were not treated with antimicrobials or other drugs.

All animals used in the present study were identified with ear tags (odd numbers, male; even numbers, female), and individually weighed on days 0, 14, 28, 42, 56, 70, 84, 98, 112, and 126. In addition, feed intake in each pen was calculated on the same days. Individual daily weight gain (DG) and daily feed intake in pens were calculated, and feed conversion ratio (FCR) in pens were calculated with the two parameters using the following equation: (FCR) = (Daily feed intake in a pen)/(total DG of two pigs in a pen). Separately, blood was collected individually when the body weights were measured. Blood was collected from the jugular vein and immediately cooled on ice. Plasma was separated by centrifugation (3,000 × *g*, 10 min, 4°C; CF9RX, HITACHI, Ibaraki, Japan) and stored at −80°C (VT-208; Nihon Freezer, Tokyo, Japan) until further use.

2.4 Slaughter procedure

On days 126 and 127, all pigs were slaughtered. For technical limitations, pigs in pens 1 and 2 were slaughtered on day 126, and the remaining pigs in pens 3 and 4 were slaughtered on day 127. All pigs were injected Mafoprazine mesylate (0.5 mg/kg; Mafropan 1% injection, Bussan Animal Health, Osaka, Japan) and Midazolam (1.0 mg/kg; Dorumicum Injection 10 mg, Maruishi Pharmaceuticals, Osaka, Japan) intramuscularly for sedation. When the pigs were sedated, pentobarbital sodium (12.96 mg/kg; Somnopentyl, Kyoritsu, Tokyo, Japan) was injected intravenously. While under deep anesthesia, the bodies of pigs were midline incised, and blood was

collected from the cecal and ileal veins. Then, after exsanguination, the intestines were resected and separated into the small and large intestines. The small intestines were equally cut into eight segments as previously described (38). Segments 2 and 8 were defined as jejunum and ileum, respectively. Digestas in ilea and ceca were collected under sterile conditions, and then immediately cooled on ice. The mucosae of jejuna, ilea and ceca were collected with clean sterile glass slides. The mucosae were soaked into RNA-*later* solution (Sigma-Aldrich Japan, Tokyo, Japan) and immediately cooled on ice.

A portion of the *longissimus dorsi* muscle was collected approximately 15 cm from the rear end of left scapula and immediately cooled on ice. The samples of blood, digestas, mucosae and muscles were brought to our laboratory under cool condition (on ice) within 10 h. Plasma was separated by centrifugation (3,000 × *g*, 10 min, 4°C). Samples were then stored at −80°C (VT-208; Nihon Freezer) until further use.

2.5 Lactulose/mannitol test

We used L/M test to evaluate the leaky gut condition in the weaning period. L/M test is the gold standard method to detect intestinal permeability in humans (39); thus, we adopted this method to evaluate the leaky gut condition in pigs without slaughter.

A solution of LM [lactulose (L; 0.5 g/mL, MONILAC powder, Chugai Pharmaceutical, Tokyo, Japan) and mannitol (M; 0.1 g/mL, Nacalai Tesque, Kyoto, Japan)] was prepared.

All pigs had their feed removed in the evening of day 28 and their body weights were measured. Next, the LM solution (1 mL/kg) was administered orogastrically the next morning (administration started at 10:00 am) with a commercial catheter (J Feed Nutrition Catheter 15F 120 cm, JMS, Hiroshima, Japan). After 4 h, blood was collected from the jugular vein of pigs and cooled on ice immediately. Plasma was separated by centrifugation (3,000 × *g*, 10 min, 4°C; CF9RX, HITACHI) and stored at −80°C (VT-208; Nihon Freezer) until further use.

The L and M concentrations were measured by an LC–MS/MS apparatus (Acquity TQD UPLC-MS/MS system; Waters, Milford, MA, USA) at the Kyoto Institute of Nutrition and Pathology (Kyoto, Japan). Briefly, an internal standard solution was prepared by mixing 500 mg/L of lactulose-$^{13}C_{12}$ (Sigma-Aldrich Japan) and D-Mannitol-$^{13}C_6$ (Sigma-Aldrich Japan) with ultrapure water (Fujifilm Wako, Osaka, Japan). Frozen plasma was thawed and 50 µL were transferred to 2-mL screw-cap tubes (Watson, Tokyo, Japan), to which 5 µL of internal standard solution was immediately added. After mixing, 495 µL of acetonitrile (Fujifilm WAKO) was added, and then homogenized at 3,000 r.p.m. for 30 s using a Micro Smash MS-100 (TOMY, Tokyo, Japan) with a 5.5 φ stainless steel ball (TOMY). After homogenization, the microtubes were kept 30 min on ice, then the tubes were centrifuged at 15,000 × *g*, for 10 min, at 4°C (Model3520; Kubota, Tokyo, Japan). The supernatants were collected and filtered with commercial filters (PTFE φ 4mm/0.2µm; As ONE, Osaka, Japan). The lactulose and mannitol concentrations in the samples were measured using an ultra-pressure liquid chromatography apparatus equipped with a binary solvent manager, an autosampler, and a column heater and tandem mass spectrometry (Acquity TQD UPLC-MS/MS system; Waters). A chromatographic separation was conducted using an Acquity UPLC BEH Amide column (2.1 × 100 mm; particle size 1.7 µm; Waters). The mobile phase, delivered at a flow rate of 0.2 mL/min at 85°C, was a gradient of solution A (acetonitrile: isopropanol (Fujifilm WAKO): ultrapure water = 90: 5: 5) and solution B (acetonitrile: ultrapure water = 80: 20). The gradient was as follows: a flow of 100% solution A for 5 min, followed by a gradual decrease to 0% solution A for 9 min, and maintenance of the 100% solution B for 8 min. The total run time of this method was 22 min. Solutes were detected using the tandem quadrupole mass spectrometer (Waters) with a Z-spray ion interface. The system was controlled using the Waters MassLynx mass spectrometry software (Waters). Ionization was achieved using an electrospray positive ionization mode. The positive ion mode was set as follows: ion source temperature, 150°C; capillary voltage, 2.0 kV; and desolvation temperature, 450°C. The desolvation and cone gas flow were 900 L/h and 50 L/h, respectively. A multiple reaction monitoring (MRM) was conducted. Transitions, m/z values, and cone voltages are shown in the Supplementary Table S2. The injection volume was 5 µL. The concentrations of lactulose and mannitol were calculated from the peak areas detected in the chromatogram with MRM in relation to the respective internal standard.

Prior to this, all pigs were confirmed to be negative in lactulose and mannitol in the blood.

2.6 Measurement of blood parameters related to liver functions

Plasma collected on days 0, 14, 28, 42, 56, 70, 84, 98, 112, and 126 had the aspartate aminotransferase (AST) and alanine aminotransferase (ALT) concentrations measured using the Japan society of clinical chemistry transferable method by a commercial laboratory (Fujifilm VET systems, Tokyo, Japan).

2.7 Measurement of short-chain fatty acid and lactate concentrations in the digesta and blood

The short-chain fatty acid (SCFA) and lactate concentrations in the cecal digesta were measured by ion-exclusion high pressure liquid chromatography (Shimadzu, Kyoto, Japan) as described elsewhere (40). The SCFA concentrations in plasma of ileal and cecal veins were measured by gas chromatography–mass spectrometry (Shimadzu) as described elsewhere (41).

2.8 mRNA expression analyses by real-time PCR

Total RNA was extracted from the mucosae in the same manner as previously described (42) with some modifications. Briefly, total RNA was extracted from the respective portions of mucosae using a QuickGene RNA tissue kit SII (KURABO, Osaka, Japan) and an RNA extraction kit for use with a semi-automated nuclear acid extraction machine (QuickGene Mini-480; KURABO). The concentration of the extracted total RNA was measured with a spectrophotometer (Infinite 200 PRO M Nano; TECAN Japan, Kanagawa, Japan), and 500 ng of the total RNA was used for reverse transcription. Afterwards, reverse

transcription was conducted using a commercial kit (PrimeScript RT Reagent Kit, TakaraBio, Shiga, Japan) with oligo (dT)20 and random primers, which were part of the kit. All procedures were conducted according to the manufacturer's instruction.

Real-time polymerase chain reaction (PCR) was conducted using a Rotor-Gene Q (Qiagen, Tokyo, Japan). Primers (FASMAC, Kanagawa, Japan) and TaqMan probes (Universal ProbeLibrary Set and Human and Extension Set; Roche Applied Science, Penzberg, Germany) used in the present study are listed in the Supplementary Table S3. Target genes were selected from our previous study (23). Optimal primers and probes were designed using freely available online tools.[1] The relative expression levels of mRNA were calculated by the $2^{-\Delta\Delta Ct}$ method (43). $\Delta\Delta Ct$ value was calculated as follows: $\Delta\Delta Ct = (Ct_{\text{target gene}} - Ct_{\text{beta-actin}})_{\text{group PP or PC}} - (Ct_{\text{target gene}} - Ct_{\text{beta-actin}})_{\text{group CC}}$.

2.9 Triglyceride concentration and fatty acid compositions in the muscle

Triglyceride concentration was measured by a previously described method (44) with some modifications. Briefly, the center segment (approximately 5 g) of the *longissimus dorsi* muscle was sub-sampled and chopped with a surgery scissor (As ONE). After mixing, 90 mg of the chopped meat of each sample was transferred to 2-mL screw-cap microtubes (Watson), then 1.710 mL of extractant [chloroform (Guaranteed reagent, cat. 038–02606; Fujifilm WAKO): methanol (for LC/MS, cat. 134–14523; Fujifilm WAKO) = 2: 1] and a 5.5 φ stainless steel ball (TOMY) were added. The muscle was homogenized at 3,800 r.p.m. for 120 s with the Micro Smash MS-100 (TOMY). After homogenization, the microtubes were vortexed for 30 min at room temperature, and then 200 µL of ultrapure water (Fujifilm WAKO) was added. The microtubes were vortexed again and then centrifuged at 10,000 × *g*, for 5 min, at 4°C (Model3520; Kubota). The chloroform layers were collected and transferred to new 2-mL microtubes (Watson). The solvent was evaporated using a centrifugal evaporator (CVE-2200, EYELA, Tokyo, Japan) at 60°C. Dried extracts were then suspended in 90 µL of isopropanol (Fujifilm WAKO). The suspension was used to measure the triglyceride concentration using a commercial kit (Triglyceride Test WAKO; Fujifilm WAKO), as per the manufacturer's instructions.

To measure the fatty acid compositions in the muscles, first, an internal standard solution was prepared by diluting 1 mg/mL of methyl heptadecanate (Sigma-Aldrich Japan) in methanol (Fujifilm Wako). A total 600 mg of the chopped muscle was inserted to 2-mL screw-cap microtubes (Watson), and then 0.3 mL of ultrapure water (Fujifilm Wako) and a 5.5 φ stainless steel ball (TOMY) were added. The muscle was minced at 3,800 r.p.m. for 120 s with the Micro Smash MS-100 (TOMY). After mincing, 100 mg of slurry were sub-collected to new 2-mL screw-cap microtubes (Watson), and 50 µL of internal standard solution, 1.6 mL of extractant [chloroform (Fujifilm WAKO): methanol (Fujifilm WAKO) = 2: 1] and a 5.5 φ stainless steel ball (TOMY) were added. The sample was homogenized at 3,800 r.p.m. for 120 s with the Micro Smash MS-100 (TOMY). After homogenization, the microtubes were vortexed at 30 min at room temperature, and then 200 µL ultrapure water (Fujifilm WAKO) was added. After further vortexing, the microtubes were then centrifuged at 10,000 × *g*, for 5 min, at 4°C (Model3520; Kubota). The chloroform layer was collected and transferred to new 2-mL microtubes (Watson), and evaporated using the centrifugal evaporator (CVE-2200, EYELA) at 37°C. The dried extracts were suspended in 400 µL of methylation reagent A and 400 µL of methylation reagent B [these reagents were part of the commercial kit named Fatty Acid Methylation Kit (Nacalai Tesque)]. The mixtures were stored at room temperature for 1 h. Next, 400 µL of methylation reagent C was added to the mixtures, vortexed, and stored at room temperature in 20 min. Finally, 400 µL of the isolation reagent was added and vortexed. The upper layer was collected and used for further analysis. Fatty acids such as methyl linoleate, methyl oleate, methyl palmitate and methyl stearate were measured using a gas chromatography apparatus equipped with a flame ionization detector (GC-2014-FID; Shimadzu). The chromatographic separation was conducted using a ZB-FFAP column (ID: 0.32 mm × 15 m; film thickness: 0.25 µm; Shimadzu). Helium (Masuda Medical Instrument, Kyoto, Japan) was used as the carrier gas, and the flow rate was set at 35 cm/s. The thermal conditions of the column oven was 210°C, the injection port temperature was 260°C, the flame ionization detector temperature was 260°C, and the split ratio was 40:1.

2.10 Microbiome analysis of the cecal digesta

Bacterial DNA was extracted from the cecal digestas using a commercial extraction kit (QuickGene DNA tissue kit; KURABO) as described elsewhere (45).

Library preparation and deep sequencing by MiSeq (Illumina K.K., Tokyo, Japan) were carried out exactly as described by Inoue et al. (46). Briefly, the V3–4 region of the 16S rRNA genes in each sample was amplified by primers 341F and 805R containing a 5′ overhang adapter sequence for PCR by a KAPA HiFi HotStart Ready Mix (Kapa Biosciences, Wilmington, MA, USA). The amplicon was purified with NucleoFast 96 PCR plates (Takara Bio). A second PCR was carried out using the KAPA HiFi HotStart Ready Mix to attach a unique combination of dual indices (I5 and I7 indices) and Illumina sequencing adapters to each sample. The amplicon of the second PCR was purified and the concentration was normalized using a SequalPrep Normalization Plate Kit (Life Technologies, Tokyo, Japan). Each of the normalized amplicons was then evenly pooled and concentrated using AMPure XP beads (Beckman Coulter, Tokyo, Japan). The quantity of the library was assessed with a Library Quantification Kit for Illumina (Kapa Biosciences). The library was denatured with 0.2N NaOH (SIGMA-Aldrich Japan) and combined with phiX Control (v3, Illumina, San Diego, CA, USA; expected 20%). Eleven picometers of the library, combined with phiX Control, was heat-denatured at 96°C for 2 min and sequenced using a 285 bp paired-end strategy on the Miseq apparatus (Illumina K.K.) as per the manufacturer's instructions.

After the sequencing, quality filtering, denoising, determination of amplicon sequence variants (ASVs), and taxonomic classification of ASVs against the SILVA 138 database, the obtained reads were analyzed by QIIME2 (ver. 2023.2) with DADA2 plugin. Denoising using DADA2 was conducted with the

1 https://primers.neoformit.com/

trimming length from the left set at 17 and that from the right at 19. The truncation length was set at 250 for both reads. Singletons and ASVs assigned to chloroplasts and mitochondria were removed in this study.

2.11 Statistical analysis

The daily clinical score in each pen was summed in the weaning, growing and fattening stages.

The parameters of growth performance (DG and FCR; CC group, $n = 4$ and P group, $n = 8$) and blood parameters (L/M ratio, AST and ALT concentration; CC group, $n = 8$ and P group, $n = 16$) in the weaning period (days 0–42) were analyzed to investigate the homoscedasticity by the F test. According to the results, either the Student's or the Welch's *t*-test was used to analyze differences between the groups. The summed clinical score in the weaning period was analyzed by the Mann–Whitney test to determine the differences between groups.

The parameters of growth performance (DG and FCR; $n = 4$), blood parameters (AST and ALT concentrations), SCFA concentrations, mRNA expression in mucosae, fatty and amino acid concentrations in muscles, and the microbiome parameters in ceca (CC group, $n = 7$; PP and PC groups, $n = 8$) were analyzed to investigate the homoscedasticity by the Bartlett test. According to the results, either a completely randomized design one-way analysis of variance or the Kruskal–Wallis test was used to analyze the differences between the groups. Either Tukey or Steel-Dwass *post-hoc* comparisons were used for multiple comparisons, as needed. The summed clinical scores in the growing and fattening periods were analyzed by the Kruskal–Wallis test to determine the differences between groups. Steel-Dwass *post-hoc* comparisons were used for multiple comparisons, as needed.

In all the statistical analyses, the values are the arithmetic means ± the standard errors. In addition, the differences between the means were considered significant if $p < 0.05$, and with a tendency to be significant if $p < 0.1$. All statistical analyses were conducted using R software version 4.1.2 (R Core Team, Vienna, Austria).

3 Results

3.1 Scoring criteria for clinical symptoms and abnormalities at slaughter

Total diarrhea scores during the study were 20.8, 17.3 and 9.8 for the pigs in the groups control, PP and PC, respectively, indicating that there were no statistical differences ($p = 0.30$). Total vitality and appetite scores during the study were 0.3, 0.0, and 0.0 for the pigs in the groups control, PP and PC, respectively, indicating that there were no statistical differences. Total respiratory difficulty scores during the study were 1.0, 0.3, and 3.5 for the pigs in the groups control, PP and PC, respectively, again, indicating that there were no statistical differences. In addition, as mentioned above, a pig died of acute pleuropneumonia on day 90.

At the slaughter day, macroscopic abnormalities of organs were checked; remarkable abnormalities were not found in any of the pigs.

3.2 Growth performance parameters

During the weaning period, the pigs in the P group had a significantly lower DG than the control pigs during the first 14 days ($p = 0.04$; Figure 2A). The reduction of DG became unclear after day 14; DG tended to be lower in P group rather than in CC group with no statistical significances on days 14–28, and then DG became similar between CC and P groups on days 28–42. During the growing period, pigs in group PP showed a significant increase in DG on days 42–56 ($p = 0.04$). In addition, pigs in groups PP and PC had higher DG on days 70–84 ($p = 0.01$; Figure 2A). Nonetheless, no differences were observed between groups on days 56–70. During the fattening period, no differences were observed between groups on days 84–98, 98–112 and 112–126.

During the weaning period (days 28–42), group P had a lower FCR than control ($p = 0.004$; Figure 2B). Nonetheless, no differences were observed between groups on days 0–14 and 14–28. During the growing period (days 70–84), pigs in group PC had a lower FCR than the CC pigs ($p = 0.03$). Nonetheless, no differences were observed between groups on days 42–56 and 56–70. During the fattening period, no differences were found between groups on days 84–98, 98–112, and 112–126.

3.3 Lactulose/mannitol ratio in weaning period

The lactulose/mannitol (L/M) ratio is used to assess the integrity of the gut. The L/M ratio in blood plasma was found to be lower in pigs supplemented with SANK70258 than in control ($p = 0.03$; Figure 3). In addition, according to a previous report, L/M ratio in blood after 4 h of L and M inoculation was approximately 0.25–2.5 (39) in weaned pigs, hence the values of our results seemed reasonable.

3.4 Aspartate and alanine aminotransferase in plasma

The ranges of AST concentration were 40–60 U/L in the weaning period, 30–50 U/L in the growing period, and 20–80 U/L in the fattening period (Figure 4A). The ranges of ALT concentration were 30–60 U/L in the weaning period, 40–50 U/L in the growing period, and 30–40 U/L in the fattening period (Figure 4B). In a previous report, the AST concentration was 55–65 U/L in the growing period and 55–70 U/L in the fattening period, whereas the ALT concentration was 20–30 U/L in the growing period and 20–33 U/L in the fattening period (47). Comparing these studies, the parameters were generally similar, but those of the present study were slightly higher than those of the previous report. Hepatitis E virus infection induces the AST secretion into the serum as high as 100 U/L in shipping pigs (48). However, the

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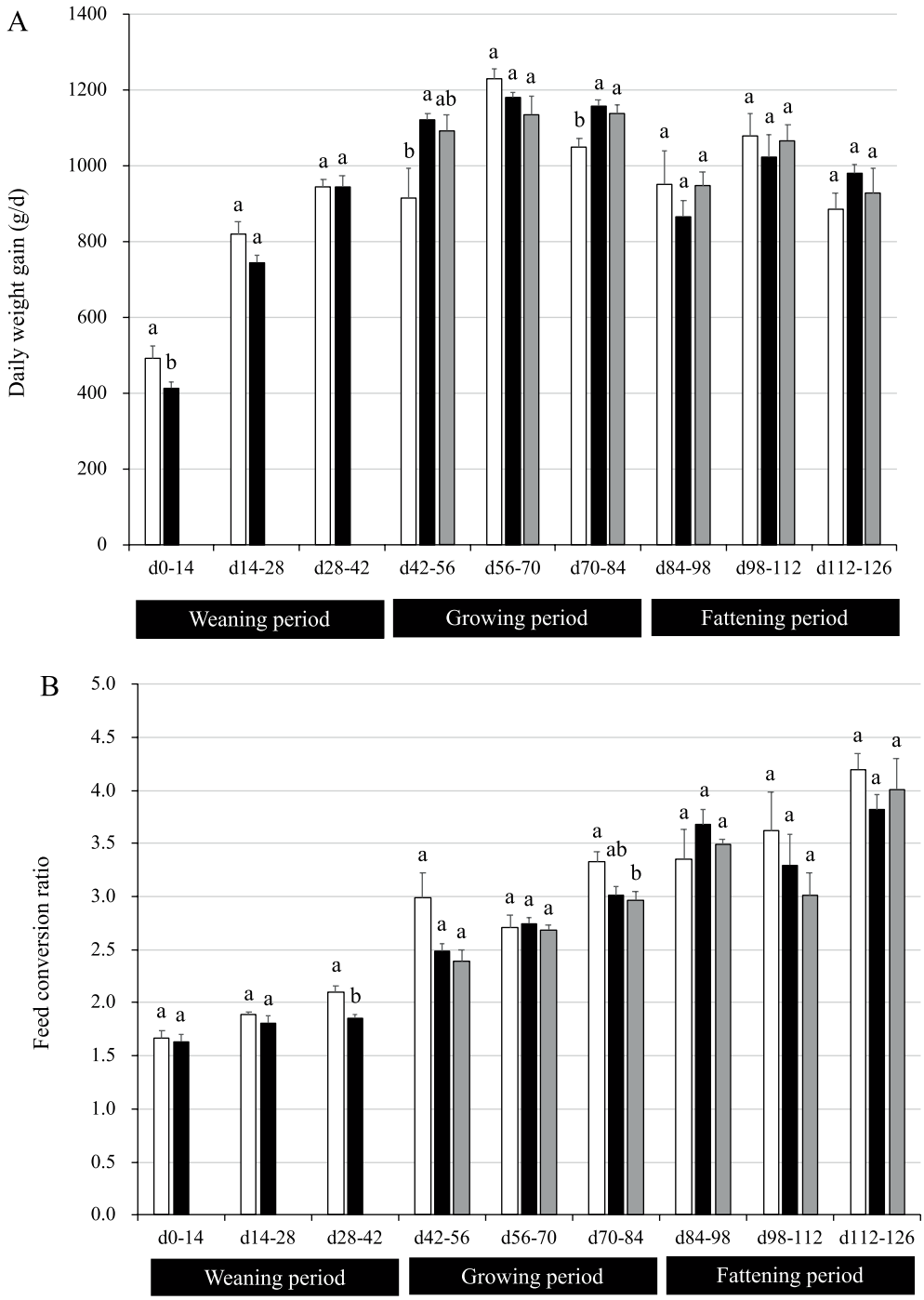

FIGURE 2
Growth performance of pigs with or without *H. coagulans* SANK70258 administration. Bars indicate the arithmetic means and error bars indicate the standard errors. Open bar, control (CC; $n = 4$); Close bar, pigs supplemented with *H. coagulans* SANK70258 at the weaning (P; $n = 8$) and growing and fattening (PP; $n = 4$) periods. Gray bar, pigs supplemented with *H. coagulans* SANK70258 at the weaning period but not at the growing and fattening periods (PC; $n = 4$). **(A)** Daily weight gain; **(B)** feed conversion ratio. The parameters in weaning period were analyzed to investigate the homoscedasticity by the F test. According to the results, either the Student's or the Welch's *t*-test was used to analyze differences between the groups. The parameters in growing and fattening periods were analyzed to investigate the homoscedasticity by the Bartlett test. According to the results, either a completely randomized design one-way analysis of variance or the Kruskal–Wallis test was used to analyze the differences between the groups. Either Tukey or Steel-Dwass *post-hoc* comparisons were used for multiple comparisons, as needed. Different letters indicate significant differences between groups ($p < 0.05$).

pigs used in the present study seemed to be within a normal range (not infected) throughout the study except for the day 126 (70–80 U/L in range).

The AST concentration in plasma did not change between groups during this study. However, the ALT concentrations significantly decreased in the blood plasma of pigs in groups P and

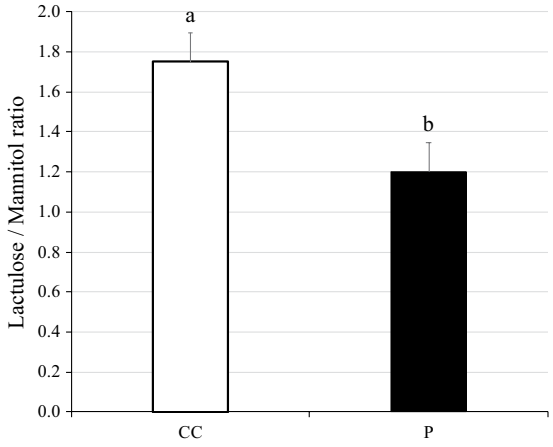

FIGURE 3
Lactulose/Mannitol ratio in plasma of weaning pigs with or without *H. coagulans* SANK70258 administration. Bars indicate the arithmetic means and error bars indicate the standard errors. Open bar, control (CC; $n = 8$); Close bar, pigs supplemented with *H. coagulans* SANK70258 (P; $n = 16$). The parameter was analyzed to investigate the homoscedasticity by the F test. According to the results, either the Student's or the Welch's *t*-test was used to analyze differences between the groups. Different letters indicate significant differences between groups ($p < 0.05$).

CC on day 28 ($p = 0.01$; Figure 4B). By day 126 (slaughter day), pigs in all pig groups had drastic increases of AST in blood plasma (Figure 4A).

3.5 Fatty acids composition in the longissimus dorsi muscles of fattening pigs

There were no differences in the triglyceride concentrations between groups (Figure 5A). However, when compared with pigs in the control group, the percentage of methyl stearate in the fatty acids of PP was higher than in the CC group ($p = 0.03$). In contrast, the percentage of methyl oleate in the fatty acids composition of pigs in group PP was lower than in the CC group ($p = 0.04$; Figure 5B). Nonetheless, no differences were observed in the percentage of methyl palmitate and methyl linoleate between groups.

3.6 Short chain fatty acid and lactate concentrations in cecal digesta and ileal and cecal vein

No differences were found in the concentrations of SCFA such as acetate, propionate, *n*-butyrate, *iso* -butyrate, *n*-valerate and *iso*-valerate in the cecal digesta between groups, although the concentration of lactate in cecal digesta was relatively high in pigs in the PP group ($p = 0.31$; Supplementary Figure S1). No differences were found in the SCFA concentrations in the plasma of ileal vein between groups. No differences were found in the acetate, propionate, *n*-butyrate, and *n*-valerate concentrations in the plasma of cecal vein between groups, although the concentrations of *iso*-butyrate and *iso*-valerate were lower in the blood in the cecal vein of pigs in the group PP than those of CC pigs significantly ($p = 0.01$ and 0.02, respectively).

3.7 Cecal microbiota of fattening pigs

No differences in the alpha diversity of the Chao 1 and Shannon indices of the cecal microbiota were detected between groups (Supplementary Figure S2).

At the genus level, the abundances of nine genera were significantly different (Supplementary Table S4). The relative abundance of genus *Subdoligranulum* was higher in the cecal digestas of pigs in the group PC than those in the other groups. The relative abundances of genera *Solobacterium, Butyricicoccus*, *Bifidobacterium* and *Lachnospiraceae*_ND3007_group were higher in the cecal digestas of pigs in the group PC than those in CC group. Order Enterobacterales unknown family and family *Pasteurellaceae* unknown genus were higher in pigs in the group PP than in the other groups. In addition, the relative abundance of family *Lachnospiraceae* unknown genus was lower in pigs in groups PP and PC than that in CC group. Uncultured family *Atopobiaceae* was higher in both groups PP and PC than that in CC group. The raw data of microbiome at the genus level is shown in the Supplementary Table S5.

3.8 Gene expression in the jejunal, ileal, and cecal mucosae

The gene expression levels in jejunal, ileal, or cecal mucosae of pigs in the present study are shown in Table 1. Gene expressions associated with antimicrobial peptide such as *PR39* and *PMAP23* in jejunal, ileal, and cecal mucosae did not change between groups. While the gene expressions associated with tight junction proteins such as *CLDN1*, *OCLN*, and *ZO1* in ileal and cecal mucosae did not change between groups, *ZO1* expression was more stimulated in the jejunal mucosae of PC pigs than in those of CC pigs. The gene expression associated with the IgA secretion such as *PIGR* in jejunal, ileal, and cecal mucosae did not change between groups. The gene expressions of cytokines such as *IFNG, IL6, IL10, IL17A, IL17F* in jejunal, ileal, and cecal mucosae did not change between groups. Nonetheless, in the jejunal mucosae, *IL22* expression was more stimulated in PC pigs than in that of CC pigs.

4 Discussion

The key mechanism of *H. coagulans* SANK70258 was "anti-inflammatory" and/or "suppression of inflammation" (23, 27). Previously, the administration of *H. coagulans* SANK70258 to broilers was shown to improve the growth performance of broilers infected with coccidiosis (21). Administering SANK70258 to broilers improved the immune (gene expressions of *IL10* and *TGFb*) and the barrier (gene expressions associated with tight junction protein) functions in the duodenal mucosa (23). We hypothesized that, similarly in pigs, the improvement of these functions could contribute to enhance the growth performance of pigs. Therefore, in the present study, we evaluated the growth performance of pigs with changes in the intestinal immune and barrier functions induced by SANK70258 administration.

In the present work, supplementation with SANK70258 during the weaning period did not significantly increase growth performance; on the contrary, for the first 14 days, pigs in group P had a lower DG

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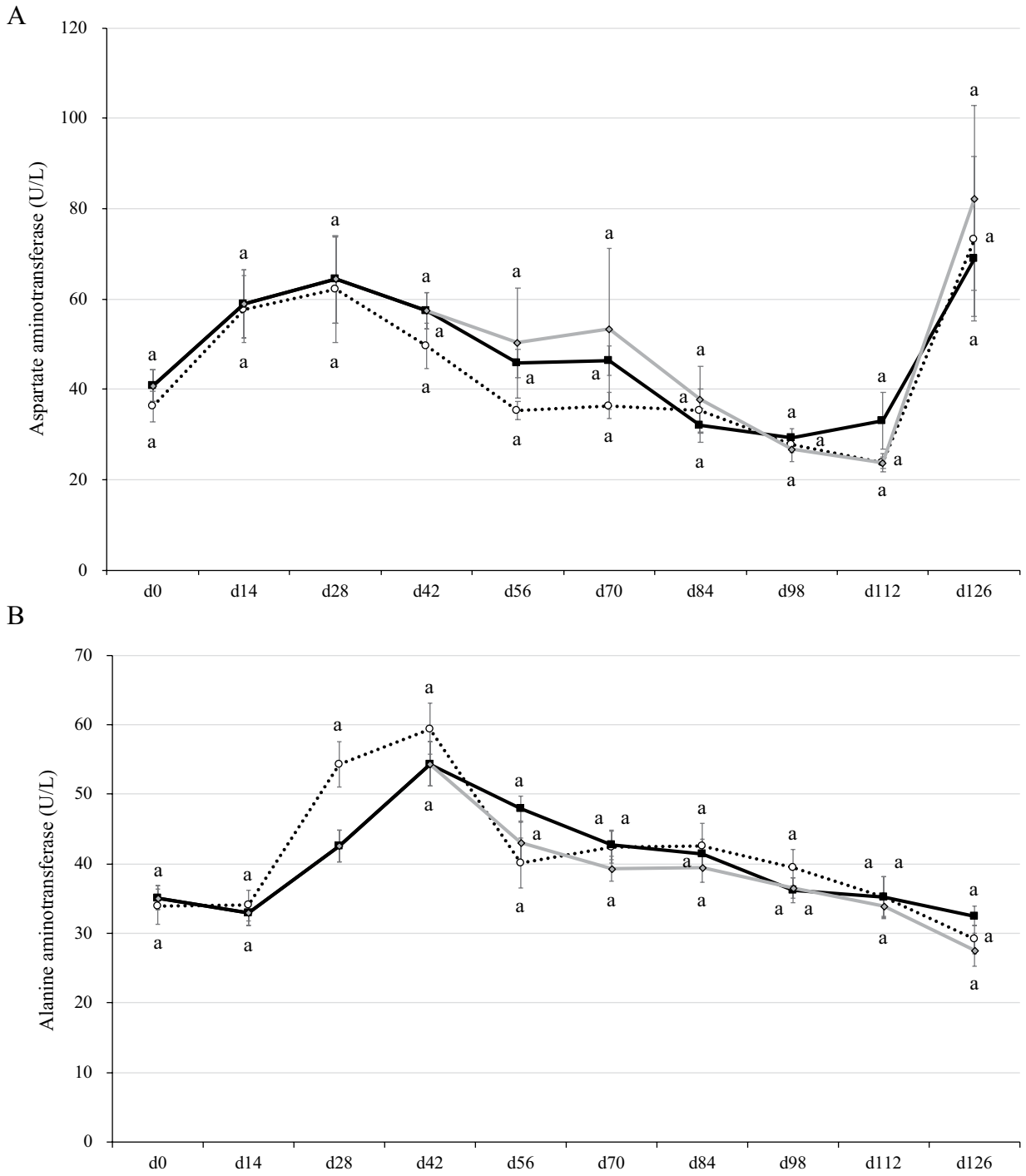

FIGURE 4
Aspartate and alanine aminotransferase concentrations in the plasma of pigs with or without *H. coagulans* SANK70258 administration. Plots indicate the arithmetic means and error bars indicate the standard errors. Dotted line, control (CC; $n = 8$); Black line, pigs supplemented with *H. coagulans* SANK70258 at the weaning (P; $n = 16$), growing and fattening (PP; $n = 8$) periods; Gray line, pigs supplemented with *H. coagulans* SANK70258 at the weaning period but not at growing and fattening periods (PC; $n = 8$). A control pig died on day 90. Afterwards, the means of CC group was calculated with 7 pigs. **(A)** Aspartate aminotransferase concentration; **(B)** alanine aminotransferase concentrations. The parameters in weaning period were analyzed to investigate the homoscedasticity by the F test. According to the results, either the Student's or the Welch's *t*-test was used to analyze differences between the groups. The parameters in growing and fattening periods were analyzed to investigate the homoscedasticity by the Bartlett test. According to the results, either a completely randomized design one-way analysis of variance or the Kruskal–Wallis test was used to analyze the differences between the groups. Either Tukey or Steel-Dwass *post-hoc* comparisons were used for multiple comparisons, as needed. Different letters indicate significant differences between groups ($p < 0.05$).

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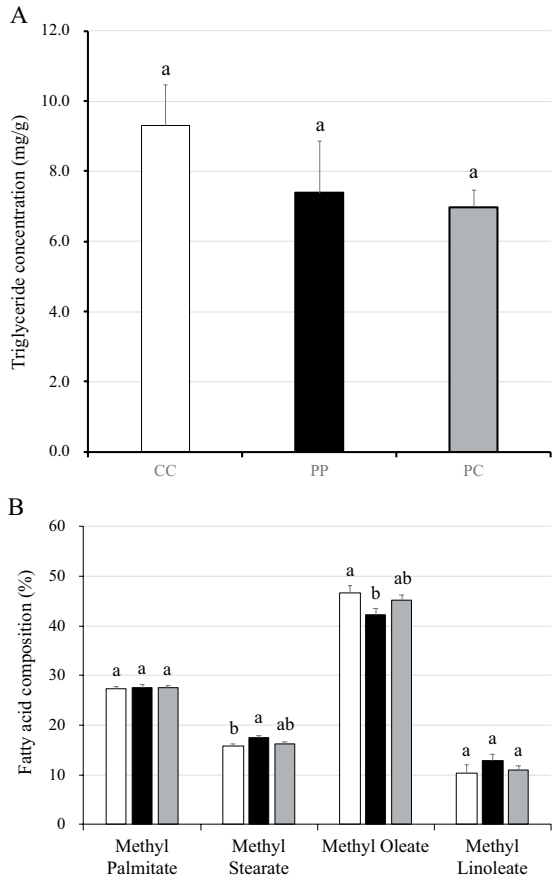

FIGURE 5
Triglyceride concentration and fatty acid composition in muscle of pigs with or without *H. coagulans* SANK70258 administration. Bars indicate the arithmetic means and error bars indicate the standard errors. Open bar, control (CC; $n = 7$); Close bar, pigs supplemented with *H. coagulans* SANK70258 (PP; $n = 8$). Gray line, pigs supplemented with *H. coagulans* SANK70258 at the weaning period but not at growing and fattening periods (PC; $n = 8$). **(A)** Triglyceride concentration in muscle; **(B)** fatty acid composition in muscle. The parameters were analyzed to investigate the homoscedasticity by the Bartlett test. According to the results, either a completely randomized design one-way analysis of variance or the Kruskal–Wallis test was used to analyze the differences between the groups. Either Tukey or Steel-Dwass *post-hoc* comparisons were used for multiple comparisons, as needed. Different letters indicate significant differences between groups ($p < 0.05$).

than control pigs. Usually, many pathogens infect commercial pigs during the weaning period. Therefore, weaned pigs are afflicted with a variety of diseases, not only gastrointestinal conditions such as colibacillosis, coccidiosis, and rotavirus infection (33), but also systemic diseases such as the porcine respiratory and reproductive syndrome (PRRS) and the porcine circovirus type 2 infection (49). To prevent pathogen infections, the acute inflammatory response is effective (35). For example, the inflammatory stimulation suppresses the PRRS virus replication in piglets (50). In our previous study, a postbiotic administration enhanced the inflammatory response and prevented colibacillosis in weaning pigs (51). On the other hand, in our previous work, SANK70258 was categorized as the anti-inflammation-inducing probiotic (23, 27). Suppression of the inflammatory response in enterotoxigenic *E. coli* challenged weaned pigs was associated with increased intestinal injury and clinical disease (52). We hypothesized that suppression of the inflammatory response induced by SANK70258 administration was also sensitive to the pathogens and/or harmful materials. Reduction of DG on days 0–14 by SANK70258 administration may have been caused by the same reason. Indeed, in the present study, although the administration of SANK70258 suppressed DG from day 0–14 (Figure 2A), the leaky gut condition improved during the weaning period (Figure 3). This effect (leaky gut improvement) was also observed in our previous broiler study (23). It is well known that weaning stress induces the leaky gut condition and this gut dysfunction causes disease susceptibility (53). An improvement of the leaky gut condition also caused an improvement in the FCR during days 28–42 in group P (Figure 2B). Leaky gut induces harmful materials translation such as pathogenic bacteria, toxins and harmful antigens (5, 54). These materials may affect the growth performance of pigs because these factors induce inflammation (55). Therefore, the outcome of the administration of SANK70258 is not always an improved growth performance but instead, an improvement of the leaky gut condition in weaning pigs.

During the growing period, supplementation with SANK70258 increased significantly the DG of pigs in PP group (Figure 2A). Previous work reported that increasing the supplementation of probiotics to pigs during the growing and fattening periods significantly improved DG (56). Pigs are less likely to be infected by pathogens during the growing period than during the weaning period (33). Furthermore, chronic inflammation, caused by stress and inflammation-inducing ingredients in feed, is always found in intensive livestock production systems; thus anti-inflammation-inducing materials are beneficial to promote growth performance in animals (35). In the present work the administration of SANK70258 stimulated an anti-inflammatory response and/or suppressed inflammatory responses (23, 27, 57), which were later reflected in the growth-promoting effect observed during the growing period because of the chronic inflammation downregulation (Figure 2A).

It has been previously shown that supplementation with probiotic *Clostridium butyricum* helped lower the ALT concentration in sera of growing pigs (58). In addition, Wang et al. (59) reported that supplementation with *Clostridium butyricum* and *Enterococcus faecalis* to weaned pigs lowered the levels of AST and ALT in the sera of weaned piglets. In the present study, there were no differences in the AST level between groups, although all pigs experienced a drastic increase by day 126. The cause of this increase remains unclear, but it can be speculated that pigs were infected with a pathogen by the end of the fattening period, because our previous study suggested that the pigs in the fattening period are likely to be infected by pathogens again in commercial swine farms (33). Furthermore, in the present study, a pig in CC group died of acute pleuropneumonia during the fattening period, which seems to supports our speculation. Although the infection was not substantial enough to make all animals in the present study symptomatically sick, acute inflammatory conditions were likely present in the fattening period, particularly on day 126.

Whereas AST is diffusely represented in the heart, skeletal muscle, kidneys, brain, and red blood cells, ALT has low concentrations in skeletal muscle and kidney. Therefore, an increase in ALT serum levels is more specific associated with liver damage (60). In the present work, the administration of SANK70258 helped lower the ALT concentration of weaned pigs in the P group on day 28 (Figure 3B). In our previous study with humans, consecutive 8-week

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TABLE 1 Gene expression levels in jejunal, ileal, and cecal mucosae.

Genes	Intestine	CC	PP	PC	*P*-value
Jejunum	*PR39*	4.05 ± 2.26a	11.43 ± 6.44a	8.90 ± 3.19a	0.80
	PMAP23	3.09 ± 1.68a	10.20 ± 5.19a	7.55 ± 2.84a	0.43
	CLDN1	1.05 ± 0.14a	1.32 ± 0.27a	1.75 ± 0.17a	0.07
	OCLN	1.03 ± 0.09a	1.79 ± 0.33a	1.75 ± 0.39a	0.20
	ZO1	1.04 ± 0.11b	1.36 ± 0.33ab	1.63 ± 0.08a	0.04
	PIGR	1.87 ± 0.82a	1.76 ± 0.72a	3.28 ± 0.64a	0.27
	IFNG	1.02 ± 0.08a	1.03 ± 0.15a	0.80 ± 0.11a	0.32
	IL6	0.80 ± 0.65a	0.29 ± 0.15a	0.59 ± 0.39a	0.90
	IL10	1.18 ± 0.25a	1.71 ± 0.47a	1.94 ± 0.34a	0.37
	IL17A	1.13 ± 0.23a	1.47 ± 0.33a	2.08 ± 0.27a	0.08
	IL17F	1.23 ± 0.29a	1.50 ± 0.32a	2.59 ± 0.48a	0.04
	IL22	1.10 ± 0.18b	1.52 ± 0.29ab	2.51 ± 0.48a	0.03
Ileum	*PR39*	0.94 ± 0.43a	4.50 ± 1.81a	6.07 ± 3.44a	0.14
	PMAP23	2.08 ± 0.86a	3.55 ± 1.00a	4.00 ± 1.71a	0.56
	CLDN1	1.29 ± 0.31a	0.91 ± 0.25a	2.56 ± 1.36a	0.73
	OCLN	1.14 ± 0.22a	0.82 ± 0.13a	2.01 ± 0.89a	0.43
	ZO1	1.03 ± 0.10a	0.84 ± 0.06a	1.14 ± 0.24a	0.34
	PIGR	1.50 ± 0.54a	0.96 ± 0.21a	1.82 ± 0.76a	0.86
	IFNG	1.25 ± 0.35a	0.78 ± 0.10a	1.08 ± 0.36a	0.77
	IL6	1.13 ± 0.50a	3.32 ± 0.79a	4.41 ± 1.42a	0.18
	IL10	1.15 ± 0.22a	1.13 ± 0.21a	3.30 ± 1.94a	0.93
	IL17A	1.12 ± 0.20a	0.96 ± 0.22a	3.50 ± 2.26a	0.71
	IL17F	1.30 ± 0.33a	1.15 ± 0.34a	2.61 ± 1.61a	0.83
	IL22	1.21 ± 0.27a	0.86 ± 0.16a	2.94 ± 1.77a	0.70
Cecum	*PR39*	1.67 ± 0.71a	2.28 ± 0.37a	6.93 ± 3.56a	0.55
	PMAP23	1.02 ± 0.31a	2.24 ± 0.79a	2.92 ± 1.37a	0.74
	CLDN1	1.28 ± 0.32a	0.98 ± 0.27a	1.68 ± 0.51a	0.43
	OCLN	1.25 ± 0.29a	1.20 ± 0.21a	3.19 ± 1.07a	0.53
	ZO1	1.14 ± 0.27a	0.73 ± 0.15a	0.75 ± 0.08a	0.10
	PIGR	1.16 ± 0.28a	1.07 ± 0.21a	0.83 ± 0.22a	0.60
	IFNG	1.12 ± 0.22a	1.23 ± 0.17a	1.79 ± 0.50a	0.60
	IL6	2.66 ± 1.54a	1.96 ± 1.12a	0.61 ± 0.33a	0.36
	IL10	1.56 ± 0.54a	1.10 ± 0.29a	2.17 ± 0.77a	0.41
	IL17A	1.33 ± 0.40a	0.96 ± 0.21a	2.07 ± 0.73a	0.74
	IL17F	1.31 ± 0.37a	0.98 ± 0.31a	2.08 ± 0.69a	0.28
	IL22	1.45 ± 0.47a	0.96 ± 0.21a	1.60 ± 0.48a	0.50

Different letters indicate significant differences between groups ($P < 0.05$). The parameters were analyzed to investigate the homoscedasticity by the Bartlett test. According to the results, either a completely randomized design one-way analysis of variance or the Kruskal–Wallis test was used to analyze the differences between the groups. Either Tukey or Steel-Dwass *post-hoc* comparisons were used for multiple comparisons, as needed.

SANK70258 administration induced a reduction of ALT but not AST (61) in the blood. It can be speculated that only the ALT reduction was the characteristic of this probiotic not only in humans but also in pigs. These results seemed to suggest that supplementation of SANK70258 improved the liver function of pigs, as injury of hepatocytes increases the level of ALT in the blood (62). Many strains of probiotic bacteria have already been applied to the non-alcoholic fatty liver disease and non-alcoholic steatohepatitis patients with AST and/or ALT reduction (18). An improving mechanism of liver dysfunction by SANK70258 was suggested as suppressing the leaky gut (12). It is well known that the leaky gut induces an inflammatory response in the liver (63), and the hepatic inflammation induces a higher ALT concentration in serum (64). SANK70258 was previously reported as an anti-inflammatory probiotic (23, 27, 57), thus, it can

be hypothesized that an improved liver function, as suggested by the decreased ALT levels in SANK70258-administered pigs on day 28, may have also meant an improved leaky gut. Another hypothesis of the improving mechanism was the stimulation of anti-oxidant parameters such as super oxide disumutase (SOD) in the liver. A different strain of *H. coagulans* stimulated the SOD activity in the small intestine (65) and liver (66) with improvement of the morphology and function of liver (66). Therefore, SANK70258 may also stimulate the anti-oxidant parameters. Nonetheless, since we did not measure the anti-oxidant, inflammatory and anti-inflammatory parameters in the blood and liver in the present study, this point needs further consideration.

The concentrations of stearate and oleate were higher and lower, respectively, in the *longissimus dorsi* muscles of pigs in the PP group than those of pigs in the PC and control groups (Figure 5). A higher concentration of stearate in pork meat can lower the serum cholesterol level (67). Moreover, oleate is also functional against the blood pressure (68). In the present work, while it remains unclear the reason a longer administration of SANK70258 modified the fatty acid composition in pork meat, it has been previously reported that the administration of certain probiotic bacteria such as *Lactobacillus johnsonii* increases the stearate levels in the fatty acid composition, with an upregulation of several lipid-related genes, including DGAT1, DGAT2, CD36, and PPARγ in the muscle (69). The effect of the administration of SANK70258 on the fatty acid lipid composition of pigs needs further elucidation.

The gene expressions of ZO1 and IL22 were higher in the jejuna of pigs in the group PC than in those in the control and PP groups (Table 1). Protein ZO1 is crucial for mucosal repair (70). IL22 is a member of the interleukin-10 family of cytokines (71). In addition, IL22 is involved in the innate host defense mechanisms against pathogens and plays a role in the protection of the barrier integrity by promoting repair, among other functions, of several tissues and organs (72). These parameters did not change in the pigs in group PP group. We can only speculate that this phenomenon may have been caused by a certain pathogen infection on day 126. For example, according to the microbiome analysis, significant increases in *Pasteurellaceae* and Enterobacterales were observed in group PP (Supplementary Table S4). These bacterial groups encompass potential pathogens. Indeed, *Pasteurellaceae* include genera *Mannheimia* and *Pasteurella* (73), and Enterobacterales include genera *Escherichia*, *Klebsiella*, *Salmonella*, and *Yersinia* (74). Our possible theoretical scenario is as follows. In the group PP, a pathogenic infection occurred in pigs, because the immune response was delayed by the continuous production of anti-inflammatory cytokines, which was caused by the administration of SANK70258. In contrast, in the bodies of pigs in groups CC and PC (pigs withdrawn at weaning from a continuous anti-inflammation response, which would have been induced by the administration of SANK70258), there was a faster immune response, which is the natural response under normal circumstances, and pathogens were quickly eliminated upon infection. Therefore, in the present work, there might have been confounding expressions of mucosal cytokines and the luminal microbiota in the fattening pigs of group PP, which was not observed in the other groups. Nonetheless, to fully elucidate this speculation, further investigation is recommended.

Some limitations were observed in this study. First, we designed a relatively small scale experiment ($n = 8$) to evaluate growth performance. Second, the growth performance of PP pigs in the fattening period became confounding with the induction of liver dysfunction. Third, only single analyses of lactulose/mannitol test were conducted on day 28 in the present study. Although strict effectiveness against leaky gut was evaluated, a pre-trial should be required to understand the internal leaky gut condition of individual pigs. Due to the above limitations, a study using SANK70258 to treat pigs from the weaning to the growing period with a sufficient scale is needed to fully understand the growth promoting effect of SANK70258 in the pigs.

5 Conclusion

The consecutive administration of SANK70258 improved the growth performance during the growing period. Pigs supplemented with SANK70258 only during the weaning period had also greater DG and lower FCR toward the end of the growing period. SANK70258-administered pigs experienced an improved liver function (as per the lower ALT levels observed) and hence, a putative improved leaky gut condition in the weaning period. As a result, these pigs also experienced an enhanced growth performance during the growing period.

Since SANK70258 is known as an anti-inflammation-inducing probiotic strain, we hypothesized that the promoting effect of growth performance by SANK70258 depended on the inflammation status of pigs. Weaned pigs are usually infected with many pathogens, therefore an acute inflammation response is required to prevent pathogen infections. The SANK70258 administration was not adequate for pigs during the acute-inflammation phase because SANK70258 suppressed the acute-inflammatory response. A consecutive SANK70258 administration during the growing period, a low risk stage of pathogen infection, resulted in a sustained anti-inflammatory response in the intestine, which may have been an additional positive effect for pigs in group PP. Nonetheless, during the fattening period, the positive effect of consecutive SANK70258 may have been confounded with the risk of pathogen infection again. To elucidate this confounding result during the growing period, it is recommended that further investigation be carried out in the future.

Data availability statement

The sequence data have been deposited in the NCBI Sequence Read Archive (SRA) under accession number PRJNA1193228. 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 approved by Ethical Committee of KYODOKEN Institute. The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

MA: Conceptualization, Data curation, Formal analysis, Validation, Writing – review & editing. RY: Conceptualization, Data curation, Funding acquisition, Project administration, Supervision, Validation, Writing – review & editing. TK: Data curation, Formal analysis, Investigation, Methodology, Software, Writing – review & editing. TM: Conceptualization, Data curation, Resources, Validation, Writing – review & editing. TT: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Validation, Visualization, Writing – original draft.

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Conflict of interest

MA, RY, and TM were employed by the Mitsubishi Chemical Corporation.

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

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Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2025.1537913/full#supplementary-material

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Effects of Pueraria extracts on growth performance, immune function, and immune-related gene expression of Wuzhishan piglets

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Pueraria extracts contain phytoestrogens, particularly isoflavones such as daidzein and genistein, which have been used in traditional Chinese medicine for centuries due to their various health benefits. Wuzhishan pig is a small pig unique to China, native to Wuzhishan, Hainan Province. Pueraria extracts have been widely utilized in the production of various animals. However, there are relatively few reports regarding their application in piglets, particularly in local pig breeds. The experiment aimed to investigate the effects of dietary Pueraria extracts on the growth performance, immune function, and immune-related gene expression of Wuzhishan piglets. Forty-eight piglets were randomly divided into four groups, each with six replicates and two piglets in each replicate. The control group (CON) was fed the basal diet, and the other groups were given 400, 600, and 800 mg/kg Pueraria extracts (PE1, PE2, and PE3, respectively). Compared with the CON, (1) the dietary Pueraria extracts increased the average daily gain and decreased the feed conversion ratio in PE2 and PE3 ($p < 0.05$); (2) the dietary Pueraria extracts increased the serum immunoglobulin M and complement 3 and 4 contents in PE2 ($p < 0.05$); (3) the serum ALB and ALP contents in groups treated with the Pueraria extracts were higher ($p < 0.05$); (4) the ALT and GOT contents were lower ($p < 0.05$) in groups treated with Pueraria extracts; (5) the dietary Pueraria extracts increased the Bacillus acidi lactici and Saccharomycetes contents, and decreased the *Escherichia coli* and Salmonella contents in the jejunum in PE1, PE2, and PE3 ($p < 0.05$); (4) the dietary Pueraria extracts ameliorated the relative expression of *Interleukin-10*, *Tumor necrosis factor-α*, *Mucoprotein 2*, *Transforming growth factor-β1*, *Zonula occludens-1* in jejunum mucosa ($p < 0.05$). In this study, adding 600 mg/kg dietary Pueraria extracts to the Wuzhishan piglets' diets improved the growth performance, immune function, and immune-related gene expression. This study laid a theoretical foundation for popularizing and applying Pueraria extracts in local pig production.

KEYWORDS

Pueraria extracts, Wuzhishan piglets, growth performance, immune function, immune-related gene expression

1 Introduction

Plant extracts, recognized for their natural and safe properties, have increasingly become a valuable feed additive in livestock and poultry breeding, driven by their wide-ranging benefits (1–3). These benefits extend across multiple dimensions of animal production, such as enhancing growth performance, boosting immune function, improving carcass traits, and elevating meat quality (2, 3). In piglet nutrition, plant extracts have garnered particular attention in recent years. Research has shown that specific plant extracts can optimize piglets' growth performance and overall health by modulating gut microbiota and enhancing immune responses (4, 5). Essential oils derived from plants like oregano and thyme have demonstrated antimicrobial properties, which can effectively reduce the incidence of gastrointestinal infections in piglets, potentially diminishing the reliance on antibiotics (6). Additionally, Galanopoulos *et al.* explored the antioxidant and anti-inflammatory effects of plant extracts, such as flaxseed oil, which can improve systemic and gut immunity in piglets (7). These findings underscore the necessity of further investigating the applications of plant extracts in piglet nutrition to foster sustainable and healthy livestock production practices. Pueraria extracts are derived from the root of the *Pueraria lobata* plant, native to parts of Asia, including China, Japan, and Korea (8–10). Pueraria extracts contain phytoestrogens, particularly isoflavones such as daidzein and genistein, which have been used in traditional Chinese medicine for centuries due to their various health benefits (9, 11, 12). Wang et al. (13) found that Puerarin improves the immune response, antioxidant capacity, and intestinal morphology of pigeons. Liu et al. (14) reported that the Pueraria crude extracts significantly changed the fecal microbiota of finishing pigs. Guo et al. (15) found that the Pueraria extracts enhanced the antioxidant status and intestinal integrity of broilers. Zhang et al. (16) studied the *Pueraria lobata* extracts, which remediated intestinal dysbiosis and promoted bile acid biosynthesis. Pueraria extracts have been widely utilized in the production of various animals. However, there are relatively few reports regarding their application in piglets, particularly in local pig breeds.

The Wuzhishan pig is a diminutive swine species endemic to China, originating specifically from Wuzhishan in Hainan Province (17). Characterized by its small stature and robust and compact physique, this pig has a relatively small and slightly elongated head, small and thin ears, and an irregular black and white fur distribution. Most individuals exhibit a black back and a white belly, earning them the colloquial name mouse pig (18, 19). The Wuzhishan pig is highly inbred, rendering it an invaluable genetic resource for elucidating the genetic underpinnings of various traits. Owing to its anatomical, physiological, and pathological similarities to humans, it is an ideal biomedical research model. Moreover, the Wuzhishan pig is a crucial breed for the development of high-quality pork products. Its unique characteristics and potential applications underscore the practical significance of studying this pig breed.

The primary aim of this study is to systematically investigate the multifaceted impacts of Pueraria extracts on Wuzhishan piglets. Specifically, we hypothesize that Pueraria extracts can significantly enhance growth performance metrics, such as weight gain and feed efficiency. Additionally, we posit that these extracts can bolster immune function by upregulating key immune-related genes and improving biochemical blood indicators. To achieve these goals, the study will (a) meticulously assess growth parameters and immune responses in piglets administered varying doses of Pueraria extracts; and (b) evaluate the practical applicability of Pueraria extracts in piglet production settings through pilot trials. By focusing on these targeted objectives, the study aims to provide robust scientific evidence supporting the integration of Pueraria extracts into pig production practices, thereby laying a solid theoretical foundation for their widespread adoption.

2 Materials and methods

2.1 Experiment material

Forty-eight Wuzhishan piglets (sows or castrated boars) with an average body weight of 10.00 ± 1.00 kg, the same genetic background, and similar birth dates were used.

Pueraria extracts (PE) were purchased from Shaanxi Baichuan Biotechnology Co., LTD., Xian, China. The active ingredient contents were 4.37% puerarin, 1.62% daidzin, 1.05% puerarin apigenin, and 0.27% genistin, detected in Liquid chromatography–tandem mass spectrometry.

Serum biochemical kits were purchased from Shanghai Sangon Biotechnology Co., Ltd., Shanghai, China.

2.2 Experiment design and sample collection

The amount of Pueraria extracts added was a single influencing factor in this study. Forty-eight Wuzhishan piglets were randomly allocated into four groups, with six replicates and two piglets per replicate. The basal experimental diets for piglets were corn-soybean meal-type diets formulated according to the NCR (2012) standards, and the composition of the basal experimental diets and nutritional levels are shown in Table 1. The control group (Con) was fed basal experimental diets, and the experimental groups were fed 400, 600, and 800 mg/kg Pueraria extracts in powder (PE1, PE2, and PE3, respectively) according to recommended usage. The experiment period was 90 days. The piglet house was maintained at a temperature of 28–30°C and 60–70% humidity. Lighting was provided for 16 h daily. Fecal waste was cleared twice daily, and the house was disinfected weekly with a 0.10% povidone-iodine solution to ensure hygiene.

At the end of the experiment, all piglets were given 12 h of forbidden feeding and were allowed to drink freely. We chose one piglet that was close to the average weight for slaughter from each replicate. We put one piglet that will be euthanized into a carbon dioxide euthanasia chamber (LC-800-S1, 80 × 70 × 60 cm^3, Shanghai, China). Once it is calm, the air in the chamber will be replaced with a mixture of 90% carbon dioxide and 10% air at a rate of 15% of the chamber's volume per minute until the piglet is in a deep state of unconsciousness. The entire process is expected to take about 45 min. Before slaughter, five mL of blood was collected from each pig's ear vein and centrifuged at 3000 r/min for 15 min. The super serum was collected and divided into 1.50 mL Ep tubes and stored at −20°C for later use. Two grams of jejunum contents were taken from each piglet in 2.00 mL frozen storage tubes and stored in a − 80°C refrigerator for future use. Another section of the jejunum was taken and cut lengthwise to expose the intestinal cavity. Sterile saline was used to clean the intestinal cavity and gently remove the surface intestinal

TABLE 1 Composition (kg/100 kg) of the basal experimental diets[1] for Wuzhishan piglets.

Ingredients	Contents	Nutrient levels, on an air-dry basis	Contents
Corn, %	32.80	Digestible energy[3], DE, MJ/kg	13.61
Soybean meal, %	15.40	Crude protein[4], CP, %	18.00
Wheat, %	8.00	Calcium[4], Ca, %	0.95
Low protein whey powder, %	6.00	Total Phosphorus[4], P, %	0.75
Fish meal, %	3.00	Available phosphorus[4], AP, %	0.53
Corn gluten meal, %	10.00	Lysine[4], Lys, %	1.25
Wheat bran, %	3.00	Methionine[4], Met, %	0.35
Soybean oil, %	5.00	Threonine[4], Thr, %	0.78
Mountain flour, %	0.80	Tryptophan[4], Try, %	0.29
Calcium hydrogen phosphate, %	2.00		
Sodium chloride, %	0.30		
L-Lysine hydrochloride, %	0.60		
Threonine, Thr, %	0.10		
Tryptophan, Try, %	0.10		
Saccharose, %	2.50		
Glucose, %	2.50		
Premix[2], %	2.00		
Feed mold inhibitor, %	1.00		
Sweetening agent, %	0.30		
Zeolite powder, %	4.60		
Total, %	100.00		

[1]Based on the NRC (2012) nutrient requirements for piglets.
[2]The premix provided the following per kg of diet: VA 2,000 IU, VD 200 IU, VE 45.00 IU, VK 0.50 mg, VB_1 1.00 mg, pantothenic acid 12.00 mg, nicotinic acid 10.25 mg, VB_6 3.85 mg, VB_{12} 15.00 ug, folic acid 1.35 mg, biotin 0.21 mg, VC 200 mg, Mn (Manganese sulfate) 20.00 mg, Fe (Ferrous sulfate) 80.00 mg, Cu (Copper sulfate) 5.00 mg, I (Potassium iodide) 0.14 mg, Se (Sodium selenite) 0.15 mg.
[3]Calculated value (NRC, 2012).
[4]Analysed content.

contents. A sterile slide was used to scrape the intestinal mucosa gently, and this process should avoid penetrating the basement membrane. The intestinal mucosa was stored in 2.00 mL frozen storage tubes and placed in a − 80°C refrigerator for later use.

2.3 Growth performance

The feed intake of piglets was carefully recorded. The amount of feed offered and the amount wasted by the piglets in each replicate were precisely measured and recorded daily. The average daily feed intake (ADFI) was calculated by subtracting the weight of leftover feed from the initial feed provided. Additionally, each pig was weighed at the end of the experiment to determine the average daily gain (ADG). ADFI, g/d = Total feed intake, g/ Experiment period, d. ADG, g/d = (Final weight, kg - Initial weight, kg) * 1,000/Experiment period, d. Feed conversion ratio (FCR) = ADFI, g/d/ADG, g/d.

2.4 Immune function

The immunoglobulin A (IgA), immunoglobulin G (IgG), immunoglobulin M (IgM), complement 3 (C3), complement 4 (C4), total protein (TP), albumin (ALB), alkaline phosphatase (ALP), alanine transaminase (ALT), glutamic oxalacetic transaminase (GOT), and urea nitrogen (UN) in the serum were determined using corresponding commercial assay kits, Shanghai Sangon Biotechnology Co., Ltd., Shanghai, China. The determining process is strictly according to the kit instructions.

2.5 Jejunal microorganism

In a sterile super-clean workbench, two grams of jejunum contents were taken and diluted to 10^{-7} with sterile normal saline. Then the total bacteria, *Escherichia coli*, Bacillus acidi lactici, Salmonella, and Saccharomycetes were identified and cultured with LB culture, Macconkey AGAR culture, MRS culture, SS AGAR culture with brilliant green, and Potato glucose AGAR with antibiotics, respectively. The number of various microorganisms was counted using the plate counting method.

2.6 RNA extraction and quantitative analysis of mRNA with real-time PCR

Jejunum mucosa samples of soybean size were taken, and RNA was extracted according to the instructions of the animal tissue RNA

TABLE 2 Primer sequence list.

Gene	Gene name	Forward and reverse primers	Product size	Accession No.
IL-10	Interleukin-10	F:5'-GTGGCAGCCAGCATTAAGTC-3′	100	NM_214041.1
		R:5'-AACTCTTCACTGGGCCGAAG-3'		
TNF-α	Tumor necrosis factor-α	F: 5'-CCAGACCAAGGTCAACCTCC-3'	89	NM_214022.10
		R: 5'-TCCCAGGTAGATGGGTTCGT-3'		
MUC2	Mucoprotein 2	F: 5'-CTGTGCGACTACAACTTCGC-3'	142	XM_021082584.1
		R: 5'-AGATGGTGTCGTCCTTGACC-3'		
TGF-β1	Transforming growth factor-β1	F: 5'-GGACCTTATCCTGAATGCCTT-3'	134	XM_021093503.1
		R: 5'-TAGGTTACCACTGAGCCACAAT-3'		
ZO1	Zonula occludens-1	F: 5'-CTGAGGGAATTGGGCAGGAA-3'	178	XM_021098896.1
		R: 5'-TCACCAAAGGACTCAGCACG-3'		
PCNA	Proliferating Cell Nuclear Antigen	F: 5'-GTGATTCCACCACCATGTTC-3'	123	NM_001291925.1
		R: 5'-TGAGACGAGTCCATGCTCTG-3'		
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	F: 5'-GTCGGAGTGAACGGATTTGG-3'	76	NM_001206359.1
		R: 5'-CAATGTCCACTTTGCCAGAGTTAA-3'		

extraction kit. The quality of RNA was assessed by a 2% agarose gel electrophoresis. An ultra-microspectrophotometer (NanoPhotometer, Implen German) was used to evaluate the total RNA concentration and purity (A260/A280 ratio). The primer sequences are shown in Table 2, and the primers corresponding to the sow gene sequence were synthesized using Sangon (Shanghai, China). The cDNA was synthesized by PrimeScript® RT regent Kit With gDNA Eraser (TaKaRa, Dalian, China) reverse transcription kit. DNA was removed from the sample using a reaction system as 2 μL 5 × gDNA Eraser Buffer, 1 μL gDNA Eraser, 1 μg RNA, then replenished the volume with RNase Free ddH_2O to 10 μL (20). The system of reverse transcription was 10 μL reaction liquid, 1 μL RT Primer Mix, 4 μL RNase Free ddH_2O, 4 μL 5 × PrimeScript® Buffer 2, PrimeScript® RT Enzyme MixI. The reaction procedure was 37°C constant temperature for 15 min, 85°C constant temperature for 5 s; 4°C store briefly. The obtained cDNA was stored at −20°C (17). The housekeeping gene of *Glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*) was used and the relative mRNA levels of *Interleukin-10* (*IL-10*), *Tumor necrosis factor-α* (*TNF-α*), *Mucoprotein 2* (*MUC2*), *Transforming growth factor-β1* (*TGF-β1*), *Zonula occludens-1* (*ZO1*), *Proliferating Cell Nuclear Antigen* (*PCNA*) were calculated using the $2^{-\Delta\Delta Ct}$ method.

2.7 Statistical analysis

Statistical analyses were conducted using SPSS 29.0 statistics software (NY, USA). The sample size for this study was determined based on the anticipated effect size, statistical power, and significance level. Drawing on prior research and pilot data, we set the expected effect size at 0.50, the significance level at 0.05, and the statistical power at 0.80. Utilizing G*Power software for the calculation, we determined that a minimum of six piglets per group was required. Accordingly, the study was structured with six replicates per group, each comprising two piglets, for a total of 48 piglets. This design ensures the robustness of our findings and achieves the desired statistical power. The Kolmogorov–Smirnov test was used to check if all data in this study followed a normal distribution. Data were expressed as mean ± SEM. Statistical comparisons between treatments were conducted using one-way ANOVA or Welch ANOVA following Kolmogorov–Smirnov and variance homogeneity tests (Barteet's or Levene's test). To mitigate the risk of Type I errors due to multiple comparisons, we employed the Bonferroni correction method to adjust the *p*-values. The test results of all analyses were considered significant at $p < 0.05$.

3 Results

3.1 Effects of Pueraria extracts on the growth performance of Wuzhishan piglets

There were no significant differences ($p > 0.05$) in initial weight and ADFI among groups. The final weight and ADG were higher ($p < 0.05$), and the FCR was lower ($p < 0.05$) in PE2 and PE3 compared with the Con. (Table 3).

3.2 Effects of Pueraria extracts on serum immune indices of Wuzhishan piglets

There was no significant difference ($p > 0.05$) in lgA contents among groups. The lgG contents in PE3 were higher ($p < 0.05$) compared with the Con. The lgM and C3 contents in PE2 and PE3 were higher ($p < 0.05$) than in the Con. The C4 contents were higher ($p < 0.05$) among groups treated with Pueraria extracts compared with the Con. (Table 4).

3.3 Effects of Pueraria extracts on serum biochemical indices of Wuzhishan piglets

The serum TP and UN contents among groups were not significantly different ($p > 0.05$). The serum ALB and ALP contents in groups treated with the Pueraria extracts were higher ($p < 0.05$) compared with the

TABLE 3 Effects of Pueraria extracts on the growth performance of Wuzhishan piglets.

Items	Con	PE1	PE2	PE3	*P*-value
Initial weight, kg	10.00 ± 1.01	10.00 ± 0.98	10.00 ± 0.96	10.00 ± 1.02	0.1206
Final weight, kg	24.62 ± 0.35^{b}	25.12 ± 0.46^{b}	26.56 ± 0.16^{a}	26.51 ± 0.22^{a}	0.0352
ADFI, g/d	583 ± 10.26	580 ± 10.26	570 ± 6.92	572 ± 8.12	0.2315
ADG, g/d	162 ± 5.76^{b}	168 ± 3.05^{b}	184 ± 5.06^{a}	183 ± 6.23^{a}	0.0238
FCR	3.60 ± 0.12^{a}	3.45 ± 0.10^{a}	3.10 ± 0.10^{b}	3.12 ± 0.11^{b}	0.0389

^{a,b}Values with different small letter superscripts in the same column mean a significant difference ($p < 0.05$). ADFI: average daily feed intake; ADG: the average daily gain; FCR: feed conversion ratio.

TABLE 4 Effects of Pueraria extracts on serum immune indice of Wuzhishan piglets.

Items	Con	PE1	PE2	PE3	*P*-value
IgA, g·L^{-1}	0.03 ± 0.01	0.04 ± 0.01	0.04 ± 0.02	0.03 ± 0.02	0.0968
IgG, g·L^{-1}	3.12 ± 0.21^{c}	3.56 ± 0.29^{b}	3.97 ± 0.12^{ab}	4.13 ± 0.17^{a}	0.0452
IgM, g·L^{-1}	0.88 ± 0.07^{b}	0.90 ± 0.03^{b}	1.12 ± 0.01^{a}	1.11 ± 0.02^{a}	0.0311
C3, mg·L^{-1}	30.52 ± 1.56^{b}	31.99 ± 2.01^{b}	36.25 ± 1.26^{a}	36.86 ± 1.37^{a}	0.0205
C4, mg·L^{-1}	26.59 ± 1.23^{b}	31.26 ± 1.56^{a}	32.23 ± 1.75^{a}	32.46 ± 2.01^{a}	0.0289

^{a,b,c}Values with different small letter superscripts in the same column mean a significant difference ($p < 0.05$). IgA, immunoglobulin A; IgG, immunoglobulin G; IgM, immunoglobulin M; C3, complement 3; C4, complement 4.

TABLE 5 Effects of Pueraria extracts on serum biochemical indices of Wuzhishan piglets.

Items	Con	PE1	PE2	PE3	*P*-value
TP, g·L^{-1}	80.17 ± 16.03	83.23 ± 8.95	81.19 ± 9.12	83.05 ± 10.12	0.0859
ALB, g·L^{-1}	36.03 ± 2.12^{b}	39.29 ± 1.06^{a}	39.72 ± 1.07^{a}	39.00 ± 2.61^{a}	0.0416
ALP, U·L^{-1}	230 ± 5.12^{c}	270 ± 10.56^{b}	262 ± 10.39^{b}	290 ± 6.31^{a}	0.0357
ALT, U·L^{-1}	99.02 ± 5.87^{a}	80.12 ± 6.95^{b}	76.25 ± 6.12^{bc}	67.19 ± 4.12^{c}	0.0267
GOT, U·L^{-1}	190 ± 10.12^{a}	156 ± 10.92^{b}	120 ± 10.11^{c}	117 ± 9.32^{c}	0.0379
UN, mmol·L^{-1}	5.87 ± 0.42	5.32 ± 1.25	5.27 ± 2.17	5.00 ± 2.29	0.9150

^{a,b,c}Values with different small letter superscripts in the same column mean a significant difference ($P < 0.05$). TP, total protein; ALB, albumin; ALP, alkaline phosphatase; ALT, alanine transaminase; GOT, glutamic oxalacetic transaminase; UN, urea nitrogen.

TABLE 6 Effects of Pueraria extracts on jejunum flora of Wuzhishan piglets.

Items	Con	PE1	PE2	PE3	*P*-value
Total bacteria, lgCFU·g^{-1}	11.62 ± 0.18	11.65 ± 0.12	11.62 ± 0.16	11.63 ± 0.25	0.0951
Escherichia coli, lgCFU·g^{-1}	6.59 ± 0.25^{a}	5.56 ± 0.26^{b}	5.00 ± 1.13^{c}	5.02 ± 1.09^{c}	0.0326
Bacillus acidi lactici, lgCFU·g^{-1}	6.24 ± 0.16^{c}	7.15 ± 0.19^{b}	7.20 ± 0.20^{b}	7.56 ± 0.18^{a}	0.0215
Salmonella, lgCFU·g^{-1}	5.12 ± 0.08^{a}	4.67 ± 0.10^{b}	4.32 ± 0.09^{c}	4.33 ± 0.01^{c}	0.0456
Saccharomycetes, lgCFU·g^{-1}	5.00 ± 0.16^{c}	5.36 ± 0.10^{b}	5.69 ± 0.12^{a}	5.72 ± 0.14^{a}	0.0411

^{a,b,c}Values with different small letter superscripts in the same column mean a significant difference ($P < 0.05$).

Con. The ALT and GOT contents were lower ($p < 0.05$) in groups treated with Pueraria extracts than in the Con. (Table 5).

3.4 Effects of Pueraria extracts on the jejunum flora of Wuzhishan piglets

There was no significant difference ($p > 0.05$) in total bacteria contents among groups. The *Escherichia coli* and Salmonella contents among groups treated with Pueraria extracts were lower ($p < 0.05$) compared with the Con. The Saccharomycetes and Bacillus acidi lactici contents among groups treated with Pueraria extracts were higher ($p < 0.05$) than in the Con. (Table 6).

3.5 Effects of Pueraria extracts on expression of immune-related genes in Wuzhishan piglets

The relative expressions of *IL-10*, *ZO1*, and *MUC2* in jejunum mucosa in groups treated with Pueraria extracts were higher ($p < 0.05$) than those in the Con, and the relative expressions of *IL-10* and *ZO1*

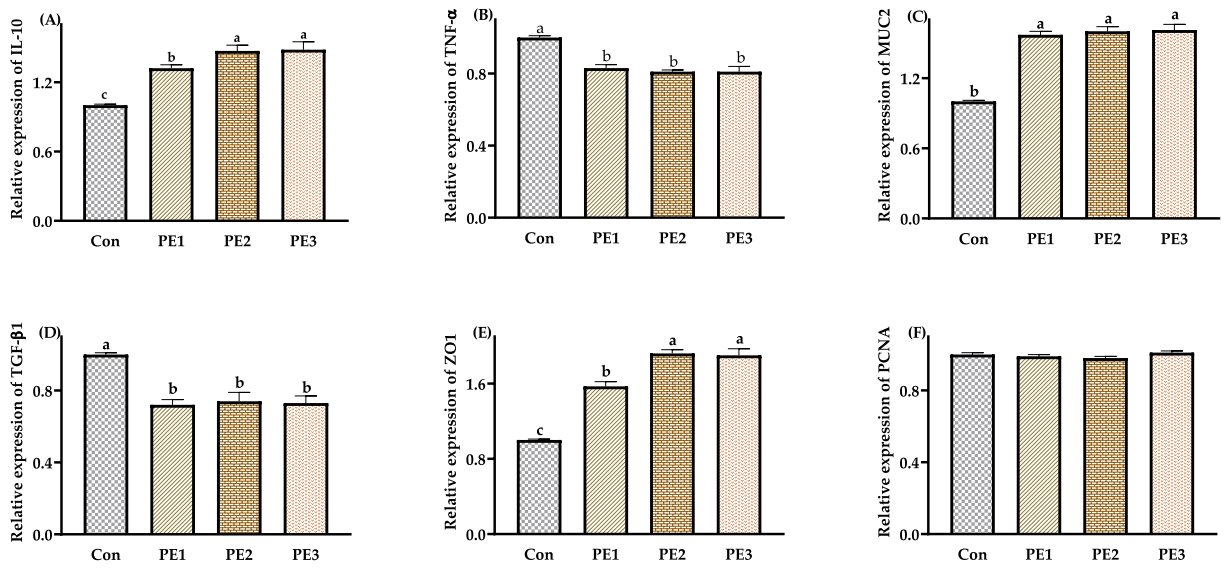

FIGURE 1
Effects of Pueraria extract on the expression of immune-related genes in jejunum mucosa of Wuzhishan piglets. **(A)** The data of relative expression levels of *IL-10* (*Interleukin-10*) in Con, PE1, PE2, and PE3 were 1.00 ± 0.01^{c}, 1.32 ± 0.03^{b}, 1.47 ± 0.05^{a}, 1.48 ± 0.07^{a}, individually, p = 0.0237; **(B)** The data of relative expression levels of *TNF-α* (*Tumor necrosis factor-α*) in Con, PE1, PE2, and PE3 were 1.00 ± 0.01^{a}, 0.83 ± 0.02^{b}, 0.81 ± 0.01^{b}, 0.81 ± 0.02^{b}, individually, p = 0.0429; **(C)** The data of relative expression levels of *MUC2* (*Mucoprotein 2*) in Con, PE1, PE2, and PE3 were 1.00 ± 0.01^{b}, 1.57 ± 0.03^{a}, 1.60 ± 0.04^{a}, 1.61 ± 0.05^{a}, individually, p = 0.0436; **(D)** The data of relative expression levels of *TGF-β1* (*Transforming growth factor-β1*) in Con, PE1, PE2, and PE3 were 1.00 ± 0.01^{a}, 0.97 ± 0.04^{b}, 0.72 ± 0.03^{c}, 0.74 ± 0.05^{c}, individually, p = 0.0268; **(E)** The data of relative expression levels of *ZO1* (*Zonula occludens-1*) in Con, PE1, PE2, and PE3 were 1.00 ± 0.01^{c}, 1.57 ± 0.05^{b}, 1.92 ± 0.04^{a}, 1.90 ± 0.07^{a}, individually, p = 0.0357; **(F)** The data of relative expression levels of PCNA (*Proliferating Cell Nuclear Antigen*) in Con, PE1, PE2, and PE3 were 1.00 ± 0.01, 0.99 ± 0.01, 0.98 ± 0.01, 1.01 ± 0.01, individually, p = 0.0689. ^{a,b,c}Values with different small letter superscripts in the same column mean a significant difference (p < 0.05).

in jejunum mucosa were higher (p < 0.05) in PE2 and PE3 compared with PE1. The relative expressions of *TNF-α* and *TGF-β1* in jejunum mucosa were lower (p < 0.05) in PE1, PE2, and PE3 compared with the Con. There was no significant difference (p > 0.05) in the relative expressions of *PCNA* in jejunum mucosa among groups (Figure 1).

4 Discussion

Growth performance is a key indicator of animal growth, which is significant for evaluating animal growth environment and feed nutrition (21–23). The isoflavones in Pueraria extract, such as puerarin, daidzein, and genistein, have essential medicinal value; they have anti-inflammatory, antioxidant, anti-tumor, and improve immunity (24, 25). Guo et al. (15) found that the Pueraria extract supplementation decreased the average daily gain during the whole period and improved the feed conversion ratio of broiler chickens. Gong et al. (26) added the *Pueraria lobata* leaf powder to the feed of broiler chickens and found it did not affect the growth and slaughter performance. Wang et al. (13) found that the puerarin treatment did not significantly alter the growth performance of pigeons. Tiyasatkulkovit et al. (27) studied the effect of Pueraria Mirifica extract and puerarin on the healthy growth of primary baboon osteoblasts. In this study, the Pueraria extract improved the final body weight, the average daily gain, and the feed conversion ratio of Wuzhishan piglets, possibly because Pueraria extract affects mammals more than birds. At the same time, the isoflavones in Pueraria extracts may improve the growth performance of pigs through multiple mechanisms, such as regulating metabolic pathways and hormone levels, improving gut health, and exerting antioxidant and anti-inflammatory effects.

The animal immune system is a set of complex biological mechanisms that enable the animal to recognize and defend against foreign pathogens, such as bacteria, viruses, parasites, etc., while also removing damaged or aging cells in the body and maintaining the stability of the internal environment (28–30). Plant extracts have received attention for their naturalness, versatility, and potential animal health and immunity benefits. They contain various bioactive substances, such as alkaloids, polyphenols, flavonoids, polysaccharides, and volatile oils, which are closely related to immune function (2, 3). Immunoglobulins are a class of globulins with antibody activity or molecular chemical structure similar to antibodies, and they play an essential role in the animal immune system. B lymphocytes secrete immunoglobulin after being stimulated by an antigen, mainly in serum, body fluid, exocrine fluid, and some cell membranes, and have the efficacy and effect of anti-infection, improving immunity, and preventing and treating diseases (31–33). The complement C3 and C4 in serum are critical immune components that play a key role in animal immune response and inflammation. Complement C3 is the most abundant complement component in serum, while C4 is involved in the classical activation pathway of complement, and their content can reflect inflammation and immune system problems (34–36). Gong et al. (26) found that *Pueraria lobata* leaf powder improved the thymus organ index, antioxidant performance, and immune functions of broiler chickens. Wang et al. (13) found that the puerarin treatment afforded a significant linear enhancement in the thymus index and significantly increased serum immunoglobulin A and immunoglobulin M levels in pigeons in a linear manner of pigeons.

Jung et al. (37) studied the effects of *Lactobacillus casei* HY2782 and *Pueraria lobata* root extract complex, and they found that it significantly reduced Th2/Th17-derived cytokines, immunoglobulin E, and leukotriene C4 in bronchoalveolar lavage fluid and serum in mice. In this study, the Pueraria extract improved the serum immunoglobulins (G and M) and complements (C3 and C4) of Wuzhishan piglets, which suggests that Pueraria extract can improve the immune performance of piglets by increasing the immunoglobulins and complements in the body.

Albumin, alkaline phosphatase, alanine aminotransferase, and glutamic oxalate aminotransferase are all critical indicators of animal immune function (38–40). Albumin is the most abundant protein in plasma, which can combine with many small insoluble molecules of organic matter and inorganic ions in the body to form soluble complexes and become the transport form of these substances in blood circulation (38). Albumin levels can reflect the nutritional status and immune function in animals, and low levels may indicate malnutrition or impaired immune function (39). Alkaline phosphatase is a nonspecific phosphohydrolase that can catalyze the hydrolysis of monophosphate and transfer phosphate groups (40). Alanine aminotransferase and glutamic oxalate aminotransferase are commonly used to assess liver health because they are highly active in liver cells (40). When liver cells are damaged, these enzymes are released into the blood, resulting in elevated serum aminotransferase activity (39, 40). In this study, the Pueraria extracts increased the albumin and alkaline phosphatase contents, and they decreased alanine aminotransferase and glutamic oxalate aminotransferase contents, which suggests that Pueraria extract has the function of protecting the animal liver, thereby directly or indirectly improving animal immune function.

Escherichia coli, Lactobacillus, Salmonella, and Saccharomycetes affect the balance of the gut microbiota through interactions with host cells, which impact the immune system (41–44). Probiotics such as Lactobacillus and Saccharomycetes usually help to enhance the immune response (45). In contrast, pathogens such as Salmonella and pathogenic *Escherichia coli* may activate the immune response to clear the infection (46, 47). Kwon et al. (48) found that *Pueraria lobata* extract increased the *Lactobacillus rhamnosus* in human fecal specimens. Xu et al. (49) found that the polyphenols extracted from *Pueraria lobata* root improved the gut microbiota in mice, and Chen et al. (50) reported that the polysaccharides from *Pueraria lobata* significantly increased the abundance of beneficial bacteria, including Oscillospira and Anaerotruncus. In this study, Pueraria extracts significantly increased the Saccharomycetes content while decreasing the content of *Escherichia coli* and Salmonella in the jejunum of Wuzhishan piglets. This finding directly demonstrates that Pueraria extracts have the ability to inhibit the growth of harmful bacteria while simultaneously promoting the growth of beneficial bacteria. These changes may indirectly enhance the immune function of pigs by improving gut barrier function and reducing the risk of pathogen infection, thereby improving the immunity of piglets, which is consistent with the improved immune indicators described above.

Interleukin-10 (IL-10) is a pleiotropic cytokine with immunosuppressive or immunostimulating effects (51). Mucin 2 (MUC2) is a significant component of the intestinal mucus layer, which has the functions of lubricating the intestinal tract, providing antimicrobial proteins and adhesion sites for commensal flora, and resisting the invasion of pathogenic bacteria and harmful substances (52). Zonula occludens 1 (ZO1) is a tight junction protein that plays a vital role in forming and maintaining tight junctions between cells (53). Tumor necrosis factor-α (TNF-α) is an important cytokine that regulates inflammation, apoptosis, and immune cell activity (54). Transforming growth factor-β1 (TGF-β1) is a multifunctional cytokine synthesized by almost all cells and has the functions of immune regulation and promoting fibrosis (55). IL-10, MUC2, ZO1, TNF-α, and TGF-β1 play essential roles in the animal immune system and intestinal health, and they are involved in regulating the immune response and maintaining the integrity of the intestinal barrier through different mechanisms. In this study, the relative expressions of *IL-10*, *MUC2,* and *ZO1* were increased, and the relative expressions of *TNF-α* and *TGF-β1* were decreased in the groups treated with Pueraria extracts, which may be because the changes in the microbial community described above, by improving gut barrier function and reducing the risk of pathogen infection, indirectly affected the function of the immune system. It can be speculated that the alterations in immune-related gene expression are likely associated with the changes in the gut microbial community, which will be further explored in future studies.

The Wuzhishan pig, a local pig breed, is distinguished by its small size and relatively slow growth rate, resulting in a longer growth cycle compared to other pig breeds. Typically, it requires a longer period to reach market weight. Although the study period allowed for the observation of significant improvements in growth performance and immune function of Wuzhishan pigs due to Pueraria extracts, it may not fully reflect the potential long-term impacts of prolonged use of Pueraria extracts on piglets. In this study, Pueraria extracts significantly enhanced the average daily gain and feed conversion ratio of Wuzhishan pigs. In the long term, these improvements may help piglets maintain better growth efficiency throughout their entire growth cycle, thereby reducing feeding costs. Pueraria extracts also increased the immunoglobulins and complement levels in piglets within a short period, suggesting that they may have a positive impact on the long-term immune function of piglets. It can be inferred that long-term use of Pueraria extracts may enhance the immune system of piglets, reduce disease incidence, and consequently improve the overall health status of the pig herd. Moreover, Pueraria extracts improved the number of beneficial bacteria in the piglets' intestines while reducing the number of harmful bacteria in the short term. These changes may further optimize the gut microbiota structure and enhance gut barrier function over the long term, thereby reducing the incidence of intestinal diseases. However, while Pueraria extracts have shown positive effects on growth and immunity in the short term, long-term use may alter the piglets' requirements and absorption of other nutrients. This could lead to the development of tolerance to Pueraria extracts, thereby reducing their effectiveness in later stages. Therefore, further research is needed to fully understand the long-term effects and safety of Pueraria extracts in piglet diets.

5 Conclusion

In this study, adding dietary Pueraria extracts at 600 mg/kg to the diets of Wuzhishan piglets significantly improved growth performance by increasing final weight and average daily gain, while decreasing the feed conversion ratio. These findings indicate that Pueraria extracts can enhance feed efficiency and promote piglet growth, potentially

leading to economic benefits for pig farming. Additionally, Pueraria extracts enhanced immune function by boosting the levels of immunoglobulin G and M and complement 3 and 4, and by promoting beneficial bacteria such as Lactobacillus and Saccharomycetes in the jejunum. These results suggest that Pueraria extracts can be used as a natural and safe feed additive to improve the health status of piglets, thereby reducing the risk of diseases and the need for antibiotics. Future research endeavors will be dedicated to unraveling the intricate mechanisms through which Pueraria extracts exert their beneficial effects. This will involve investigating their role in regulating metabolic pathways and modulating the gut microbiota.

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 approved by All procedures involving care and management were approved by the Institutional Animal Care and Use Committee of the Chinese Academy of Tropical Agricultural Sciences (Approval No.: CATAS-20231101-23). The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

HW: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Software, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing. WP: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Software, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing. XY: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Software, Supervision, Validation, Visualization, Writing – original draft. XZ: Conceptualization, Data curation, Formal Analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Software, Supervision, Validation, Visualization, Writing – original draft. FJ: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Software, Supervision, Validation, Visualization, Writing – original draft. QS: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Software, Supervision, Validation, Visualization, Writing – original draft. RL: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Software, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing.

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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 essential oils on calf growth, ruminal fermentation, and antioxidative status: a meta-analysis

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Introduction: Essential oils (EO) have gained significant attention in the calves industry due to their antimicrobial properties. This meta-analysis aimed to evaluate the efficacy of EO on calves to provide better guidance for cattle feed.

Methods: We conducted a comprehensive search of relevant studies published from inception to February 6, 2022, using PubMed, CENTRAL,Web of Science, and EMBASE. The quality of included studies was assessed using the CAMARADES checklist. Effect sizes were calculated using weighted mean differences (WMD) for continuous variables and summary risk ratios (RR) for binary variables. Subgroup and sensitivity analyses were also performed.

Results: This systematic review and meta-analysis included 10 animal studies with 226 calves. The average quality score was 5.8 (range: 5–7). EO improved milk production (WMD = 0.30; 95% CI 0.13 to 0.47; I^2 = 0%, p = 0.985) and beta-hydroxybutyric acid levels (WMD = 0.01; 95% CI 0.04 to 0.16; I^2 = 0%, p = 0.472). However, EO did not significantly improve rumen fermentation characteristics or overall performance index.

Discussion: While EO may have beneficial effects on specific outcomes like milk production and beta-hydroxybutyric acid levels,its impact on rumen fermentation and overall performance remains inconclusive. Future large-scale randomized controlled trials (RCTs) are needed to better assess the effects of EO on ruminal fermentation efficiency, anti-oxidative status, and overall performance.

KEYWORDS

meta-analysis, essential oil, calves, ruminal fermentation, anti-oxidative status

Introduction

In the feed diets of beef cattle, cereal grains are usually used to increase performance and feed efficacy. However, highly fermentable substances in these diets can decrease ruminal pH and increase the risk of acidosis and bloat (1). Therefore, antibiotics have been successfully used in beef diets to improve nutrient utilization efficiency and reduce the incidence of ruminal acidosis and bloat. Nevertheless, the use of antibiotics often results in residues in milk and meat, which can affect human health (2). As public demand for reduced use of additives in animal feed diets, the European Union proposed regulations to ban antimicrobials in 2003. This has put enormous pressure on scientists and health authorities to reduce the use of

antibiotics in feed production. Effective alternatives to antimicrobials are receiving increasing attention (3–5).

Essential oils (EO) are naturally occurring minor component metabolites and volatile components extracted from plants by distillation methods, primarily by steam distillation (6). Chemically, EO is a complex mixture of monoterpenes and sesquiterpenes and biologically relevant phenols or monophenols (7). EO has antimicrobial activities against gram-negative and gram-positive bacteria. The antimicrobial properties of essential oils regulate rumen fermentation (8). EO, for example, oregano oil containing high concentrations of phenolic compounds, was evaluated as a modulator of rumen fermentation (9). EO has attracted much attention due to its favorable antimicrobial properties as an alternative to commonly used antimicrobial agents in livestock production (10, 11).

Some *in vivo* studies have identified the efficacy of EO. In 2001, Landete-Castillejos et al. (9) observed that EO increased total volatile fatty acids (VFA) concentration in 24 h batch fermentation, which energetically benefits the ruminant animal. In 2013, Vakili et al. (12) reported an increase in the molar proportion of propionate in ruminal fluid collected from beef cattle-fed diets supplemented with EO. Several short-term *in vitro* studies have shown that EO affects N metabolism via the reduction of protein degradation and ammonia production (13, 14). In 2008, Macheboeuf et al. (15) observed that EO decreased methane production (up to a 98% decrease). In the same study, the authors reported lower anti-methanogenic activity of carvacrol, suggesting that other components present in lower concentrations in EO may have acted antagonistically with carvacrol, thereby attenuating the anti-methanogenic properties of EO.

Due to the limitations of the *in vitro* technique (i.e., short-term culture, buffered media, and inability to replicate the diversity and viability of rumen microbial populations), the data should be interpreted cautiously. High-quality meta-analysis has always been regarded as the best evidence and provides credible suggestions. Therefore, this systematic review and meta-analysis aimed to determine the efficacy and safety of essential oils on ruminal fermentation, anti-oxidative status, and calf performance as comprehensively as possible and to provide better guidance for cattle feed.

Methods and method

This study was reported in line with Preferred Reporting Items for Systematic Reviews and Meta-Analyses(PRISMA).

Data sources and searches

CENTAL, Embase, PubMed, and Web of Science were searched from the earliest publication date to February 06, 2023. We also screened the reference lists of relevant reviews. The search terms included related text words and medical subject headings regarding "calf" and "essential oil." We tailored search strategies for each database. Details of the search strategies were provided in Supplementary Table S1.

Study selection

Two independent reviewers screened titles, abstracts, and full texts and agreed on the final included studies. When disagreements arose, a third investigator was consulted. Studies were considered eligible if they (1) included cow or calf, (2) included the intervention group using essential oils as feed additives, (3) included the control group using no additive feed, (4) the research results need to include the effect of essential oil addition on rumen fermentation. There are no restrictions on the research design. Non-English literature, *in vitro* studies, single-arm studies, studies without full-text and statistical methods, and literature with the repeated publication of research results were excluded.

Justification for inclusion and exclusion criteria

The inclusion and exclusion criteria were designed to ensure the studies included were relevant and of sufficient quality to provide meaningful insights. The criteria were as follows: Inclusion criteria: Studies were included if they (1) included cow or calf, (2) included the intervention group using essential oils as feed additives, (3) included the control group using no additive feed, and (4) reported the effect of essential oil addition on rumen fermentation. Exclusion criteria: Studies were excluded if they were (1) *in vitro* studies, (2) single-arm studies without a control group, (3) lacked full-text availability, (4) had incomplete statistical data, or (5) were duplicate publications.

Given the involvement of live animals in the studies included in this meta-analysis, it is crucial to address the ethical considerations and regulatory compliance related to animal experimentation. All studies included in this meta-analysis were conducted in accordance with the ethical guidelines for the use of animals in research, which emphasize the principles of Replacement, Reduction, and Refinement (the 3Rs). These guidelines aim to minimize the use of animals and ensure their well-being throughout the experimental procedures.

Data extraction and quality assessment

Reviewers independently extracted data in a standardized form. The following data were extracted: (1) general information of the included studies, including the first author, published year, type of intervention, composition, duration, and dose of intervention, and baseline characteristics of the calf; (2) rumen fermentation characteristics, including PH, NH3-N (mg/dL), Total VFA (mM), Butyrate, Acetate: propionate, ammonia (mg/dL), Protozoa. (3) blood metabolites, including Urea N (mg/dl), beta hydroxyl butyric acid (mM), and Glucose (mg/dL). (4) performance, including Body weight (kg), Withers height (cm), and Heart girth (cm). (5) Feed efficiency (milk/dry matter intake), Milk production (kg/d). Two authors independently assessed the risk of bias. Any disagreements were resolved via discussion among the authors. The quality of included studies was evaluated using the "Collaborative Approach to

Abbreviations: EO, Essential oil; CAMARADES, Collaborative approach to meta-analysis and review of animal data from experimental studies; RR, Risk ratio; PRISMA, Preferred reporting items for systematic reviews and meta-analyses; VFA, Volatile fatty acids; WMD, Weighted mean difference; CI, Confidence intervals; RCT, Randomized controlled trials; GIT, Gastrointestinal tract.

Meta-Analysis and Review of Animal Data from Experimental Studies" (CAMARADES) checklist with ten items. The CAMARADES checklist is used to perform a combined assessment of the reporting of several measures to reduce bias and several indicators of external validity and study quality.

Data synthesis and analysis

STATA Version 14.1 (Stata Corporation, College Station, Texas, USA) was used to calculate the weighted mean difference (WMD) as the effect size for the continuous variables. Summary RR with 95% confidence intervals (CI) were presented if the results were binary variables. Using a fixed effects model, the summary RR and 95% CIs were calculated. The random effects model was used if high heterogeneity. The extent of heterogeneity was interpreted by the total percentage of variation between the studies concerned, measured by the I^2 statistic. The I^2 value was categorized as low if I^2 was 0%e25%, moderate if I^2 was 25%e50%, and high if I^2 was >50%. Additionally, Q-statistic was used to assess the presence of heterogeneity. P statistic 0.05 was considered to indicate no significant heterogeneity among the included studies. Subgroup analysis was conducted according to the different subtypes of interventions.

The publication bias test would not be necessary to analyze if the number of included trials was less than ten.

Results

Search results

A total of 1,186 publications were identified, and 995 publications were excluded. Among them, 265 publications were excluded for duplication, and 730 were excluded after screening titles and abstracts. Ultimately, 44 full-text publications were assessed for eligibility. Of the 44 retrieved publications, 34 were excluded, and five studies were identified as eligible for inclusion in this review (Figure 1).

Study characteristics

The key characteristics of all included studies were summarized in Table 1. All included studies were published from 2013 to 2021. A total of 226 calves were included in this meta-analysis, 142 in EO intervention groups and 84 in placebo control groups. The interventions varied in their dose and intervention periods. The duration of the intervention ranged from 21 days to 115 days.

Risk of bias in included studies

The average was 5.8, ranging from 5 to 7. Three studies were rated as five scores, six studies were rated as six scores, and only one study was rated as seven scores. No studies reported allocation concealment, blinded assessment of outcome, use of animals with cancer, sample size calculation, statement of compliance with regulatory requirements, physiological monitoring, and reporting animals excluded from the analysis. The results were shown in Table 2.

Rumen fermentation characteristics

The merged effects of Rumen fermentation characteristics were shown in Figure 2. Seven studies reported Acetate: propionate ratios. No significant difference was observed between EO and control groups (WMD = −0.08; 95% CI −0.17 to 0.01; I^2 = 64.9%, p = 0.009). However, the effect size (WMD) of −0.08 indicates a small but potentially meaningful difference in the ratio of Acetate to propionate, which could suggest a slight shift in rumen fermentation pathways. This effect size, although not statistically significant, may still be biologically relevant in certain contexts and warrants further investigation. Butyrate levels were analyzed in seven studies. The meta-analysis showed no significant difference between EO and control groups (WMD = −0.28; 95% CI −0.66 to 0.10; I^2 = 73.9%, p = 0.004). The effect size (WMD) of −0.28 suggests a moderate decrease in butyrate levels in EO-treated calves, which could have implications for rumen health and energy metabolism. However, the wide confidence interval and high heterogeneity (I^2 = 73.9%) indicate that this effect is not consistent across studies and requires further exploration. Four studies involving 103 calves reported NH3-N. Similarly, no significant difference between EO and control groups (WMD = −0.51; 95% CI −2.13 to 1.12; I^2 = 59.8%, p = 0.059) were shown in Figure 2. The effect size (WMD) of −0.51 indicates a small reduction in NH3-N levels, which could be beneficial for reducing ammonia emissions and improving air quality in calf housing. However, the wide confidence interval and moderate heterogeneity (I^2 = 59.8%) suggest that this effect is not robust and needs to be confirmed in larger studies.

Blood metabolites

Figure 3 showed the meta-analysis results for blood metabolites. EO could significantly improve the Beta hydroxyl butyric acid (WMD = 0.01; 95% CI 0.04 to 0.16; I^2 = 0%, p = 0.472). The effect size (WMD) of 0.01 indicates a small but statistically significant increase in beta-hydroxybutyric acid levels, which could be biologically relevant for improving energy metabolism and reducing the risk of ketosis in calves. This effect size, although small, suggests a potential benefit of EO supplementation in enhancing metabolic health. For Glucose and Urea N, there was no significant difference between EO and control groups (WMD = 0.34; 95% CI −16.45 to 17.12; I^2 = 18.9%, p = 0.939), (WMD = 0.00; 95% CI −1.16 to 1.16; I^2 = 0%, p = 0.983). The effect sizes (WMD) of 0.34 for Glucose and 0.00 for Urea N indicate no meaningful differences in these blood metabolites, suggesting that EO supplementation does not significantly impact glucose metabolism or nitrogen balance in calves.

Performance

The merged effect for the performance of calves was presented in Figure 4. EO could not significantly improve the heart girth from three studies (WMD = 1; 95% CI −3.33 to 5.34; I^2 = 0%, p = 0.982). The effect size (WMD) of 1 indicates no meaningful difference in heart girth, suggesting that EO supplementation does not significantly impact this measure of calf growth. Besides, the data from two and five studies showed that EO could not significantly improve the Withers height (WMD = 0.51; 95% CI −3.09 to 4.12; I^2 = 0%,

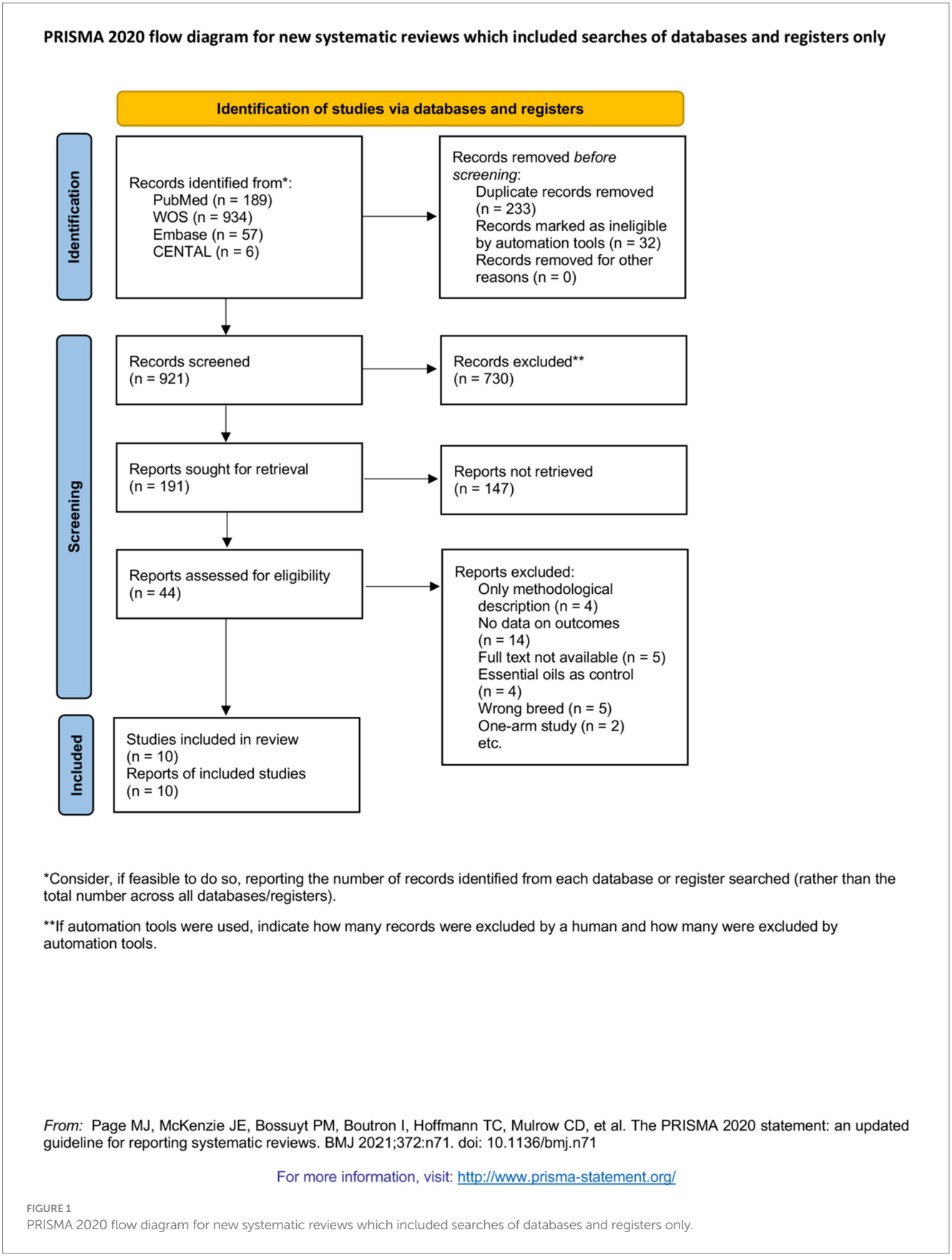

FIGURE 1
PRISMA 2020 flow diagram for new systematic reviews which included searches of databases and registers only.

$p = 0.765$) and Body weight (WMD = −1.16; 95% CI −6.48 to4.17; $I^2 = 0\%$, $p = 0.100$). The effect sizes (WMD) of 0.51 for Withers height and −1.16 for Body weight indicate no meaningful differences in these measures of calf performance, suggesting that EO supplementation does not significantly impact growth and development in calves.

TABLE 1 The characteristics of included studies.

Author	Year	Control group			Intervention group			Period (day)
		Control	Dose	Sample size	Intervention	Dose	Sample size	
M. Akbarian-Tefaghi	2017	without essential oils	NA	11	Phytogenic feed additive containing EO	3 g/kg /day	11	67
Olga Teresa Barreto Cruz	2014	without essential oils	NA	10	EO from cashew and castor	3 g/animal/day	10	115
C. Benchaar	2019	without essential oils	NA	2	Oregano oil	50 mg/kg	2	26
C. Benchaar	2020	without essential oils	NA	2	Oregano oil	50 mg/kg	2	28
C. Benchaar	2006	without essential oils	NA	2	Oregano oil	50 mg/kg	2	28
Joana Palhares Campolina	2021	without essential oils	NA	14	Commercial blend of EO	1 g/day/calf	29	90
S. N. S. e Silva	2021	without essential oils	NA	2	Natural EO with carvacrol, cinnamaldehyde, and limonene	16 g/cow/day	8	21
Matteo Mezzetti	2021	without essential oils	NA	18	EO	50 g/cow/day	36	35
F. H. R. Santos	2015	without essential oils	NA	15	EO with milk replacer	400 mg/kg	30	70
A. R. Vakili, B. Khorrami	2013	without essential oils	NA	8	basal diet supplemented with thyme oil	5 g/calf daily	12	45

This table provides detailed information on the included studies, including the first author, publication year, intervention details, sample size, and intervention period. Statistical significance is indicated where applicable.

TABLE 2 The quality assessment of included studies.

Items	1	2	3	4	5	6	7	8	9	10
Publication in peer-reviewed journal	1	1	1	1	1	1	1	1	1	1
Statement of control of temperature	1	0	1	1	1	1	1	0	0	1
Randomization to treatment and control	1	1	1	1	1	1	1	1	1	1
Allocation concealment	0	0	0	0	0	0	0	0	0	0
Blinded assessment of outcome	0	0	0	0	0	0	0	0	0	0
Avoidance of intrinsically neuroprotective aesthetics	1	0	0	0	0	0	0	0	0	0
Use of animals with cancer	0	0	0	0	0	0	0	0	0	0
Sample size calculation	0	0	0	0	0	0	0	0	0	0
Statement of compliance with regulatory requirements	0	0	0	0	0	0	0	0	0	0
Statement regarding possible conflict of interest	1	1	1	1	1	1	1	1	1	1
Physiological monitoring	0	0	0	0	0	0	0	0	0	0
Prespecified inclusion and exclusion criteria	1	1	1	1	1	1	1	1	1	1
Reporting animals excluded from analysis	0	0	0	0	0	0	0	0	0	0
Reporting of study funding	1	1	1	1	1	1	1	1	1	1
Total score	7	5	6	6	6	6	6	5	5	6

This table evaluates the quality of included studies using the CAMARADES checklist. Each item is scored, and the total score is provided. Statistical significance is indicated where applicable.

Feed efficiency

The meta-analysis showed that EO could not significantly improve milk production (WMD = 0.30; 95% CI 0.13 to 0.47; I^2 = 0%, p = 0.985), but EO did not have efficacy on milk/dry matter intake (WMD = −0.02; 95% CI −0.05 to 0.01; I^2 = 0%, p = 0.835). The effect size (WMD) of 0.30 for milk production indicates a small but statistically significant increase in milk production, which could be biologically relevant for improving feed efficiency and economic returns in calf rearing. However, the lack of significant effect on milk/dry matter intake suggests that this improvement in milk production may not be directly related to increased feed intake but could be due to other factors such as improved metabolic efficiency. The forest plot was shown in Figure 5.

Publication bias

The publication bias test was unnecessary since the included trials were less than ten in analyzed outcomes. The funnel plot of the OR for publication bias suggested the absence of bias because of plot symmetry (Figure 6).

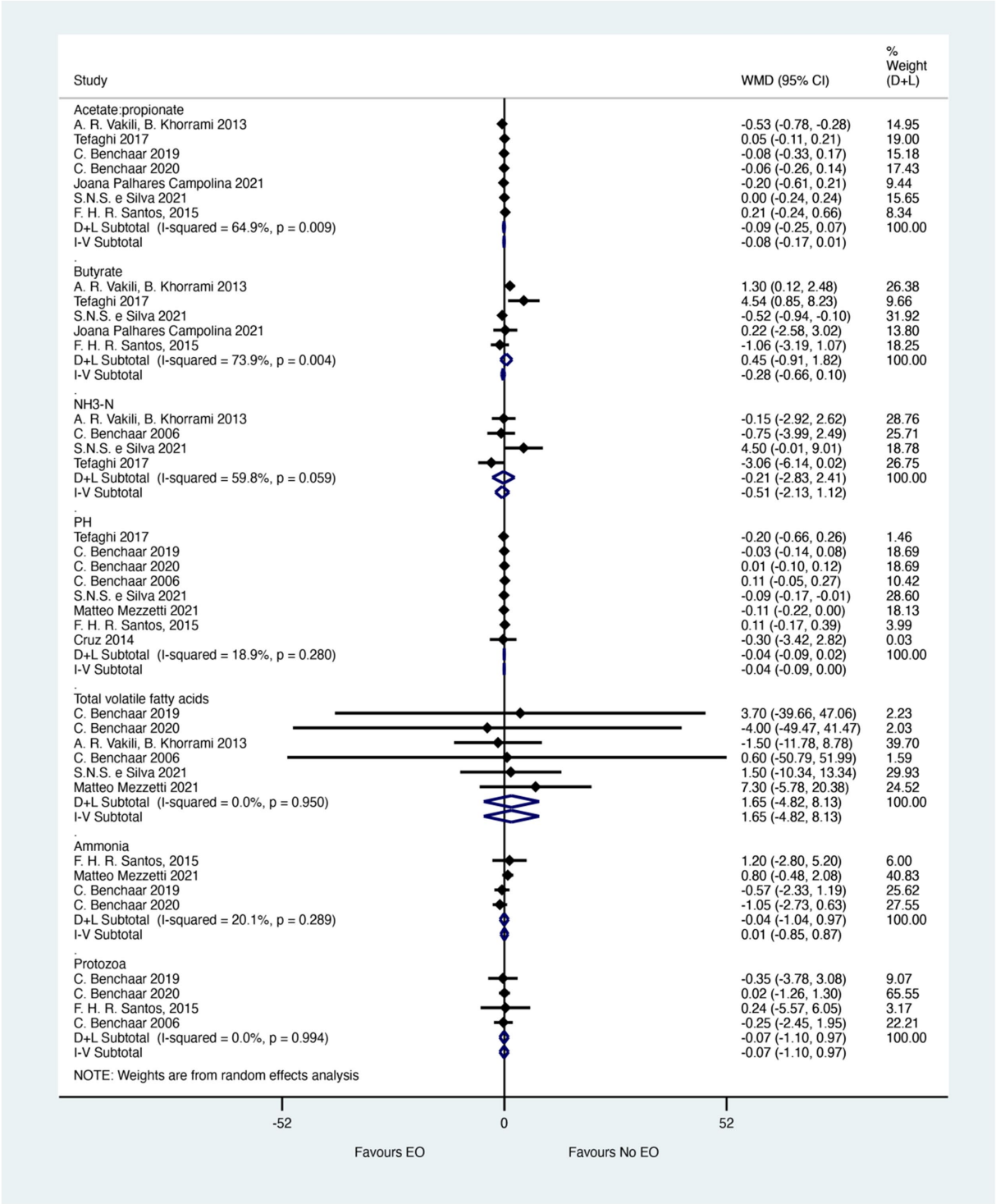

FIGURE 2
Merged effects of essential oils on rumen fermentation characteristics. The figure shows the weighted mean differences (WMD) and 95% confidence intervals (CI) for various rumen fermentation parameters. The effect sizes indicate the magnitude of change in each parameter due to essential oil supplementation. Asterisks (*) denote statistical significance ($p < 0.05$).

Discussion

EO have a variety of probiotic benefits in animal husbandry, including anti-inflammatory, antioxidant and *in vitro* deworming (16–18). EO may have antimicrobial properties, potentially representing a methane mitigation strategy suitable for organic production (19–21). EO has a complex mix of many compounds and has a major role in an antimicrobial activity tested for its effect on rumen fermentation using a batch culture technique (22, 23). EO has antimicrobial activity against a broad spectrum of Gram-positive and Gram-negative bacteria, and its potential effect on

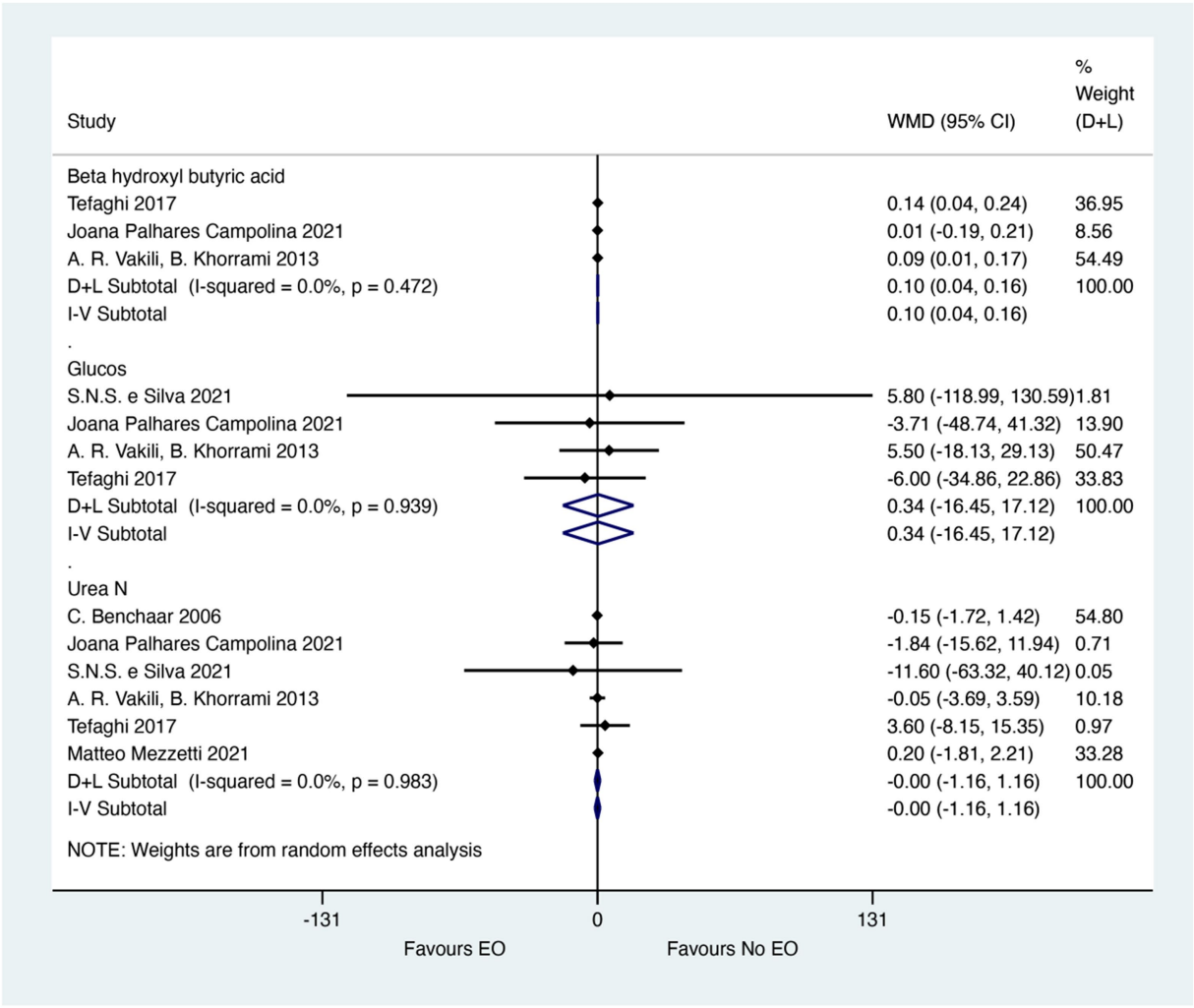

FIGURE 3
Meta-analysis results for blood metabolites. The figure displays the weighted mean differences (WMD) and 95% confidence intervals (CI) for key blood metabolites, including beta-hydroxybutyric acid, glucose, and urea nitrogen. Asterisks (*) indicate statistically significant differences ($p < 0.05$).

modifying rumen microbial fermentation has been recently studied (24–26). In this systematic review and meta-analysis of 10 animal studies, including 226 calves, EO could improve milk production and beta hydroxyl butyric acid. However, EO did not significantly improve rumen fermentation characteristics and performance index.

In small ruminants, several studies have evaluated the effect of dietary inclusion of EOs on animal performance, nutrient digestibility, ruminal fermentation, blood biochemistry, meat quality, and milk production and composition (27–29). According to the meta-analysis conducted by Dorantes-Iturbide et al., EOs could improve the taste and palatability of livestock foods with beef cattle (30). Besides, various EOs have been shown to increase the abundance of fungi and ruminal bacteria associated with fiber degradation in the rumen (21, 31, 32). In a meat analysis for six kinds of small ruminants, EOs were found to increase dry matter intake (6). Consistent with these findings, our results show that EO improved milk production, but did notsignificantly affect milk/dry matter intake, possibly due to the indirect role of dry matter intake (33).

Butyric acid is a short-chain C2–5 organic fatty acids (34), is the primary end-product of anaerobic bacterial carbohydrate fermentation in the rumen of certain bovine species. *In vivo* studies have shown that adding Butyric acid to acidified milk could affect gastrointestinal tract metabolism and development in calves (7). Butyric acid supplements can improve small intestine development in calves, reducing diarrhea rate and alleviating weaning stress (35, 36). In our study, EO improve beta-hydroxyl butyric acid, which may have indirect efficacy on digestibility with better intestinal development for calves.

Although most of the included studies were not RCTs and lacked high methodology quality, they exhibited similar biases and limitations to those in our current study (22, 37). The small size may have weakened the strength of the evidence. Additionally, the varying intervention periods and observations could have acted as potential confounding factors in assessing efficacy. For most outcomes, EO did not significantly alter performance or rumen fermentation characteristics (38, 39). However, these previous studies primarily focused on the synergistic effects of EO and other nutrient intakes. A larger-scale trial would provide a better assessment of EO's impact on milk production efficiency.

Compared to no EO addition, supplementing cattle feed with essential oils demonstrated positive effects, primarily through impacts on the gastrointestinal tract (GIT). EO supplementation increased

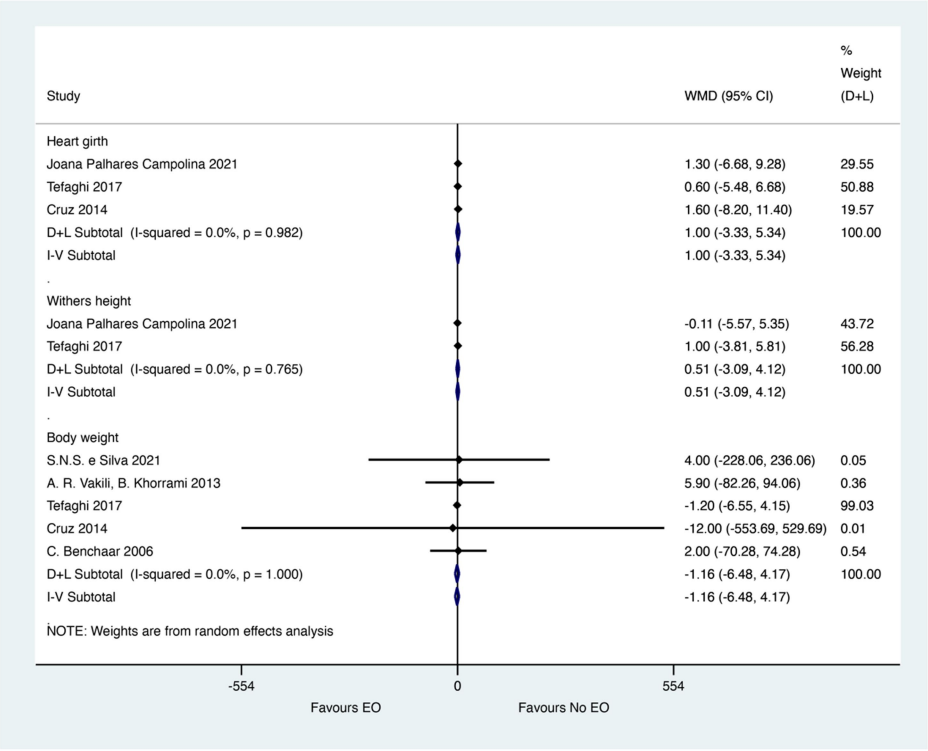

FIGURE 4
Merged effects of essential oils on calf performance. The figure presents the weighted mean differences (WMD) and 95% confidence intervals (CI) for performance indicators such as heart girth, withers height, and body weight. Asterisks (*) denote statistical significance ($p < 0.05$).

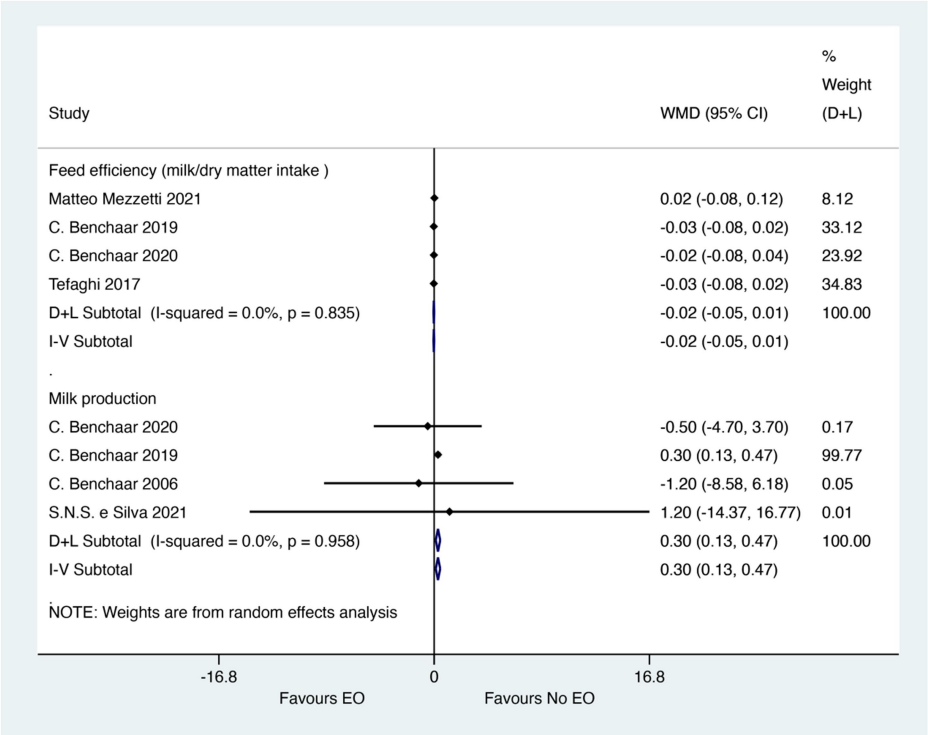

FIGURE 5
Forest plot of essential oils on feed efficiency and milk production. The figure shows the weighted mean differences (WMD) and 95% confidence intervals (CI) for milk production and milk/dry matter intake. Asterisks (*) indicate statistically significant differences ($p < 0.05$).

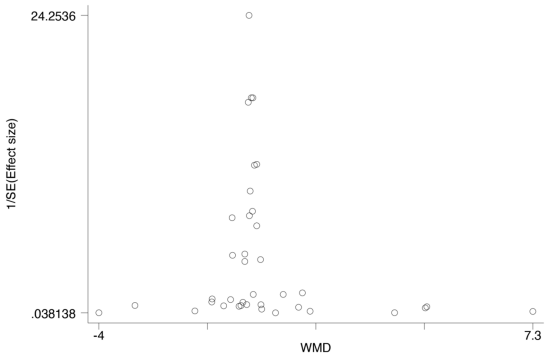

FIGURE 6
The funnel plot of the OR for publication bias.

digestibility, improved pancreatic enzyme activity, changed microbiota, affected amino acid absorption in the intestines, and consequently, improved feed conversion rates (39–41). In our study, EO improved milk production and beta-hydroxyl butyric acid levels. EOs represent a viable health additive option for modern production systems and can serve as an alternative to improve calf health and performance.

In summary, EO supplementation showed potential benefits in improving milk production and beta-hydroxybutyric acid levels in calves. However, its effects on rumen fermentation and overall performance were not significant. Further large-scale RCTs are needed to comprehensively evaluate the efficacy of EO in enhancing ruminal fermentation, anti-oxidative status, and performance in calves.

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

WL: Writing – original draft, Writing – review & editing. FW: Conceptualization, Data curation, Writing – review & editing. YH: Conceptualization, Data curation, Formal analysis, Funding acquisition, Writing – original draft, Writing – review & editing. FS: Writing – review & editing. CL: Data curation, Investigation, Methodology, Writing – review & editing. YZ: Data curation, Formal analysis, Methodology, Writing – review & editing. PZ: Data curation, Funding acquisition, Writing – original draft, Writing – review & editing.

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Conflict of interest

CL was employed by Liaoning Feidi Feeding Technology 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.

Generative AI statement

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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.2025.1573846/full#supplementary-material

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The effects of Astragalus, Epimedium, and Fructus Ligustri Lucidi extract on the antioxidant capacity, and immune status of goslings under stress conditions

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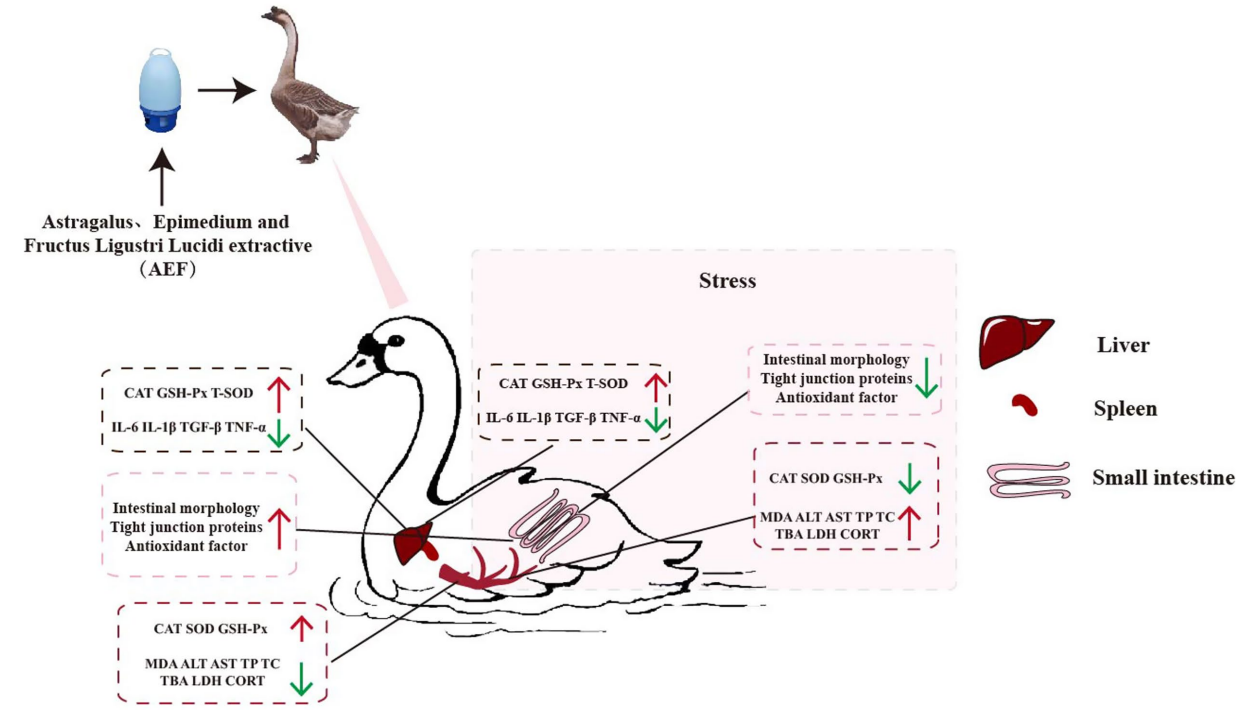
With the development and improvement of the scale, intensification, and production level of the goose farming industry, oxidative stress often occurs during the process of producing meat geese. This study employed the intraperitoneal injection method with a concentration of 2.96 mmol/mL H_2O_2 to establish a model of oxidative stress in goslings. The effects of Astragalus, Epimedium, and Fructus Ligustri Lucidi (AEF) extract on the immune function, antioxidant capacity, meat quality, intestinal morphology, and intestinal barrier integrity of goslings were investigated. Twenty-four 1-month-old Magang geese from the same batch were randomly divided into four groups: control group (C group), AEF treatment group (AEF group), H_2O_2 stress group (S group), AEF and H_2O_2 stress group (S + AEF group). The experiment lasted for 30 days, during which the AEF group and the S + AEF group were fed 0.1 g/mL of AEF aqueous solution, once daily. Hydrogen peroxide (H_2O_2) was employed as the stressor. On the 25th day of the experiment, the goslings were weighed and administered an abdominal cavity injection of H2O2. The results showed that compared with the control group, the body weight, spleen weight, bursa index of fabricius, spleen index, thymus index, the activity of glutathione peroxidase (GSH-PX), total superoxide dismutase (T-SOD), and catalase (CAT) in serum, liver, and jejunum were decreased in S group ($p < 0.05$). Meanwhile, compared with the S group, the body weight, spleen weight, bursa index of fabricius, spleen index, thymus index, the activity of glutathione peroxidase (GSH-PX), total superoxide dismutase (T-SOD), and catalase (CAT) in serum, liver, and jejunum were enhanced in S + AEF group and S + AEF + LP group ($p < 0.05$). However, the content of cortisol (CORT) and blood glucose were increased in S group ($p < 0.05$), AEF and AEF + LP effectively alleviated these growth trend. Supplementing with AEF and LP post-stress alleviated stress-induced the expression of inflammatory factors (TGF-β, TNF-α, IL-1β, IL-6), and improve L*, a*, b*, Ph45 (min), Ph24 (h) and drip loss in pectoral ($p < 0.05$). The intestinal morphology results showed that the villus structures in the duodenum and jejunum of goslings were short and sparse under stress conditions, with a significant decrease in the villus height/crypt depth ratio (Vh/Cd). After AEF treatments, the villus height increased, leading to an improved Vh/Cd in the small intestine. In conclusion, AEF can enhance the immune performance, and antioxidant capacity of goslings, thereby improving intestinal health. These findings offer new insights into enhancing immune performance and preventing stress in goslings.

KEYWORDS

geese, stress, growth performance, inflammation, immune function



GRAPHICAL ABSTRACT

Introduction

Geese in the modern poultry business are subjected to prolonged periods of intense pressure, high nutrition, and fast growth because to the rapid advancement of high-density farming practices. Stress factors have a significant impact on the antioxidant function and health of geese (1). Existing literature has reported that oxidative stress can have adverse effects on the health of geese. Oxidative stress depletes poultry antioxidant defenses, elevates free radicals, thereby compromising disease resistance and accelerating senescence or mortality (2). However, there are many factors that can cause stress in animals, including transportation, restraint, density, temperature variations, and the presence of loud and aggressive animals (3).

Existing literature has reported that H_2O_2 can induce oxidative damage by increasing the levels of reactive oxygen species (ROS) in cells and organisms (4, 5). Therefore, in this experiment, H_2O_2 was directly administered to the animal body to elevate the levels of reactive oxygen species (ROS) and establish an oxidative stress model in geese. H_2O_2 has been confirmed to cause damage to the intestinal epithelial barrier, reduce the immune levels of the organism, and induce oxidative damage (6, 7). However, after the onset of disease, farms prefer to use antibiotics to treat and control diseases (8). Prolonged use of antibiotics can lead to bacterial resistance, resulting in the emergence of "superbugs," causing a decline in animal immune function, an increase in mortality, and other consequences (9). However, there are also some other commonly used anti-stress drugs. Due to stress being an extremely complex physiological process, these drugs are easily oxidized by the body during their action, rendering them ineffective. Therefore, finding a safe and effective therapeutic drug is urgent. Current literature reports that the use of probiotics and plant extracts can improve the intestinal microbial community of poultry, maintain intestinal health. The intestinal microbial community has been designated as a hidden metabolic "organ" because of its significant impact on host metabolism, physiology, nutrition, and immune function (10, 11). Plant extracts can effectively enhance human resistance, and they have promising prospects. As the demand for alternatives to antibiotics has become crucial, plant extracts are currently a viable option to partially replace antibiotics (12, 13). The interest in plant extracts is due to their natural properties, environmental friendliness, safety, and characteristics such as no drug residues, stable drugs, and minimal adverse reactions. They can improve animal immunity, increase egg production, and prevent and treat stress (14). In addition, probiotics are also an excellent choice for antibiotic replacement and are beneficial microbial strains for health. The animal gut is a complex ecosystem of microorganisms, host cells, etc. Probiotics regulate the immune system to prevent diseases in both humans and animals by interacting with the gut microbiota (15).

Astragalus, with its main components being polysaccharides, flavonoids, saponins, etc., is a commonly used traditional Chinese medicine in many herbal formulations. It is widely applied in clinical settings for its antioxidative, anti-inflammatory, anti-apoptotic properties, enhancing immunity, and alleviating aging (16, 17). As a green feed additive, Astragalus is utilized as a supplement for live microbial feed, improving intestinal barrier, maintaining microbial balance, and promoting gut health (18).

Epimedium is an herbal plant rich in flavonoid compounds, offering various beneficial effects to animals. Epimedium improves the

composition of the gut microbiota by modulating the core genus Lactobacillus, thereby enhancing antioxidant capacity, intestinal permeability, and strengthening tight junction proteins (19).

Ligustrum lucidum, as a high-quality feed additive, can reduce the mortality of animals, cholesterol in cracked eggs, low-density lipoprotein cholesterol, triglycerides, and aspartate aminotransferase. Additionally, it increases the rate of high-density lipoprotein in serum and the cholesterol function in serum levels (20).

Lactobacillus plantarum is a kind of lactic acid bacterium that does not produce gas. *Lactobacillus plantarum* has long been employed for fermentation in vegetables, meat, and dairy products, but its significance as a probiotic is now being more widely acknowledged. It plays a crucial role in intestinal health, immune function, and brain health (21). Previous studies have demonstrated that feeding plant Lactobacillus to weaned piglets can enhance piglet growth performance, immune function, and regulate intestinal microbiota, among other significant effects (22).

A combination of Astragalus, Epimedium, and Fructus Ligustri Lucidi (AEF) with Plant Lactobacillus (LP) has the ability to boost the immune system of the host and promote the growth performance of animals. The therapeutic and preventative benefits of incorporating AEF into drinking water for pigeons. These benefits include increased stress tolerance, enhanced egg production, improved immunity, and better digestive health (19). Nevertheless, there is a scarcity of studies regarding the impact of incorporating AEF and AEF + LP into drinking water on stress resilience, antioxidant capability, immune response, and gastrointestinal well-being in goslings. Hence, we evaluated the effects of AEF and AEF + LP on the growth performance, antioxidant capacity, immunological function, and intestinal microbiota of goslings. This work establishes a theoretical foundation for utilizing AEF and AEF + LP to enhance the production performance and immunity of goslings. This experiment aims to investigate the impact of incorporating AEF (antioxidant-enriched feed) and AEF + LP (antioxidant-enriched feed combined with low protein) on the production performance of goslings. The evaluation will focus on assessing alterations in immunological and antioxidant capacity.

Materials and methods

Animals

Thirty-six 30-day-old goslings (from Guangdong Golden Livestock and Poultry Breeding Co., Ltd., China) were placed in a pathogen-free environment and raised freely. The goslings were randomly divided into 6 groups (6/group): Control group (C group), water supplemented with Astragalus, Epimedium, and Fructus Ligustri Lucidi extract (AEF) group, water supplemented with AEF and *Lactobacillus plantarum* (AEF + LP) group, stress group (S group), stress plus AEF group (S + AEF group), and stress plus AEF plus LP group (S + AEF + LP group) (Table 1).

TABLE 1 Experimental grouping and design for goslings.

Item	Add category	Give stimulation
C	Normal water	Nothing
AEF	Water with AEF	Nothing
S	Normal water	H_2O_2 stress
S + AEF	Water with AEF	H_2O_2 stress

Preparation of hydrogen peroxide solution

Select a 30% hydrogen peroxide solution, perform gradient dilution with 0.75% saline, and calculate the final working concentration for use.

Plant extracts and plant lactobacilli

Take 100 g of Astragalus, 60 g of Epimedium, 60 g of Ligustrum, 40 g of Chaihu, 40 g of Poria, and 30 g of Licorice. Decoct in water 10 times for 30 min. Combine the decoction, filter, concentrate the filtrate to about 330 mL, add 3 times ethanol, let it stand at 4°C for 20 h. Filter, recover ethanol from the filtrate, concentrate to a clear paste with a relative density of 1.25 to 1.35 (25°C). Add 3 parts of sugar and 1 part of starch according to the amount of clear paste, mix well, granulate, low-temperature drying, and then package separately. The concentration of AEF in water is 0.2 g/mL, 0.2 g per kilogram of goose body weight, gradually increasing with body weight, once a day.

The plant lactobacillus is purchased from Weikai Haisi (Shandong) Biotechnology Co., Ltd., administered to each goose once a day, with a dosage of 1 g per time.

Animal treatment and tissue collection

The experiment lasted for 30 days, with AEF dissolved in physiological saline at 0.2 g/mL, LP dissolved in physiological saline at 1 g/mL, and geese treated with AEF, AEF + LP for 30 days. The basic diet used in the experiment was formulated in accordance with the Nutrient Requirements of Poultry (NRC) (1994) and that of the lion-head goose production industry in Guangdong Province. The composition and nutritional levels of the feed are listed in Table 2. In the last 5 days of the experiment, geese were subjected to H_2O_2 stress (H_2O_2 concentration of 2.96 mmol/kg). Geese had free access to feed and water. Blood was collected from the wing veins of each group of geese ($n = 6$) into blood collection tubes for serum preparation. 5 mL of blood was collected from the sub wing vein and placed at an angle until the serum was precipitated; subsequently, the blood was centrifuged at 3000 r/min for 15 min to separate the serum which was stored at −20°C for the determination of serum biochemical indexes and antioxidant indexes. After anesthesia, each group of 6 geese was euthanized, and the intestines were separated and rinsed with PBS solution. Tissues from the intestine, hypothalamus, liver, and spleen were used for real-time quantitative polymerase chain reaction (RT-qPCR) analysis. The intestines were fixed with a 4% paraformaldehyde solution and stained with HE for morphological observation.

Determination of serum indicators

Blood collected from the brachial vein of each group of geese was transferred to centrifuge tubes. Serum levels of Ca, alanine aminotransferase (ALT), alkaline phosphatase (ALP), aspartate aminotransferase (AST), total protein (TP), albumin (ALB), GLB,

TABLE 2 Composition of basal diets.

Items	Content (%)
Ingredient	
Corn	58.00
Soybean meal	24.00
Bran	6.50
Rice bran	8.20
CaHPO4	1.20
Limestone	1.10
Premix[1]	1.00
Total	100.00
Nutrient levels[2]	
Metabolizable energy (M/kg)	11.50
Crude protein	17.46
Ether extract	4.07
Calcium	0.85
PhosphorusLysine	0.83
Metabolizable energy (M/kg)	0.89

[1]The premix provided the following per kg of diets: 5000 [U, VB, 5.0 mg, VB, 8 mg, VB.5.0 mg, VB, 12 μg, VD, 800 1 U, VE 50 1 U, VK, 2.5 mg, folic acid 0.5 mg, nicotinamide 40mgbiotin 0.3 mg, pantothenic acid 25 mg, choline 1,500 mg, Fe (as ferrous sulfate) 85.2 mg, Cuas copper sulfate) 10 mg, Zn (as zinc sulfate) 50 mg, I (as potassium iodide) 0.3 mg, Se (as sodium selenite) 0.25 mg].
[2]Metabolizable energy was a calculated value, while the others were measured values.

A/G, triglyceride (TG), total cholesterol (TC), creatinine (CREA-S), UREA, creatine kinase (CK), total bilirubin (T-Bil-V), total bile acids (TBA), lactate dehydrogenase (LDH), and P were measured using the BS-240VET fully automated biochemical analyzer (Mindray Biomedical, Shenzhen). Goose serum CORT was assayed using an Elisa kit (Bolai Biotechnology Co., Ltd., Jiangsu, China). Specific procedures were performed following the manufacturer's instructions.

Morphological observations

All tissues were fixed in 4% paraformaldehyde at 4°C for 24 h, followed by processing and embedding in paraffin. Hematoxylin and eosin (H&E) staining was performed using standard protocols. Images of the slides were observed using a Leica microscope (DM500, Leica, Wetzlar, Germany). Villus height (Vh) and crypt depth (Cd) were measured using Image Pro Plus 6.0 software (Media Cybernetics, Rockville, MD).

DNA extraction and sequencing

Collect fecal samples from each group of 5 geese for nucleic acid extraction. Following the manufacturer's instructions, use the RNeasy Power Microbiome KIT (Qiagen, Milan, Italy) to extract total DNA. Amplify the gDNA of the V3-V4 region of the 16S rRNA gene to assess the microbial community (23). PCR products are tested on the Illumina MiSeq platform (Majorbio, Shanghai, China) according to the instructions (24).

RT-qPCR analysis

Total RNA was extracted from the small intestine and liver using Trizol reagent (Invitrogen, Carlsbad, CA). The RNA concentration and quality were measured using a Q3000 Microspectrophotometer (Quawell Technology, Inc., Sunnyvale, CA). cDNA was synthesized using a commercial RT-PCR kit (Takara Shuzo Co., Ltd., Kyoto, Japan). The qPCR conditions were as follows: 95°C for 30 s; 40 cycles of 95°C for 5 s, 60°C for 34 s; 95°C for 15 s; 65°C for 5 s; 95°C for 5 s. Subsequently, real-time PCR was performed using the SYBR Green QuantiTect RT-PCR kit (Roche, South San Francisco, CA), and each sample was analyzed in triplicate. The $2^{-\Delta\Delta Ct}$ formula method was used to calculate elative fold-change values between samples. The primers used are provided in Table 3.

Determination of growth performance

During the growth performance assessment, fasting geese were weighed on the first and thirtieth days.

Measurement of transpiration loss

Weigh muscles of similar size on the same side, place them in self-sealing bags, hang them in a refrigerator at 4 degrees overnight, and after removal, gently wipe the muscles with filter paper before weighing and calculating.

Muscle mass assessment

Left pectoral muscle were measured. Measure the pH value and color of the muscles using a pH meter (Lunz Technology, Model 205) and a colorimeter (China Corporation, nr20xe) after 45 min and 24 h (stored at 4°C).

Determination of the immunological organ index

Weighing of the thymus, spleen, and bursa of Fabricius during sampling (organ weight/body weight × 100%).

Determination of antioxidant capacity

According to the instructions of the kit (Nanjing Jiancheng Bioengineering Institute), the activities of superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GSH-Px), as well as the content of malondialdehyde (MDA) and the total antioxidant capacity (T-AOC), were determined in serum, liver, and duodenum.

Statistical analysis

Statistical analysis was conducted on table data using IBM SPSS Statistics V25.0 software (SPSS, Inc., Chicago, IL) through one-way

TABLE 3 Gene-special primers used in the real-time quantitative reverse-transcription PCR.

Gene	Primer (5′-3′)
β-Actin	Forward: GCACCCAGCACGATGAAAAT Reverse: GACAATGGAGGGTCCGGATT
IL-1β	Forward: CCGCTTCATCTTCTACCG Reverse: TGTAGGTGGCGATGTTGAC
IL-6	Forward: AAGTTGAGTCGCTGTGCT Reverse: GCTTTGTGAGGAGGGATT
TNF-α	Forward: GTTCTATGACCGCCCAGTTC Reverse: CACACGACAGCCAAGTCAA
TJP1	Forward: AGACCATTCCAGACATTCTCCACAG Reverse: CGCCTGCCACCTCTTCCATAG
Occluding	Forward: TGCTCTGCCTCATCTGCTTCTTC Reverse: CGTTCTTCACCCACTCCTCCAC
GSH-Px	Forward: ACAGATTAAGGCGTTTGCTGAGAAC Reverse: GGCTGCTCCTTCATCCACTTCC
SOD1	Forward: CATCTCTCTGACTGGACCACACTG Reverse: AGTTAGCGTGCTCTCGTTATCTCC
TGF-β	Forward: TCATCTCCATCTACAACAGCACCAG Reverse: GCATAATACTCCTCGTCGCTCCTC

analysis of variance (ANOVA). Differences between groups were determined by Duncan's multiple range test. Data were presented as mean ±SD, and differences between treatments were considered statistically significant when $p < 0.05$ and highly significant when $p < 0.01$. Quantitative PCR was performed in triplicate, and representative results are shown. Statistical analysis was performed with Student's *t*-test or one-way ANOVA, using GraphPad Prism 7.0 (GraphPad Software, San Diego, CA). A p value < 0.05 was considered statistically significant.

Results

Effects of AEF and AEF + LP on goose body weight, spleen weight, energy metabolism, serum antioxidant capacity, liver and intestinal antioxidant capacity under H_2O_2-induced stress

As shown in Figure 1A, the stress group exhibited the greatest reduction in body weight. Compare with C group, the body weight was decreased in S group ($p < 0.05$). Furthermore, compared with the S group, the body weight was enhanced with AEF and LP treatment ($p < 0.05$). Subsequently, the weight of the spleen was measured. Stress-induced spleen atrophy was inhibited by AEF and AEF + LP treatments ($p < 0.05$) (Figure 1B). Serum antioxidant indicators were also detected by ELISA. Compared to the control group, the content of CAT, GSH-PX, and T-SOD in goose serum decreased ($p < 0.05$), while MDA increased under stress conditions ($p < 0.05$). AEF and AEF + LP treatments enhanced the antioxidant capacity of geese ($p < 0.05$) (Figure 1C). According to Figure 1D, compared to the control group, CAT, GSH-PX, and T-SOD content in the goose liver decreased, and MDA content increased under stress conditions ($p < 0.05$). AEF and AEF + LP treatments improved the antioxidant capacity of geese. In the comparison with the control group, CAT, GSH-PX, and T-SOD content in the goose intestine decreased, and MDA content increased under stress conditions ($p < 0.05$). AEF and AEF + LP treatments enhanced the antioxidant capacity of geese ($p < 0.05$) (Figure 1E). Serum biochemical indicators in geese were determined, showing that ALT, AST, TC, CREA-S, UREA, CK, TBA, LDH, and P levels increased after stress, while ALP, TP, ALB, GLB, A/G, and TG levels decreased. Metabolic capacity decline was relieved after AEF and AEF + LP treatments ($p < 0.05$) (Table 4).

The impact of AEF and AEF + LP on corticosteroids and blood glucose in goslings under H_2O_2 stress

Figure 2A demonstrates a considerable rise in CORT levels under stress situations as compared to the control group ($p < 0.05$). Treatment with AEF and AEF + LP effectively alleviated the content of CORT, and at the same time, AEF and AEF + LP reduced the elevation of blood glucose caused by stress ($p < 0.05$) (Figure 2B).

The alleviating effect of AEF and AEF + LP on gosling intestine under H_2O_2 stress

Under stress conditions, the villous structure of the duodenum and jejunum in goslings becomes sparse and short. Statistical data on VH (villous height) and CD (crypt depth) in the duodenum and jejunum show a significant reduction in VH/CD in the small intestine under stress. Treatment with AEF and AEF + LP effectively improves VH, thus restoring the VH/CD ratio ($p < 0.05$) (Figure 3A). The expression of several inflammatory factor mRNAs was detected to assess the alleviating effect of AEF and AEF + LP on the jejunum under H_2O_2-induced stress. Compared to the control group, the mRNA expression levels of TGF-β and TNF-α in the jejunum significantly increased under stress, and their expression was reduced with the administration of AEF and AEF + LP ($p < 0.05$) (Figure 3B). Furthermore, RT-qPCR analysis revealed that, compared to the control group, AEF and AEF + LP significantly increased the expression of Occludin and TJP1 in the jejunum after stress treatment ($p < 0.05$) (Figure 3C). Finally, the expression of antioxidant genes in the jejunum was evaluated, and the administration of AEF and AEF + LP significantly enhanced the mRNA expression of GSH-Px and SOD1 after stress treatment ($p < 0.05$) (Figure 3D).

The impact of AEF and AEF + LP on the gosling liver under H_2O_2 stress

Under stress conditions, the liver undergoes certain damage. Therefore, we examined the expression levels of several common inflammatory factor mRNAs in the liver under stress conditions after administration of AEF and AEF + LP. Compared to the control group, the mRNA expression levels of TGF-β, IL-1β, TNF-α, and IL-6 were significantly increased under stress

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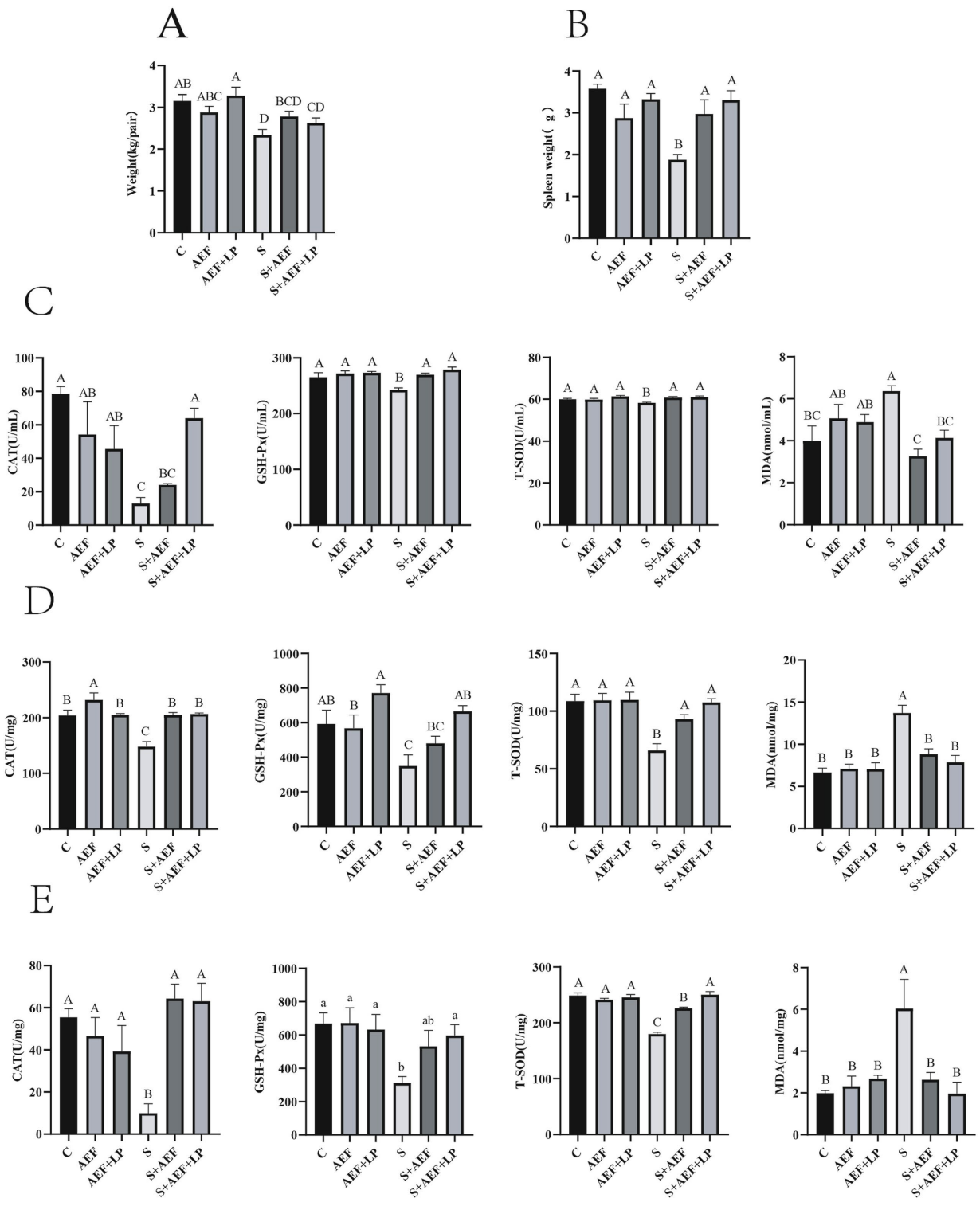

FIGURE 1
Impact of AEF and AEF + LP on the body weight and antioxidant capacity of goslings under H_2O_2 stress. **(A)** Gosling body weight reduction. Values are expressed as mean ± SEM (n = 4). **(B)** Gosling spleen weight reduction. Values are expressed as mean ± SEM (n = 4). **(C)** Impact of AEF and AEF + LP on the serum antioxidant enzyme activity in goslings. Values are expressed as mean ± SEM (n = 4). **(D)** Impact of AEF and AEF + LP on the liver antioxidant enzyme activity in goslings. Values are expressed as mean ± SEM (n = 4). **(E)** Impact of AEF and AEF + LP on the intestinal antioxidant enzyme activity in goslings. Values are expressed as mean ± SEM (n = 4). [ab; ABCD] In the same column, no letters or letters with the same superscript indicate no significant difference ($p > 0.05$), while letters with different lowercase superscripts indicate significant differences ($p < 0.05$), and letters with different uppercase superscripts indicate highly significant differences ($p < 0.001$).

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TABLE 4 The effects of AEF on the serum biochemical parameters of goslings.

Item	C	AEF	S	S + AEF	SEM	*p*-value
Ca	2.45 ± 0.04	2.59 ± 0.24	2.22 ± 0.13	2.44 ± 0.17	0.038	0.063
ALT	17.67 ± 2.09^{B}	16.63 ± 5.37^{BC}	24.70 ± 5.17^{A}	10.98 ± 1.88^{D}	1.229	<0.001
ALP	920.67 ± 247.67^{A}	754.58 ± 207.44^{A}	391.35 ± 24.58^{B}	322.43 ± 55.09^{B}	52.697	<0.001
AST	13.40 ± 2.34^{BC}	13.85 ± 2.35^{BC}	24.53 ± 7.27^{A}	8.50 ± 1.73^{C}	1.295	<0.001
TP	56.85 ± 6.49^{AB}	55.90 ± 5.41^{AB}	49.38 ± 1.63^{B}	63.68 ± 6.91^{A}	1.354	0.008
ALB	15.85 ± 0.93^{a}	15.85 ± 0.54^{a}	12.82 ± 0.78^{b}	15.20 ± 0.96^{a}	0.366	0.044
GLB	41.57 ± 6.48^{ab}	41.38 ± 8.28^{ab}	35.25 ± 1.60^{b}	49.07 ± 6.94^{a}	1.476	0.013
A/G	0.43 ± 0.05^{ab}	0.47 ± 0.05^{a}	0.35 ± 0.06^{b}	0.40 ± 0.00^{ab}	0.014	0.031
TG	0.99 ± 0.14^{A}	0.75 ± 0.07^{B}	0.45 ± 0.06^{C}	0.58 ± 0.11^{BC}	0.041	<0.001
TC	3.79 ± 0.18^{B}	3.99 ± 0.14^{AB}	4.39 ± 0.18^{A}	3.58 ± 0.46^{B}	0.077	0.006
CREA-S	17.38 ± 1.87^{B}	19.50 ± 2.51^{B}	24.03 ± 3.38^{A}	17.50 ± 1.30^{B}	0.656	0.001
UREA	0.34 ± 0.12^{bc}	0.40 ± 0.17^{ab}	0.54 ± 0.13^{a}	0.18 ± 0.05^{c}	0.032	0.012
CK	548.50 ± 113.09^{B}	659.30 ± 85.84^{B}	903.17 ± 323.37^{A}	277.32 ± 54.91^{C}	52.105	<0.001
T-Bil-V	0.68 ± 0.24^{a}	0.62 ± 0.13^{a}	0.23 ± 0.18^{b}	0.58 ± 0.15^{a}	0.044	0.028
TBA	24.13 ± 6.36^{B}	23.25 ± 11.81^{B}	40.50 ± 6.98^{A}	19.28 ± 4.94^{B}	2.148	0.004
LDH	176.42 ± 28.06^{C}	197.17 ± 34.72^{BC}	289.25 ± 32.86^{A}	154.15 ± 33.49^{C}	11.561	<0.001
P	2.00 ± 0.42^{ab}	2.07 ± 0.29^{ab}	2.28 ± 0.13^{a}	1.80 ± 0.08^{b}	0.055	0.23

^{abc}; ^{ABCD} In the same column, values without letters or with the same superscript letters indicate no significant difference ($p > 0.05$), while values with different lowercase superscript letters indicate significant differences ($p < 0.05$), and values with different uppercase superscript letters indicate extremely significant differences ($p < 0.01$). The values are presented as mean ± SD ($n = 4$). calcium (Ca), aminotransferase (ALT), alkaline phosphatase (ALP), aminotransferase (AST), total protein (TP), albumin (ALB), globulin (GLB), albumin/globulin (A/G), triglyceride (TG), total cholesterol (TC), creatinine (CREA-S), carbamide (UREA), creatine kinase (CK), total bilirubin (T-Bil-V), total bile acid (TBA), lactate dehydrogenase (LDH), and phosphorus (P).

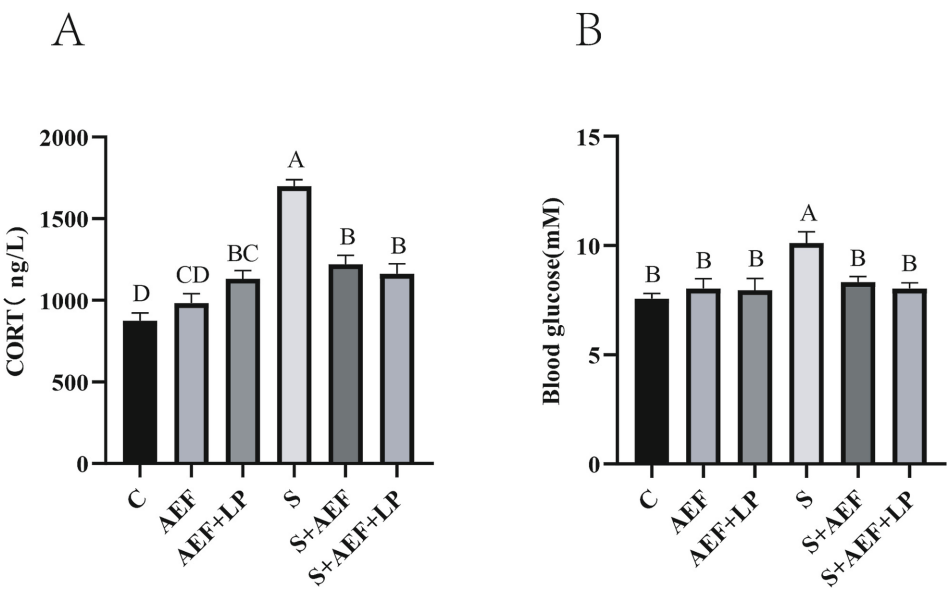

FIGURE 2
The effects of AEF and AEF + LP on corticosterone and blood glucose in goslings under H_2O_2 stress. **(A)** The content of corticosterone in gosling serum. Values are expressed as mean ± SEM ($n = 4$). **(B)** The content of blood glucose in gosling serum. Values are expressed as mean ± SEM ($n = 4$). The same letters or letter superscripts in the same column indicate no significant difference ($p > 0.05$), while different uppercase letter superscripts indicate extremely significant differences ($p < 0.001$).

conditions, and AEF and AEF + LP had a certain mitigating effect on inflammation, reducing their expression levels ($p < 0.05$) (Figure 4A). At the same time, the expression of antioxidant genes in the liver was also measured. Administration of AEF and AEF + LP significantly increased the antioxidant capacity after stress ($p < 0.05$) (Figure 4B).

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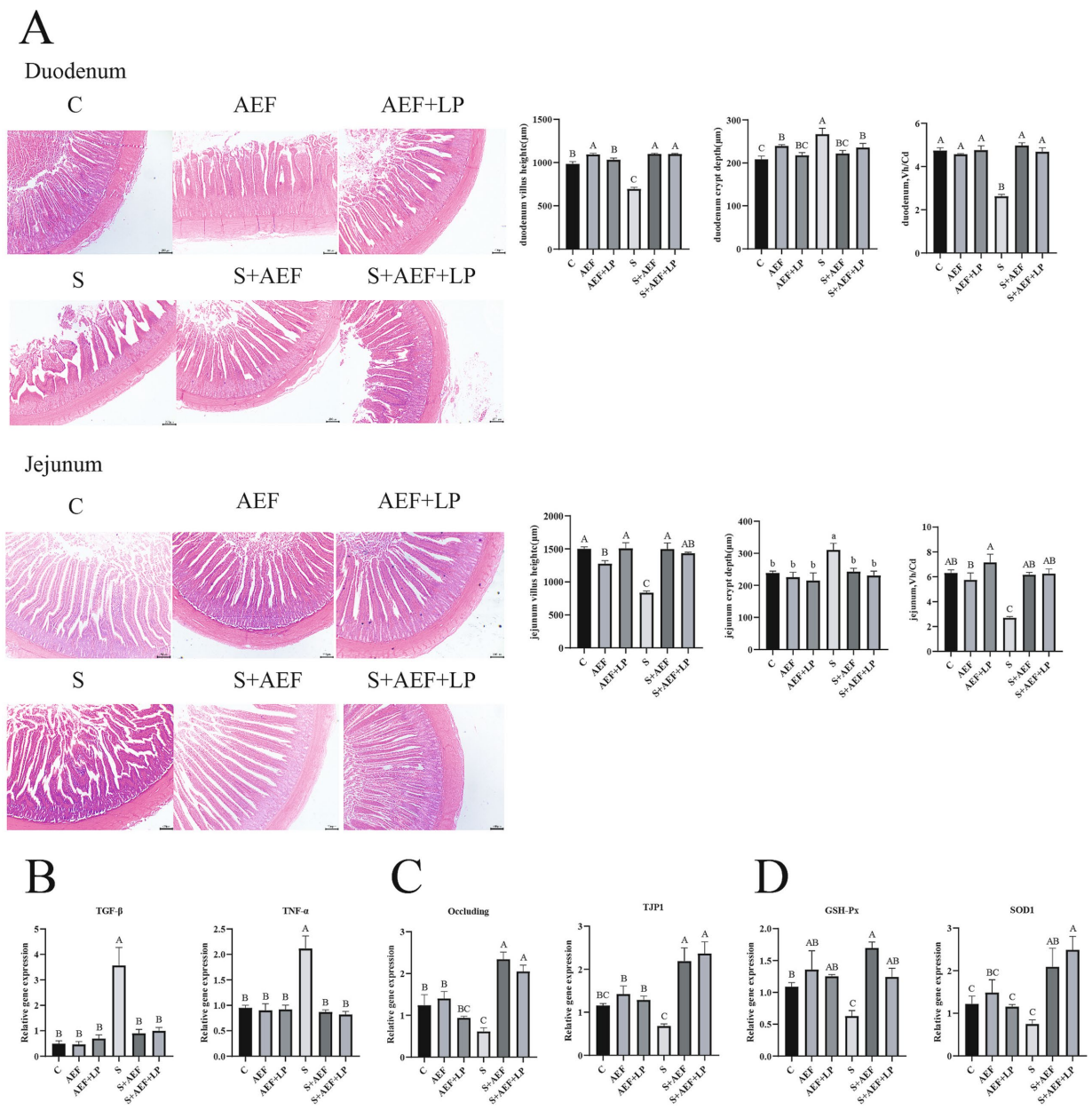

FIGURE 3
The impact of AEF and AEF + LP on the duodenum of goslings under H_2O_2 stress. **(A)** Typical morphology and Vh/Cd of the gosling duodenum. Scale: 200 mm. Values are presented as the mean ± SEM (n = 3). **(B)** RT-qPCR detection of mRNA expression levels of TGF-β and TNF-α in the jejunum. Values are presented as the mean ± SEM (n = 4). **(C)** RT-qPCR detection of mRNA expression levels of tight junction protein-related genes Occludin and TJP1 in the jejunum. Values are presented as the mean ± SEM (n = 4). **(D)** RT-qPCR detection of mRNA expression levels of antioxidant-related genes GSH-Px and SOD1 in the jejunum. Values are presented as the mean ± SEM (n = 4). $^{ab;\ ABC}$ In the same column indicate no significant difference ($p > 0.05$), while different lowercase letters indicate significant differences ($p < 0.05$), and different uppercase letters indicate extremely significant differences ($p < 0.001$).

The impact of AEF and AEF + LP on goose immune organ index, meat quality, and meat color under H_2O_2 stress

Compared to the control group, under stress conditions, the bursa of Fabricius index, spleen index, and thymus index of geese significantly decreased. Simultaneously, compared to the stress group, after AEF and AEF + LP treatment, the bursa of Fabricius index, spleen index, and thymus index showed a significant increase ($p < 0.05$) (Table 5). Compared to the control group, the L*, a*, and b* of geese showed no significant changes under stress conditions. However, drip loss and pH45 min significantly increased, while pH24 h decreased. In comparison with the stress group, after AEF and AEF + LP treatment, gosling's drip loss significantly decreased, pH45 min significantly decreased, and pH24 h significantly increased ($p < 0.05$) (Table 6).

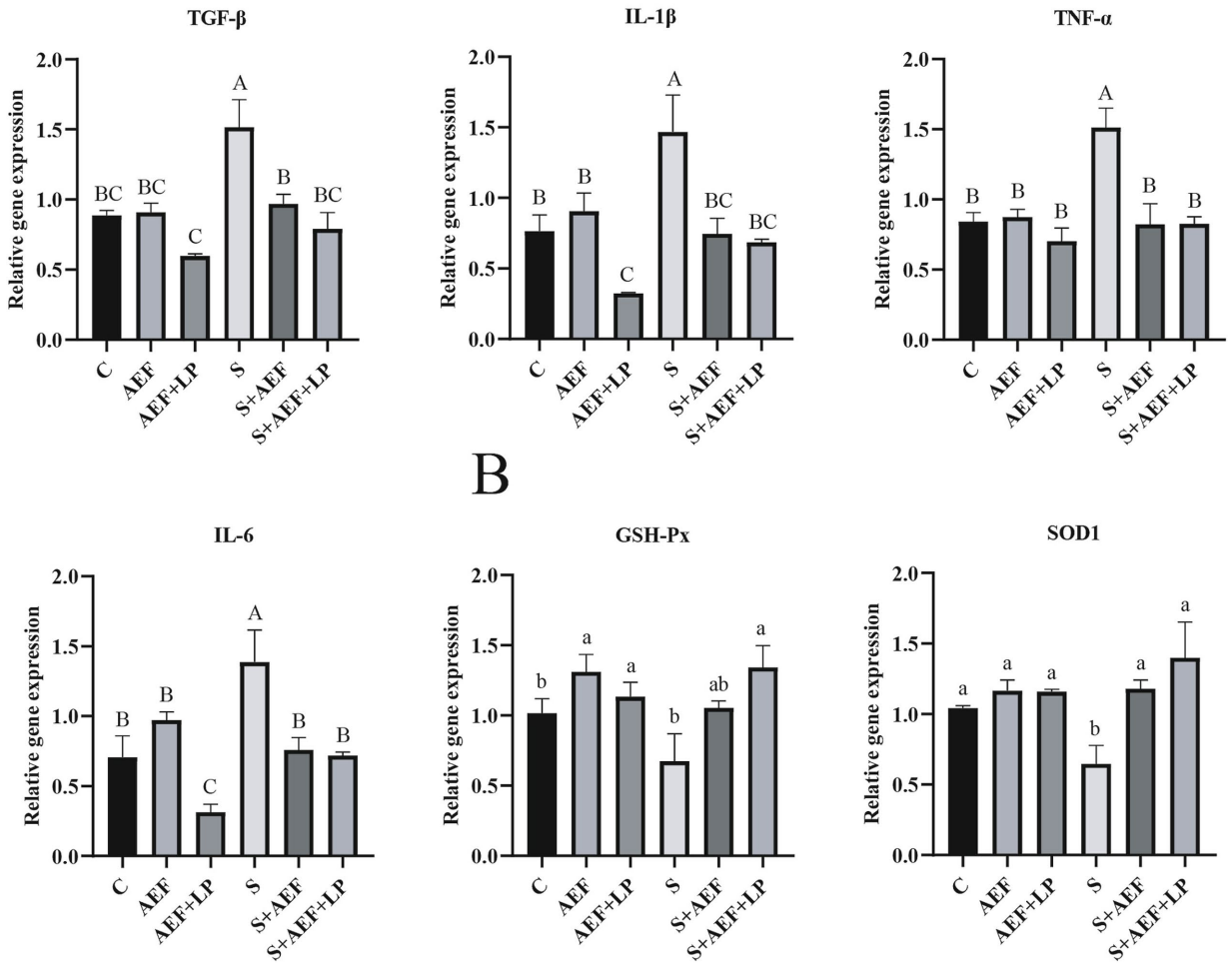

FIGURE 4
AEF and AEF + LP on the gosling liver under H_2O_2 stress. **(A)** The mRNA expression levels of TGF-β, IL-1β, TNF-α, and IL-6 in the liver were detected by RT-qPCR. Values are presented as the mean ± SEM (n = 4). **(B)** The mRNA expression levels of antioxidant enzyme-related genes GSH-Px and SOD1 in the liver were measured by RT-qPCR. Values are presented as the mean ± SEM (n = 4). $^{ab; ABC}$ In the same column, values with the same letter or superscript indicate no significant difference ($p > 0.05$), while values with different lowercase superscripts indicate significant differences ($p < 0.05$), and values with different uppercase superscripts indicate extremely significant differences ($p < 0.001$).

The impact of AEF and AEF + LP on the abundance of gosling cecal contents under H_2O_2 stress

Detection of the microbial composition of gosling cecal contents using 16S rRNA Illumina MiSeq. In this study, 1,103,008 effective sequences were selected from 15 samples for subsequent analysis. Each sample had an average of 993 operational taxonomic units (OTUs). There were significant differences at the phylum and family levels among these six groups. The bar chart of phylum-level microbial structure showed that the relative abundance of Firmicutes in the six groups was 46, 47, 51, 41, 50, and 48%, respectively. At the family level, the bar chart of microbial structure showed that the relative abundance of Ruminococcaceae in the six groups was 11.7, 11.4, 14.3, 8.9, 13.9, and 11% (Figure 5A). When the sample size was sufficiently large in the species accumulation curve, the total number of species in the community would no longer significantly increase with an increase in sample size, and the curve would become flat (Figure 5B). In the dilution curve, a flat curve indicated a reasonable sample size (Figure 5C). As the sequencing depth increased, the Shannon index curve flattened, indicating that the sequencing depth was sufficient to reflect the majority of microbial information in the samples (Figure 5D). The relative abundance of 16S rRNA gene sequences in gosling cecal contents is shown, and the heatmap depicts the 20 most important bacterial genera in the contents (Figure 5E). Observing the species count, Simpson index, and Shannon index reflected the richness and evenness of species. Compared to the control group, Simpson and Shannon indices of goslings under stress showed a decreasing trend, indicating a decrease in species richness and abundance, which to some extent was restored after AEF and AEF + LP treatment (Figure 5F). Compared to the control group, there was a large degree of community dispersion among the samples in the S group, and this dispersion was somewhat reduced after the administration of AEF and AEF + LP (Figure 5G). We obtained 2001, 2,770, 1702, 1868, 2,676, and 2,104 samples from groups C, AEF,

TABLE 5 The effects of AEF and AEF + LP on the immune indices of goslings.

Item	C	AEF	AEF + LP	S	S + AEF	S + AEF + LP	SEM	*p*-value
Bursa index of Fabricius (%)	0.09 ± 0.01^{A}	0.09 ± 0.01^{A}	0.09 ± 0.01^{A}	0.05 ± 0.00^{B}	0.08 ± 0.02^{A}	0.08 ± 0.01^{A}	0.004	<0.001
Spleen index (%)	0.13 ± 0.02^{A}	0.11 ± 0.01^{AB}	0.11 ± 0.01^{B}	0.08 ± 0.01^{C}	0.13 ± 0.01^{AB}	0.12 ± 0.01^{AB}	0.004	0.001
Thymus index (%)	0.14 ± 0.02^{AB}	0.15 ± 0.02^{A}	0.12 ± 0.01^{BC}	0.08 ± 0.00^{D}	0.11 ± 0.01^{C}	0.10 ± 0.01^{C}	0.006	<0.001

^{ABCD} In the same column, values without letters or with the same superscript letters indicate no significant difference ($p > 0.05$), while values with different lowercase superscript letters indicate significant differences ($p < 0.05$), and values with different uppercase superscript letters indicate extremely significant differences ($p < 0.01$). Values are presented as mean ± SD ($n = 4$).

TABLE 6 The influence of AEF and AEF + LP on goose meat quality.

Item	C	AEF	AEF + LP	S	S + AEF	S + AEF + LP	SEM	*p*-value
L*	37.84 ± 3.73	37.03 ± 3.23	38.11 ± 2.45	38.88 ± 1.55	38.27 ± 3.15	38.59 ± 5.53	0.643	0.981
a*	10.11 ± 2.85	10.50 ± 2.64	10.13 ± 3.26	10.18 ± 1.85	10.01 ± 1.77	10.09 ± 2.27	0.452	1
b*	7.15 ± 1.08	7.78 ± 1.24	7.00 ± 1.77	9.25 ± 2.15	7.65 ± 1.09	7.38 ± 1.66	0.32	0.396
Ph45 (min)	6.44 ± 0.08^{B}	6.36 ± 0.30^{B}	6.47 ± 0.18^{B}	7.20 ± 0.25^{A}	6.59 ± 0.16^{B}	6.59 ± 0.24^{B}	0.069	<0.001
Ph24 (h)	6.11 ± 0.14^{A}	6.03 ± 0.04^{A}	6.09 ± 0.05^{A}	5.46 ± 0.34^{B}	6.06 ± 0.14^{A}	6.03 ± 0.07^{A}	0.055	<0.001
Drip loss	1.41 ± 0.44^{B}	1.17 ± 0.34^{B}	0.78 ± 0.29^{B}	2.23 ± 0.23^{A}	1.13 ± 0.80^{B}	0.74 ± 0.19^{B}	0.13	0.001

^{AB} In the same column, values without letters or with the same superscript letters indicate no significant difference ($p > 0.05$), while values with different lowercase superscript letters indicate significant differences ($p < 0.05$), and values with different uppercase superscript letters indicate extremely significant differences ($p < 0.01$). Values are presented as mean ± SD ($n = 4$). *No practical significance, the meat quality detector displays L*.

AEF + LP, and S, S + AEF, S + AEF + LP, respectively, with 269 OTUs shared among the six groups (Figure 5H).

Discussion

The cattle sector has experienced significant growth in recent years, leading to the widespread adoption of intensive farming as the predominant agricultural strategy. Nevertheless, poultry and cattle are susceptible to stress responses in this agricultural approach, resulting in a decrease in animal output, compromised immunological function, and potentially fatal outcomes, hence resulting in substantial financial losses for farms. The hydrogen peroxide model is a classical model for studying immune stress in poultry and livestock (25). Previous studies have found that the negative effects of environmental changes directly impact the health and productivity of poultry, resulting in decreased feed intake, body weight, and immune function (26). However, oxidative stress also severely affects poultry production. Injection of hydrogen peroxide can lead to a decrease in poultry antioxidant capacity, feed-to-weight ratio, and muscle quality, indicating that hydrogen peroxide can directly impact the production performance and immune function of goslings (27). Prior research has indicated that extracts derived from Astragalus, Epimedium, and Ligustrum had therapeutic and preventative properties that enhance stress tolerance, egg production, immunology, and intestinal health in pigeons (28). Meanwhile, feeding weaned piglets with plant-derived Lactobacillus can enhance piglet growth performance, immune function, and regulate intestinal microbiota, among other critical effects (22). However, the therapeutic and preventive effects of the combined use of Astragalus, Epimedium, Ligustrum extracts, and plant-derived Lactobacillus on hydrogen peroxide stress in goslings remain unclear. Therefore, this experiment investigated the impact of Astragalus, Epimedium, Ligustrum extracts, as well as the combination of Astragalus, Epimedium, Ligustrum extracts, and plant-derived Lactobacillus on the immune and antioxidant capabilities of goslings. The results indicate that adding 0.2 g/mL AEF and 0.2 g/mL AEF + 1 g/mL LP per kilogram of gosling body weight to the water can enhance the goslings' stress resistance. Thus, the addition of AEF and AEF + LP to drinking water can promote the growth of goslings and alleviate stress.

When the organism is influenced by external or internal stressors, oxidative stress is generated, leading to the destruction of prooxidants and antioxidants in cells. The reduced activity of antioxidant enzymes in the body results in DNA hydroxylation, protein denaturation, increased lipid peroxidation, and impairment of cellular functions, disrupting the conditions necessary for cell survival. Catalase (CAT), glutathione peroxidase (GSH-PX), and total superoxide dismutase (T-SOD) are important antioxidant markers in the body. In response to external or internal stress stimuli, the organism activates the antioxidant defense system to maintain the oxidative-reductive balance within cells and uphold internal homeostasis (29, 30). When the organism undergoes oxidative stress, MDA, as a biomarker of lipid and protein oxidation, directly reflects the degree of lipid damage in the body (31). Previous studies have shown that *Forsythia suspensa* extract can improve oxidative damage in broiler breast muscles, and the combination of cinnamon and probiotics can regulate oxidative stress and alleviate kidney toxicity in broilers (32, 33). Therefore, this study explores the effects of AEF and AEF + LP on the antioxidant capacity of goslings under stress conditions. The activity of key antioxidant enzymes in serum, liver, and ileum was measured, and the results showed that adding AEF and AEF + LP to drinking water could increase the levels of CAT, T-SOD, and GSH-PX in serum and reduce MDA levels. Adding AEF and AEF + LP to drinking water could increase the levels of CAT, T-SOD, and GSH-PX in the liver and ileum while reducing MDA levels. In conclusion, adding AEF and AEF + LP to drinking water

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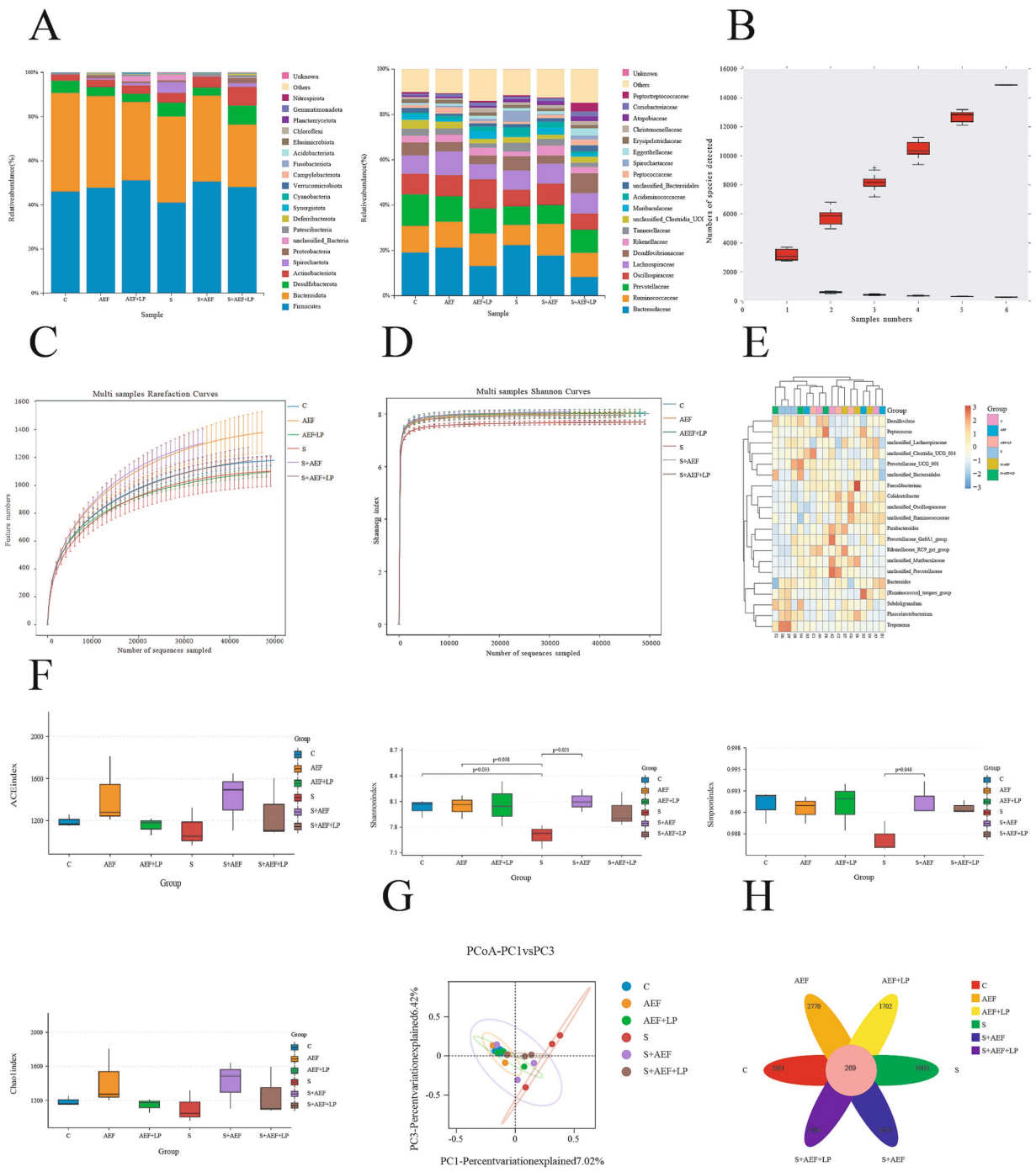

FIGURE 5
Abundance and diversity of cecal contents. **(A)** Relative abundance of 16S rRNA gene sequences at the phylum and family levels in gosling cecal contents (n = 3). **(B)** Species accumulation curve (n = 3). **(C)** Dilution curve (n = 3). **(D)** Shannon index curve (n = 3). **(E)** Heatmap of species composition (n = 3). **(F)** Alpha diversity indices (n = 3). **(G)** Principal coordinate analysis (PCoA) (n = 3). **(H)** Venn diagram (n = 3).

has antioxidant effects and can enhance the oxidative capacity of goslings. Under stress conditions, Astragalus can increase SOD activity and reduce MDA levels in lactating sows (34). Epimedium has the ability to remove extracellular free radicals, decrease levels of reactive oxygen species, boost the activity of antioxidant enzymes, and improve antioxidant function (35). *Lactobacillus plantarum* improves oxidative damage caused by weaning in young goats, effectively reducing MDA content in the small intestine and increasing duodenal GSH-PX activity (36). This experiment evaluated the expression of antioxidant genes in the duodenum and liver, and AEF and AEF + LP significantly enhanced their antioxidant capacity after stress treatment.

Certain components in the serum can reflect the physiological status of animals under stress. Total protein (TP) in the serum comes

from hepatic synthesis and intestinal absorption, with TP mainly comprising albumin (ALB) and globulin (GLB). The more common cause of TP reduction is the decrease in ALB, which is the major protein component of serum TP, playing a role in maintaining plasma osmotic pressure and transporting nutrients (37). GLB is produced by immune organs and has a regulatory role in immune response, anti-inflammation, and antibacterial functions. GLB tends to decrease when the organism is attacked by pathogens. Studies have shown that enzymatic activity and oxidative stress markers, including aspartate aminotransferase (AST) and alanine aminotransferase (ALT), which are important indicators reflecting liver function (38). When the liver is damaged, the levels of ALT and AST significantly increase (39). The concentration variation of serum creatinine (CREA-S) is mainly determined by the glomerular filtration capacity (glomerular filtration rate) of the kidneys. Decreased filtration capacity leads to an elevated concentration of creatinine. Increased blood creatinine levels often signify renal impairment. Studies have demonstrated that Chinese herbal medicine has the ability to significantly promote growth performance, improve blood chemical indicators, exhibit anti-inflammatory qualities, prevent damage, and boost antioxidant capacity (40). Probiotics are commonly employed to enhance blood biochemical parameters in animals. *Lactobacillus acidophilus* is a beneficial bacterium that lives in the intestines, developing a mutually beneficial relationship with the host. On the other hand, *Bacillus subtilis* is a kind of bacterium that may create spores but does not typically reside in the intestines. Supplementing *Lactobacillus acidophilus* and *Bacillus subtilis* in broilers can reduce plasma concentrations of ALT and AST (41). Therefore, this study explores the impact of AEF and AEF + LP on blood biochemical indicators in stressed goslings. The results show that after stress, serum levels of ALT, AST, TC, CREA-S, UREA, CK, TBA, LDH, and P increase, while ALP, TP, ALB, GLB, A/G, and TG levels decrease. The treatment with AEF and AEF + LP induced positive changes in blood biochemical indicators. Studies have shown that astragalus can enhance antioxidant capacity in fish and also has a positive alleviating effect on blood biochemistry (42). Epimedium can improve liver damage caused by the elevation of serum aspartate aminotransferase after oxidative stress (43). Extracts of ligustrum can improve the elevation of lactate dehydrogenase and creatinine in the serum of rats caused by oxidative stress (44). Plant lactobacillus has been confirmed to improve glycerophospholipid metabolism disorder induced by oxidative stress in mice (45).

When the organism is influenced by exogenous or endogenous factors, oxidative stress occurs in the organism. Corticosterone (CORT) is a crucial indicator for assessing oxidative stress. CORT is a glucocorticoid secreted by the adrenal glands, and changes in CORT can reflect the physiological stress of animals. When animals are under stress, CORT levels increase (46). This is consistent with our findings. When the organism is exposed to hydrogen peroxide stress, CORT levels increase, but with AEF and AEF + LP treatment, CORT levels decrease. In a stressed state, the organism activates the hypothalamus-pituitary–adrenal axis, leading to elevated blood glucose levels due to the secretion of glucogenic hormones (glucocorticoids, adrenaline, growth hormone, etc.). Our results are also in line with this pattern, showing a reduction in blood glucose levels with AEF and AEF + LP treatment.

The intestine is the primary organ for nutrient absorption in animals, with nutrients primarily absorbed from the small intestine. The intestinal epithelium is a unique single-layer structure arranged in the intestinal cavity, forming two distinct tissue domains, namely intestinal crypts and villi. Villi are finger-like projections that protrude from the epithelium and lamina propria into the intestinal lumen of the small intestine, serving the function of nutrient absorption. Morphological features of the intestine, such as villus height, crypt depth, and V/C, reflect information about the function and health of the intestinal mucosa. When laying hens are exposed to stressors, the proportion of decreased villus height and crypt depth in the jejunum indicates changes in intestinal morphology (47). In this study, the addition of AEF and AEF + LP to drinking water increased the villus height of the duodenum and jejunum, as well as the V/C of the jejunum. The results indicate that AEF and AEF + LP can restore the villus height of the stressed gosling's intestinal mucosa. We speculate that AEF and AEF + LP may enhance the proliferation and differentiation of intestinal epithelial cells and the renewal rate of intestinal stem cells. Additionally, AEF and AEF + LP promote the expression of Occludin and TJP1 in the stressed gosling's jejunum. Astragalus improves the villus length of the Mexican duck's small intestine and effectively stimulates the mucosal immune barrier, enhancing mucosal immune function (48). Studies suggest that AEF and AEF + LP enhance mucosal integrity, providing a favorable environment for host intestinal health and improving immune resistance to pathogen attacks.

Innate immunity is the first line of defense against the invasion of pathogenic microorganisms in the body. When the body is subjected to external stimuli, it activates a battle mode, increasing the inflammatory response. Inflammatory factors are essential indicators in the process of immune response. When piglets and chickens undergo oxidative stress, it can lead to certain important intestinal diseases and inflammatory reactions (49). Pro-inflammatory factors such as TGF-β, TNF-α, IL-1β, IL-6 significantly increase during oxidative stress (50). The results indicate that when goslings are subjected to H_2O_2 stress, TGF-β, TNF-α, IL-1β, IL-6 are consistent with the above research results. Simultaneously, when AEF and AEF + LP are administered, it can alleviate the inflammatory response. Early weaning can cause intestinal inflammation and diarrhea in animals or humans, and Astragalus has anti-inflammatory activity. They pointed out that Astragalus can improve the expression of IL-6, IL-Iβ, and TNF-α after weaning, and improve the morphology of the jejunum villi (51). *Lactobacillus plantarum*, by optimizing the intestinal barrier, regulating the intestinal microbiota, and inflammatory pathways, reduces IL-1β, TNF-α, and improves the inflammatory response (52).

Poultry has three primary immunological organs: the spleen, thymus, and bursa of Fabricius. The spleen, thymus, and bursa of Fabricius are significant immunological markers that might represent the immune condition of an organism when it experiences a stress reaction (53). The spleen is responsible for filtering pathogens and waste in the blood, clearing old red blood cells, and participating in the regulation of immune reactions in the body. The thymus of poultry plays important roles in immune regulation, metabolic regulation, and developmental regulation. The bursa of Fabricius is the central immune organ in birds, capable of producing B lymphocytes, thereby generating specific antibodies to accomplish specific immune responses. Research indicates that when broilers undergo oxidative stress, the immune organ index and humoral immunity tend to decrease (54). This experiment investigated the

impact of AEF and AEF + LP on immune organ indices in goslings under stress. Our findings suggest that immune organ indices decrease when the organism experiences stress, and this condition is alleviated by the administration of AEF and AEF + LP. Additionally, we investigated the effects of H_2O_2 stress on the meat quality and color of goslings. In this context, L* represents brightness, a* represents redness, and b* represents yellowness. The results indicate that there is no significant change in L*, a*, and b* when subjected to stress or treated with AEF and AEF + LP. The pH value of the stress group is significantly higher than that of the control group at 45 minutes, and there is a decreasing trend in the pH value of the stress group at 24 hours compared to the control group. Drip loss significantly increases under stress. When treated with AEF and AEF + LP, goslings exhibit a decreasing trend in pH at 45 minutes and an increasing trend at 24 hours, significantly alleviating the exacerbation of drip loss caused by stress and improving meat quality.

The gut microbiota can modulate the immune system, regulate intestinal absorption, thereby improving host health and enhancing productivity, playing a crucial role in livestock and poultry health management (55). Piglets who have been weaned and are under oxidative stress suffer from compromised intestinal function, which in turn causes diarrhea. Consequently, this modifies the composition of the intestinal microbiota and affects the overall growth performance (56). Bacteria belonging to the phylum Firmicutes are mostly Gram-positive bacteria, and they play a crucial role in the host's nutrition and metabolism through the synthesis of short-chain fatty acids. Through their metabolic byproducts, bacteria of the phylum Firmicutes indirectly connect with other tissues and organs, regulating hunger and satiety. However, when broilers undergo heat stress, the abundance of Firmicutes is significantly reduced (57). Our research indicates that the Firmicutes phylum is the most abundant bacteria in the cecal microbiota of goslings, constituting over 50% of the total bacterial population. Due to the addition of AEF and AEF + LP to drinking water, there is a shift in the relative abundance of the Firmicutes phylum at the phylum level, which is beneficial for gut health. Ruminococcus is a Gram-positive, anaerobic bacterium that appears spherical and does not produce spores. Ruminococcus is one of the earliest discovered bacteria in the stomach, playing a crucial role in metabolism. It has functions such as stabilizing the intestinal barrier, reversing diarrhea, and increasing energy. Our results indicate that, compared to the control group, there is a significant reduction of Ruminococcus at the genus level in the stress group. However, the relative abundance of Ruminococcus is increased when administered with AEF and AEF + LP. Ruminococcus is one of the most efficient bacteria in carbohydrate decomposition, and in the human intestine, it can reduce dietary resistant starch, thereby regulating intestinal homeostasis and improving gut health (58).

This study demonstrates that adding AEF extract and AEF + LP to drinking water can enhance the intestinal health and growth performance of H_2O_2-stressed goslings. Treatment with AEF and AEF + LP enhances the intestinal bacterial composition, intestinal morphology, immune response, and growth performance of stressed goslings. Furthermore, AEF and AEF + LP exhibit a greater impact on the immune response, intestinal microbiota, and antioxidant capacity of stressed goslings. AEF and AEF + LP can be used as a health product to improve stress in goslings. This study makes a significant contribution to the sustainable development of animal husbandry.

Data availability statement

The data presented in the study are deposited in the NCBI repository, accession number PRJNA1262860.

Ethics statement

The animal study was approved by Zhongkai University of Agriculture and Engineering. The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

QQ: Writing – original draft. YS: Conceptualization, Data curation, Investigation, Methodology, Resources, Validation, Visualization, Writing – review & editing. YL: Formal analysis, Methodology, Supervision, Writing – review & editing. YN: Formal analysis, Methodology, Supervision, Writing – review & editing. MG: Formal analysis, Methodology, Supervision, Writing – review & editing. YY: Data curation, Methodology, Writing – review & editing. WW: Funding acquisition, Writing – review & editing. SZ: Resources, Supervision, Writing – review & editing, Funding acquisition.

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Conflict of interest

YN was employed by Crown Bioscience (Zhongshan) Co., Ltd. YY was employed by Shenyang Weijia Biotechnology 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.

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Effects of folic acid on body folate status, ovarian function, and organ indexes of offspring during embryonic stage

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This experiment aimed to investigate the effect of dietary folic acid supplementation on maternal folate status and ovarian function in female broiler breeders. Fifty 30-week-old Arbor Acres female broiler breeders were randomly divided into two groups (5 pens/group, 5 hens/pen) after 2 weeks of pre-feeding: the control group (Con, supplementation 0 mg/kg folic acid) and the folic acid group (FA, supplementation 4 mg/kg folic acid). The trial lasted 16 weeks. Folate content in eggs, maternal plasma, and offspring plasma (1-day-old) was measured, alongside expression levels of folate transporters in the ovary, liver, duodenum, and jejunum. Ovarian transcriptome analysis was performed. Results showed that the FA group had significantly increased folate deposition in eggs ($p < 0.05$) and offspring plasma folate levels ($p < 0.01$). No significant differences were observed in embryonic organ indexes ($p > 0.05$). The FA group had downregulated mRNA expression of proton-coupled folate transporter (*PCFT*) in the jejunum, folate transporter (*RFC*) in the liver, and folate receptors (*FR*) in the ovary ($p < 0.05$). However, they had upregulated *RFC* in the duodenum ($p < 0.05$). Transcriptome analysis identified 326 differentially expressed genes (217 up-regulated, 109 down-regulated; $p < 0.05$). KEGG enrichment revealed 10 pathways, including cell cycle, Wnt signaling, and steroid biosynthesis. These findings suggest that folic acid enhances ovarian reproductive gene expression and improves folate transfer to eggs and offspring.

KEYWORDS

folic acid, female broiler breeder, folic acid transporter, offspring, ovarian function

1 Introduction

Feed restriction has been necessary for broiler breeders during the rearing and laying periods to prolong breeding duration, improve egg quality, and maintain metabolic disturbances (1, 2). As oviparous species, poultry embryos rely entirely on nutrients deposited during egg formation, making maternal nutrition a critical determinant of offspring embryonic development and post-hatch growth (3). Vitamins play indispensable roles in poultry development, health, and reproductive efficiency (4). Among these, folic acid (vitamin B9), a synthetic form of folate, is essential for DNA synthesis, methylation, and cellular proliferation. Unlike plants and microorganisms, humans, animals, and birds lack the capacity for *de novo* folate synthesis, necessitating lifelong exogenous intake. Beyond folic acid systemic benefits, folate metabolism critically regulates ovarian function, embryogenesis, and pregnancy

maintenance (5). For instance, embryonic folic acid administration alters epigenetic modifications, thereby promoting developmental trajectories (6) and improving post-hatch immune competence and productivity (7). These findings underscore the potential of early folic acid intervention to modulate offspring phenotypes. However, current research on folic acid in poultry nutrition predominantly focuses on commercial broilers during growth phases, with limited exploration of its role in maternal-fetal folate transfer or ovarian transcriptional regulation in breeders. This study aimed to bridge this gap by investigating how dietary folic acid supplementation influences maternal folate status, ovarian transcriptome dynamics, and offspring developmental outcomes, thereby providing insights into optimizing the nutrition of female broiler breeders.

2 Materials and methods

The animal experimental procedures were approved by the Institutional Animal Care and Use Committee of Southwest University of Science and Technology (Permit Number: L2023013).

2.1 Experimental design

The experimental design of this study is shown in Figure 1, and detailed descriptions are as follows: a total of 50 Arbor Acres female broiler breeders were purchased from Shaanxi Huaqin Broiler Chicken Co. Ltd. (Lantian, China). After 2 weeks of pre-feeding, female broiler breeders were stratified by body weight and randomly assigned to two groups using a random number generator (Microsoft Excel). Each group contained 5 pens (5 female broiler breeders/pen), with pens distributed across the housing facility to minimize environmental bias. Each replicate was randomly assigned to receive one of two dietary folic acid levels: (1) a control diet with no supplemental folic acid or (2) a control diet + 4 mg folic acid/kg of diet. The treatment time was 16 weeks. The basal diet was a corn-soybean meal ration formulated to feed the requirements of female broiler breeders consuming 160 g/day. Experimental diets were referenced to the nutritional requirements of NRC (1994); the composition and nutritional levels are shown in Table 1. The light duration was 16 h, and artificial insemination was performed every 3 days at a ratio of 1 male to 10 females. The 7-day-old eggs were collected at 44 weeks of age for incubation, and the eggs were stored in a constant temperature refrigerator at 16–18°C and a relative humidity of 60–65% before hatching. The eggs were incubated using an automatic 96 incubator (9TV-2A, Beijing Blue Sky Electronic Technology Co., Ltd.). The incubation procedure is shown in Table 2.

2.2 Samples collection

At the 48th week, one female broiler breeder per replicate was randomly selected using a randomization protocol. Blood samples were collected from the wing vein, and birds were humanely euthanized following approved animal welfare guidelines. Ovary, liver, duodenum, and jejunum were promptly dissected, and residual blood or intestinal contents were thoroughly rinsed with pre-chilled phosphate-buffered saline (PBS, 4°C). Tissues were flash-frozen in liquid nitrogen to prevent RNA degradation and stored at −80°C until mRNA extraction and analysis.

2.3 Chemical analysis

The content of 5-methyltetrahydrofolate (5-MTHF) in egg yolk was determined according to the method of House et al. (8). The yolks of egg samples were separated, freeze-dried, and stored at −20°C until analysis. Quantitation of 5-MTHF content of egg yolk samples after extraction into an ascorbate buffer (pH 7.4) was analyzed for 5-MTHF via reverse-phase HPLC with fluorescence detection using the method. The purified 5-MTHF folate external standard curve was used to quantify egg folate concentration. The amount of egg folate was expressed as micrograms of folate per egg.

The analytical standard of 5-MTHF (CAS Number: 26560-38-3, Purity: 98%) was purchased from Sigma (St. Louis, MO, United States). HPLC separation was performed with a Shimadzu LC-20 instrument equipped with HPLC-UV system. Folate was separated on an HC-C18 column (250 mm × 4.6 mm, 5 μm)

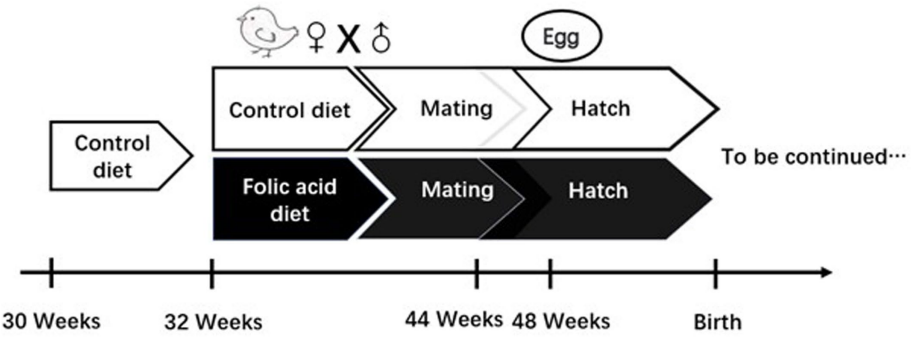

FIGURE 1
Study design investigating effects of folic acid on female broiler breeders.

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TABLE 1 Composition and nutrient levels of the diet for female broiler breeders (air-dry basis).

Items	Control group	FA group
Ingredients (%)		
Corn	60.00	60.00
Soybean meal	21.00	21.00
Wheat bran	6.93	6.93
Soybean oil	2.20	2.20
Limestone	7.70	7.70
Dicalcium phosphate	1.20	1.20
Salt	0.30	0.30
L-Lys·HCl	0.12	0.12
DL-Met	0.13	0.13
Thr	0.07	0.07
Mineral premix[a]	0.15	0.15
Vitamin, including	0.20	0.20
Vitamin A (IU/kg)	12000.00	12000.00
Vitamin D 3 (IU/kg)	3500.00	3500.00
Vitamin E (IU/kg)	100.00	100.00
Vitamin B 1 (mg/kg)	6.60	6.60
Vitamin B 2 (mg/kg)	12.00	12.00
Vitamin K (mg/kg)	4.40	4.40
Niacin (mg/kg)	50.00	50.00
Pantothenic acid (mg/kg)	15.50	15.50
Vitamin B 6 (mg/kg)	4.40	4.40
Biotin (μg/kg)	220.00	220.00
Folic acid (mg/kg)	0.00	4.00
Vitamin B 12 (μg/kg)	22.00	22.00
Choline (mg/kg)	1210.00	1210.00
Nutrition composition[b]		
Metabolizable energy (MJ/kg)	11.34	11.34
Crude protein	15.45	15.45
Lys	0.75	0.75
Met + Cys	0.62	0.62
Thr	0.55	0.55
Calcium	3.20	3.20
Total phosphorus	0.63	0.63
Available P	0.36	0.36

[a]Mineral premix provided the following per kg of diets: Mn 100 mg, Zn 80 mg, Fe80 mg, Cu 8 mg, I 1 mg, Se 0.30 mg.
[b]Nutrient levels were calculated values.

(Agilent, United States), and its temperature was set at 27°C. The mobile phase was phosphate buffer at pH 2.3 (A) and acetonitrile (B) at a flow rate of 0.8 mL/min. The gradient was as follows: 0–4 min, 9% B, 4–12 min, 24% B, 12–14 min, 24% B, and 14–15 min, 9% B. The injection volume was 20 μL, and the autosampler temperature was 27°C. The contents of 5-MTHF and homocysteine (Hcy) in plasma were detected using Elisa (ED-60659, ED-60216, Lun Chang Shuo Biotech, Xiamen, China).

2.4 Embryonic weight and organ indexes

After the embryo or chick was slaughtered, the weight of the embryo or chick, residual yolk, and liver was measured. The remaining yolk weight was expressed relative to the initial egg weight. The organ index was described as the relative weight of the organ weight to the embryo weight without yolk.

2.5 qRT-PCR and RNA-seq analysis

Total RNA of the ovary, liver, and intestinal mucosa (duodenum and jejunum) was extracted using the Trizol Reagent protocol (AG21102, AG, Changsha, China). The concentration, purity, and integrity of RNA samples were verified, and cDNA was synthesized using the *Evo M-MLV* RT Kit for qPCR (AG11707, AG, Changsha, China). The mRNA expression of reduced folate carrier (*RFC*), folate receptor (*FR*) in the ovary, and the mRNA expression of proton-coupled folate transporter (*PCFT*) and RFC in duodenum and jejunum were analyzed with a SYBR® Green Premix Pro Taq HS qPCR Kit (AG11701, AG, Changsha, China) on the iCycler IQ5 (Bio-Rad, Hercules, CA, United States). The primers are listed in Table 2. The total volume of the reaction system was 10 μL: 5 μL of SYBR Green Premix Pro Taq, 0.5 μL upstream primers (10 pmol/μL), 0.5 μL downstream primers (10 pmol/μL), and 4 mL cDNA. The reactions of real-time PCR were carried out at 95°C for 30 s, followed by 40 cycles at 95°C for 5 s, and at 60°C for 30 s. All samples were run in triplicate, and the average cycle threshold (Ct) values were normalized to *β-actin* and quantified by the $2^{-\Delta\Delta Ct}$ method (9). Finally, $2^{-\Delta\Delta Ct}$ values were normalized to the control group. In total, 10 RNA-seq libraries (5 control groups and 5 folic acid groups) were constructed. Clean reads were obtained by removing contained adaptor contamination, low-quality, and undetermined bases. Then, the clean reads sequence quality was assessed by Q20, Q30, and GC content. All downstream analyses were based on clean high-quality data. The difference expression analysis between the two comparison combinations was conducted using DESeq software (1.20.0). The conditions for screening differentially expressed

TABLE 2 Incubation procedures.

Incubation phase	Temperature (°C)	Humidity (%)	Egg turning
E1–E6	38.0	60	Turn the eggs every 1.5 h, for 180 s each time
E7–E12	37.8	55	
E13–E18	37.6	60	
E19–E21	37.2	70	Stop turning

genes were expression difference multiple |log2FoldChange| >2, significance $p < 0.05$. Personalbio Co., Ltd. commissioned the detection and analysis.

2.6 qRT-PCR verification

To ensure the reproducibility of differentially expressed genes obtained from RNA-seq data, six differential expressed genes (DEGs) were randomly selected for qRT-PCR verification in this experiment. The RNA samples used for qRT-PCR were the same as for RNA-seq. The primers are listed in Table 3.

2.7 Statistical analysis

A t-test examined the significant differences between the control and the folic acid groups. All data were analyzed using SPSS 21.0 and expressed as mean ± SEM. Significance levels: $^{*}p < 0.05$ and $^{**}p < 0.01$.

3 Results

3.1 5-MTHF content of yolk and plasma

As shown in Figure 2, dietary folic acid supplementation significantly increased folate deposition in eggs ($p < 0.01$). As shown in Figure 3, for 32 weeks of age, there were no significant differences in plasma 5-MTHF and Hcy contents between the control group and the FA group. At 44 weeks of age, the plasma Hcy concentration of female broiler breeders in the folic acid supplementation group was significantly lower than that in the control group ($p < 0.05$); 5-MTHF content had no significant difference ($p > 0.05$) but was higher than the control group.

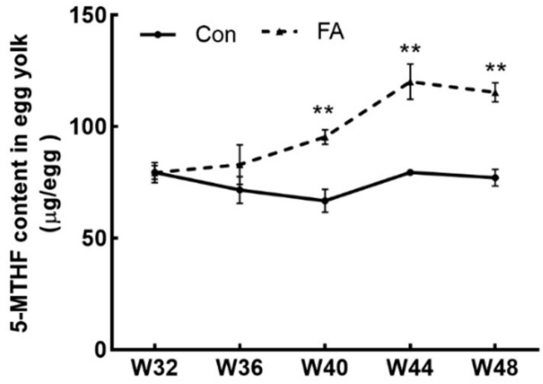

FIGURE 2
Effects of dietary folic acid on 5-MTHF content in eggs of female broiler breeders ($n = 5$). Values are means ± SEM, $^{**}p < 0.01$. Con, control group, FA, folic acid.

TABLE 3 Primer sequence of target genes.

Gene	Accession number	Primer sequences (5′–3′)	Product size (bp)
β-actin	NM_205518.1	F: ATTGTCCACCGCAAATGCTTC	113
		R: AAATAAAGCCATGCCAATCTCGTC	
PCFT	NM_001205066.1	F: GGCTGTGCTCACTTGTGGCTA	153
		F: TGAAGATCCGTTGGGCACTG	
RFC	NM_001006513.1	R: GCTATCTGGAAAATAGCGATGGG	175
		R: GAAGGTTGGAGTCCTGGATTTCTAT	
FR	XM_015280910	F: CATCCAGGATATGTGCTTGTATGA	180
		R: CAGCCCTTGTGCCAGTTCTC	
MYL3	NM_205159.2	F: ACCTAAGAAGGCGCCTGAAC	153
		R: TGAGAACGCTTCCTTAAATTCTTCA	
IHH	NM_204957.3	F: ACAGGGACCGCAACAAGT	120
		R: CAGCCGAGTGCTCTGACT	
WNT4	NM_204783.1	F: TGTGACCACGACCTCAAGAA	160
		R: ACCAGTGGAATTTGCAGCTG	
IL7	NM_001398241.1	F: AGCCTTCGATCTTGCTGGAAT	161
		R: ATCCAAATCCGGCACAAGGT	
GDF6	XM_015282935	F: ACCGGACGGTACTCCAACTA	102
		R: CAGAGCTGCTGAACCGAAGA	
MYH11	NM_205274.3	F: AGCACAACTGCCTTGTCTCC	196
		R: CCTGCAACAGTCCTACAAATCC	

PCFT, proton-coupled folate transporter; *RFC*, reduced folate carrier; *FR*, folate receptor; *MYL3*, myosin light chain 3; *IHH*, Indian hedgehog; *WNT4*, WNT family member 4; *IL7*, interleukin-7; *GDF6*, growth differentiation factor 6; *MYH11*, myosin heavy chain 11.

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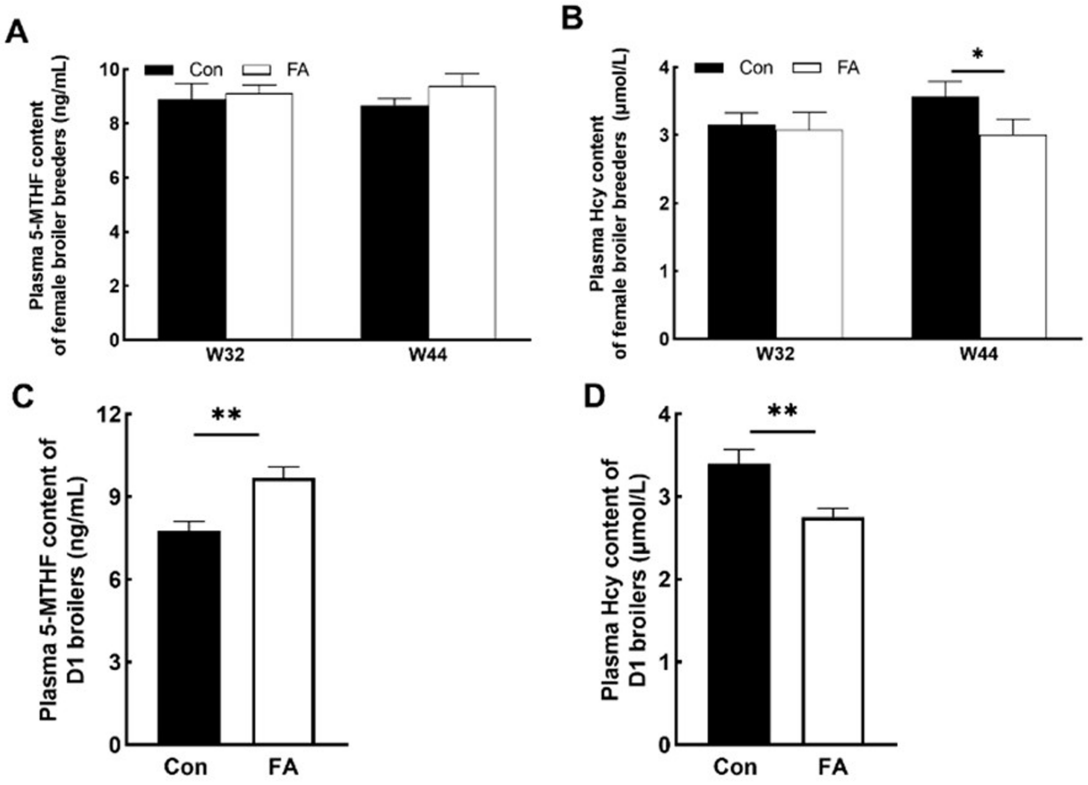

FIGURE 3
Effects of folic acid on 5-MTHF and Hcy content of female broiler breeders and offspring (n = 5). Values are means ± SEM, *p < 0.05 and **p < 0.01. Con, control group, FA, folic acid. **(A)** Plasma 5-MTHF content of female broiler breeders. **(B)** Plasma Hcy content of female broiler breeders. **(C)** Plasma 5-MTHF content of D1 broilers. **(D)** Plasma Hcy content of D1 broilers.

TABLE 4 **Effects of folic acid levels of female broiler breeders on offspring embryonic organ index.**

Items	Age	Con	FA	p-value
Egg weight (g)	—	70.21 ± 0.91	69.29 ± 0.86	0.625
Relative weight of the embryo or chick (g)	E15	14.02 ± 0.68	14.30 ± 0.44	0.738
	E19	34.48 ± 0.66	32.45 ± 1.01	0.119
	D1	42.51 ± 0.96	40.20 ± 0.74	0.199
Liver index (%)	E15	1.57 ± 0.16	1.73 ± 0.06	0.384
	E19	1.79 ± 0.07	1.90 ± 0.06	0.281
	D1	3.05 ± 0.07	3.17 ± 0.08	0.276
Relative weight of the residual yolk (%)	E15	29.17 ± 2.28	25.85 ± 3.40	0.432
	E19	21.43 ± 0.64	20.88 ± 0.95	0.648
	D1	7.43 ± 0.48	8.27 ± 0.52	0.247

3.2 Embryonic development characteristics

As shown in Table 4, there was no significant difference in the embryo weight, liver index, or residual yolk index of the offspring on embryonic day 15 (E15), embryonic day 19 (E19), or postnatal 1st day (D1) when folic acid was added to the diet of female broiler breeders ($p > 0.05$).

3.3 Regulation of folic acid on expression of folic acid transporters

Folic acid absorption in the foregut is mainly dependent on *PCFT* and *RFC*. As shown in Figure 4, the mRNA relative expression of *RFC* in the liver and *PCFT* in the jejunum was significantly decreased in folic acid ($p < 0.05$). In the duodenum, the relative expression of

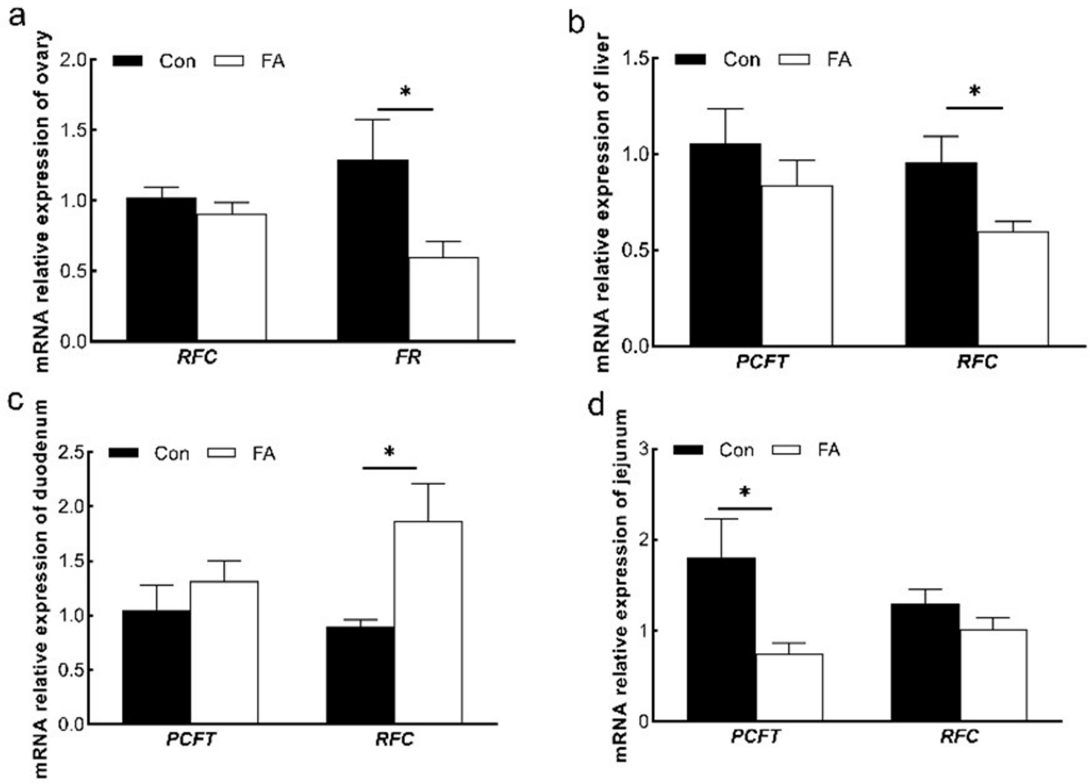

FIGURE 4
Effects of dietary folic acid on expression of folic acid transporter in ovary **(a)**, liver **(b)**, duodenum **(c)**, and jejunum **(d)** of female broiler breeders (n = 5). Values are means ± SEM, *$p < 0.05$. Con, control group, FA, folic acid group; *FR*, folate receptor; *PCFT*, proton coupled folate transporter; *RFC*, reduced folate carrier.

mRNA of *RFC* was increased considerably. *FR* and *RFC* play major roles in the ovary; the mRNA relative expression of *FR* was significantly decreased ($p < 0.05$).

3.4 Effects on the ovary transcriptome profile of female broiler breeders

In the study, we considered and determined the transcriptome profile of the ovary to investigate the effect of folic acid on ovary function because the folic acid supplementation was not significant for female broiler breeder's performance.

This test uses $p < 0.05$ and a difference multiple of more than 2 times as the screening criteria, as shown in Figure 5. (A) The PCA model can effectively separate the differences between the folic acid addition and control groups in this experiment. (B) A total of 326 differentially expressed genes were screened out in the ovaries of female broiler breeders. Compared with the control group, there were 109 up-regulated differential genes and 217 down-regulated genes in the ovaries of the folic acid-supplemented group. (C) Heat map analysis characterizes the differential genes between the two groups, indicating good reproducibility between the repeated samples.

To define the functions of differentially expressed genes (DEGs) caused by folic acid, GO and KEGG were combined to examine the pathways involved (Figure 6). The most enriched GO terms were biological processes related to cell differentiation, signaling, and tissue development. Results of KEGG pathway analysis revealed that 10 signaling pathways were significantly enriched. The 10 considerably enriched signaling pathways involved in the regulation of folic acid are caffeine metabolism, steroid hormone biosynthesis, Wnt signaling pathway, gonadotropin signaling (GnRH) pathway, drug metabolism, adrenergic signaling in cardiomyocyte, melanogenesis, calcium signaling pathway, linoleic acid metabolism, and MAPK signaling pathway. Among them, Wnt signaling pathway, steroid biosynthesis signaling pathway, and GnRH pathway production may be involved in the reproductive process of female broiler breeders.

3.5 Validation of transcriptome by qRT-PCR

To validate the RNA-seq data, the expression levels of six DEGs (namely, *MYL3*, *IHH*, *WNT4*, *IL7*, *GDF6*, and *MYH11*) were determined by qRT-PCR. Although the expression amplitudes of RNA-seq and qRT-PCR are different, the expression regulation trends in the two technologies are consistent (Figure 7). Correlation analysis showed that the expression levels of the validated genes were highly correlated between RNA-seq and qRT-PCR. Therefore, the results of RNA-seq are reliable and consistent with the results of qRT-PCR analysis.

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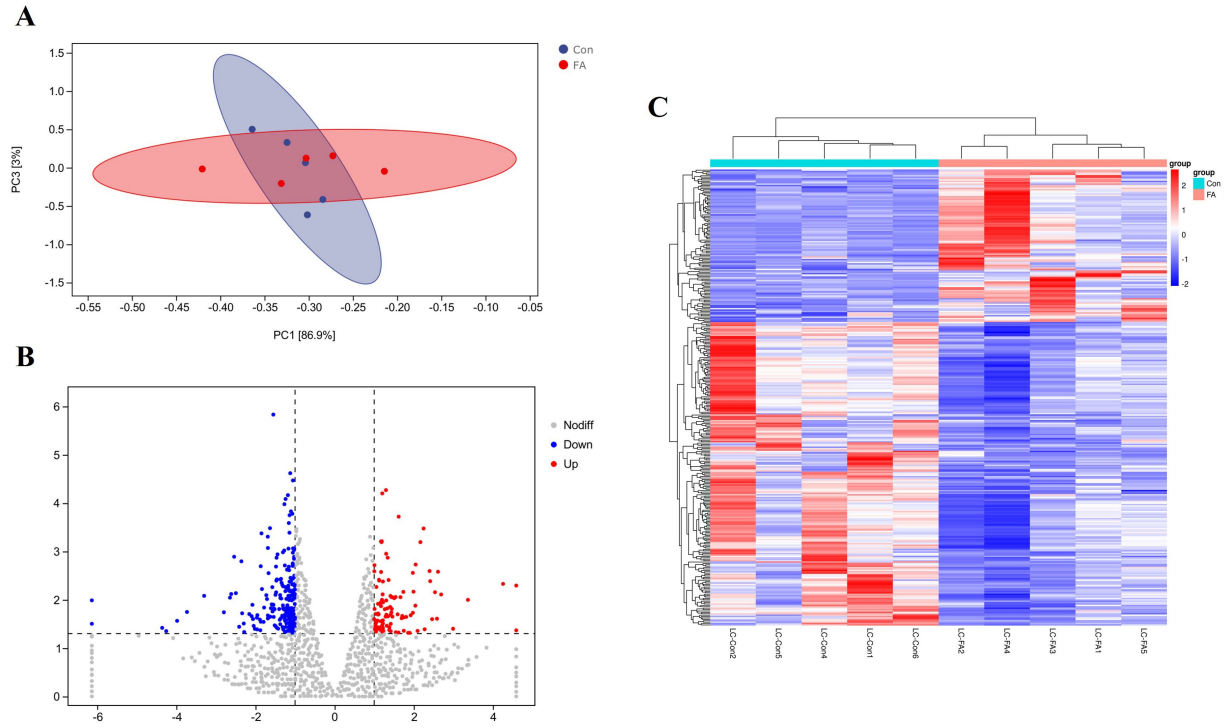

FIGURE 5
Differential expression analysis of folic acid on ovary transcriptome of female broiler breeders (n = 5). **(A)** Principal component analysis plot. **(B)** Volcano plot of differentially expressed mRNAs. **(C)** Clustering heat map of differentially expressed mRNAs. Red indicates highly expressed genes and green indicates lowly expressed genes.

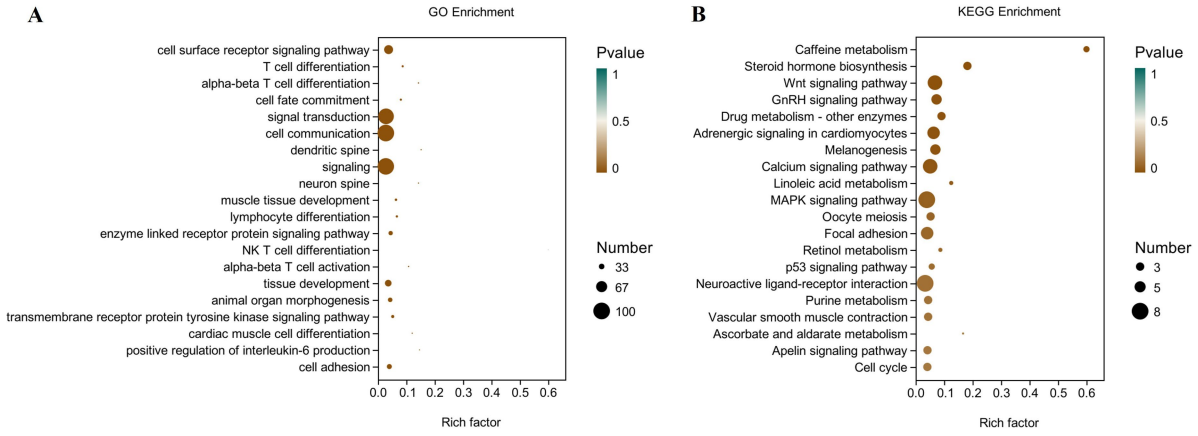

FIGURE 6
Scatter plot of GO and KEGG enrichment analysis of differentially expressed genes in the ovary of female broiler breeders (n = 5). **(A)** GO enrichment. **(B)** KEGG enrichment.

4 Discussion

This study demonstrates that dietary folic acid supplementation significantly increases folate deposition in hatching eggs, consistent with prior findings (10–12). Specifically, yolk 5-methyltetrahydrofolate (5-MTHF) increased by 120% in the FA group compared to controls (Con), reaching 120 μg/egg. While earlier studies reported lower deposition levels (54.5 μg/egg with 15 mg/kg supplementation) (13), this discrepancy likely stems from differences in detection methodologies (HPLC vs.

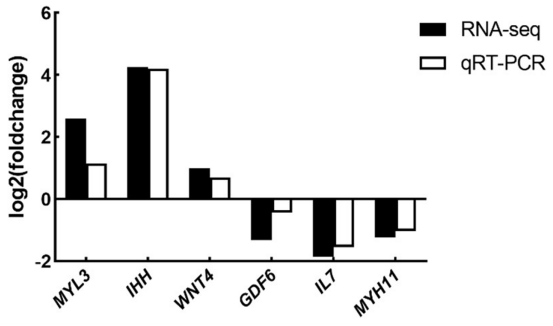

FIGURE 7
Expression levels of selected DEGs quantified by qRT-PCR (n = 5). Differential expression analysis of folic acid on ovary transcriptome of female broiler breeders (n = 5). *MYL3*, myosin light chain 3; *IHH*, Indian hedgehog; *WNT4*, WNT family member 4; *IL7*, interleukin-7; *GDF6*: growth differentiation factor 6; *MYH11*, myosin heavy chain 11.

ELISA) and egg weight variations. Notably, folate deposition exhibited saturation kinetics, as higher dietary folate levels did not proportionally increase egg folate content, suggesting a transport-limited mechanism. Blood folate serves as the precursor pool for egg deposition, and the elevated plasma-to-egg folate ratio observed here confirms efficient maternal-fetal transfer (8, 10). High levels of Hcy can easily lead to follicular atresia, reduce the quantity and quality of oocytes, and affect early embryonic development (14). In this experiment, folate metabolism reduced plasma Hcy levels while elevating methionine availability.

Intestinal folate absorption is dynamically regulated by substrate concentration. In this study, folic acid supplementation downregulated mRNA expression of *RFC* and *PCFT* in the jejunum and liver, contrasting with duodenal *RFC* upregulation. This aligns with reports showing dose-dependent duodenal *RFC* responses (15, 16) and jejunal suppression under high folate (17, 18). We propose that folate concentration dictates transporter prioritization, with *RFC* dominating at moderate levels and passive diffusion prevailing at saturation. In ovarian tissue, folic acid significantly reduced *FR* expression, potentially reflecting epigenetic silencing via folate-driven DNA hypermethylation (19). Paradoxically, despite *FR* downregulation, ovarian folate deposition increased, implying alternative transport mechanisms. Elevated *FR* in ovarian cancer (20) and its suppression here suggest folate sufficiency may mitigate pathological overactivation, preserving follicular integrity.

The ovary orchestrates egg production through folliculogenesis and hormonal regulation. Egg weight significantly influences both hatching rates and chick viability (21). RNA-seq analysis identified 326 DEGs, with significant enrichment in five pathways. Among them, neuroactive ligand-receptor interaction may be the most important pathway leading to the difference in egg production rate between high- and low-producing laying hens. Six DEGs (*GRPR*, *GRP*, *P2RX2*, *GALR1L*, *ADORA1*, and *MTNR1A*) were implicated in modulating neurotransmitter signaling, potentially enhancing hypothalamic-pituitary-ovarian (HPO) axis activity and ovulation frequency (22, 23). The steroid biosynthetic pathway, a known target of endocrine-disrupting chemicals (24), was significantly enriched in high-yielding ducks (25, 26). Ovarian steroid hormones, such as estrogen, regulate follicular dynamics by balancing proliferation and apoptosis signals (27). Concurrently, gonadotropin-releasing hormone (GnRH) coordinates the HPO axis to regulate gonadotropin secretion and sex hormone biosynthesis (28–30). GnRH pathway activation may explain improved follicular maturation in the FA group female broiler breeders. Mechanistically, upregulation of *WNT4* and GnRH pathway genes suggests that folate promotes follicular maturation and steroidogenesis. *WNT4* activates β-catenin signaling, which synergizes with FSH to stimulate granulosa cell proliferation and estrogen synthesis (31). Paradoxically, folate supplementation suppressed *FR* expression, likely via DNA hypermethylation at CpG islands in the *FRα* promoter, a phenomenon linked to reduced ovarian cancer risk (19). Despite *FR* downregulation, ovarian folate deposition increased, suggesting compensatory mechanisms such as *RFC*-mediated transport or passive diffusion at high folate concentrations. Collectively, these mechanisms enhance follicular reserve and oocyte quality, aligning with increased plasma folate deposition in eggs. Our findings position folic acid as a multi-target regulator of ovarian function, enhancing reproductive efficiency through epigenetic, transcriptional, and metabolic pathways.

5 Conclusion

Folic acid can increase the expression of genes related to ovarian reproductive function and promote the deposition of folic acid in breeding eggs and offspring. In summary, folic acid has a positive regulatory effect on ovarian function in female broiler breeders.

Data availability statement

The data presented in the study are deposited in the NCBI SRA repository, accession number PRJNA1269787.

Ethics statement

The animal study was approved by Institutional Animal Care and Use Committee of Southwest University of Science and Technology. The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

JZ: Conceptualization, Data curation, Funding acquisition, Methodology, Writing – original draft, Writing – review & editing. ZL:

Data curation, Software, Writing – original draft. JW: Data curation, Methodology, Writing – original draft. YZ: Conceptualization, Writing – original draft.

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Conflict of interest

YZ was employed by Shanxi Dayu Bioengineering 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.

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Effects of maternal and post-weaning supplementation with microbe-derived antioxidants on sow and piglet performance, oxidative status, and gut microbiota

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The antioxidants were found to improve inflammatory responses and redox status. This study investigated the effects of maternal and post-weaning supplementation with microbe-derived antioxidants (MA) on sow performance, redox status, and fecal microorganisms, as well as the growth performance, inflammatory responses and intestinal microbiota of weaned piglets. Sixty multiparous sows were randomly allocated to the control group (CON, basal diet) and the MA group (basal diet supplemented with 2.0 g MA/kg) from d 90 of gestation to d 24 of lactation, according to the parity and body condition. At weaning, a total of 80 piglets per group were selected and randomly assigned to either the basal diet or the MA-supplemented diet, with 10 pens per group and 4 piglets per pen, for a period of 21-day trial. Results showed that maternal MA supplementation increased litter size at weaning ($p < 0.05$) and the milk contents of dry matter ($p = 0.08$) and fat ($p = 0.09$), while decreasing the plasma activities of alanine aminotransferase and aspartate aminotransferase in sows on d 24 of lactation ($p < 0.05$). Moreover, maternal MA supplementation reduced plasma malondialdehyde concentration ($p \leq 0.01$) in sows at farrowing and weaning, as well as catalase activity at weaning ($p = 0.01$), and tended to increase total antioxidant capacity at farrowing ($p = 0.08$). Additionally, the fecal contents of butyrate ($p = 0.04$) and propionate ($p = 0.09$) were higher in sows receiving the MA diet at d 24 of lactation. In post-weaning piglets, maternal MA supplementation increased average daily gain ($p = 0.07$) and average daily feed intake ($p < 0.05$) throughout the period, and increased plasma immunoglobulin G and interleukin-10 concentrations ($p < 0.05$). Additionally, either maternal or post-weaning MA supplementation positively influenced the gut microbiome of both sows and weaned piglets. In conclusion, maternal MA supplementation during late gestation and lactation increased litter size at weaning, which may be associated with the improved milk quality and redox status. Furthermore, maternal MA supplementation may enhance the growth performance of post-weaning piglets, potentially linking to the improvements in immunological parameters and gut microbiome.

KEYWORDS

sows, piglets, reproductive performance, antioxidant, microorganisms

1 Introduction

During late gestation and lactation, sows experience stress due to environmental and physiological changes, which include feeding restrictions, housing modifications, and farrowing transitions. The perinatal period is particularly critical for both dam and offspring, as it brings significant changes to maternal metabolism, redox status, and microbiota (1, 2). Physiological changes that occur in sows during farrowing, such as the production of prostaglandins, prostacyclin, and tissue damage, can lead to inflammatory reactions in sows (3). Additionally, the levels of pro-inflammatory factors during early lactation are significantly higher than those during early pregnancy or late lactation (4). Furthermore, oxidative stress is observed during late gestation and lactation, which would adversely affect milk production, reproductive efficiency, and sow longevity (5).

Weaning represents a pivotal stage for the growth and development of piglets. Weaning stress, which includes dietary changes, maternal separation, environmental alterations, and more, can lead to a series of issues in piglets, such as diarrhea, morbidity, growth check etc. (6, 7). Various feed additives, such as cysteamine, plant extracts, probiotics, and postbiotics, had been shown to alleviate weaning stress by reducing diarrhea, improving redox status and microbiota composition (8–10). Recent studies have demonstrated that maternal nutrition or supplementation with functional components (such as postbiotics) can promote improvements in both the growth performance and health of offspring (11–13).

Postbiotics, also called inactivated probiotics, are a complex mixture of inactivated bacterial cells generated through fermentation and probiotic metabolites, such as secreted proteins, peptides, enzymes, proteins, vitamins, and exopolysaccharides (EPSs), which provide overall health benefits to animal (14, 15). Studies have shown that maternal supplementation with yeast-derived postbiotics can decrease piglet mortality and the diarrhea index (16). The postbiotic consisting of heat-inactivated *Lactobacillus fermentum* and *Lactobacillus delbrueckii* improved the growth performance of nursery pigs, which was related to enhanced intestinal health and increased diversity and abundance of beneficial microbiota in pigs challenged with F18$^+$ *E. coli* (17). Meanwhile, *Lactobacillus reuteri* postbiotics can also improve antioxidant function and reduce piglet mortality by regulating the gut microbiota (18).

Microbe-derived antioxidants (MA) are a mixture of fermented and inactivated *Sea buckthorn* and *Rosa roxburghii* combined with probiotics, containing numerous bioactive compounds such as isoflavones, flavone, glutathione, and polyphenols etc. (19, 20). As a type of postbiotic, studies have been reported that MA exhibits free radical scavenging ability (21), inhibits oxidative stress and inflammation (22), possesses hypolipidemic and anti-inflammatory properties (12, 23). Dietary supplementation with MA during gestation and lactation in rats can alleviate high-fat diet-induced production of hepatic nitrogen radical, increase antioxidant enzyme activity, and attenuate lipid accumulation and NLRP3 inflammasome levels (12). Considering these potential benefits, we hypothesized that MA could enhance the productive performance and health status of sows and their offspring. Therefore, this study aimed to investigate the effects of maternal and post-weaning MA supplementation on the productive performance of sows and their offspring, gut microbiota, redox status and immunology-related parameters.

2 Materials and methods

2.1 Experimental materials

Microbe-derived antioxidants was provided by Shanghai Chuangbo Ecological Engineering Co., Ltd. (Shanghai, China). Briefly, it was produced through a multi-stage complex fermentation process involving *Rosa roxburghii* and *Sea buckthorn* with *Bacillus Subtilis*, *Lactobacillus*, *Clostridium Butyricum*, and *Saccharomyces cerevisiae*. The process included extraction, concentration, inactivation, and freeze-drying. MA exhibited a fermented taste, with the main bioactive compounds being 1.37% isoflavones, 194,000 U/100 g superoxide dismutase, 886 mg/100 g glutathione, and 82.4 mg/100 g total saponins (19, 20).

2.2 Animals, experiment design and management

Sixty Landrace × Yorkshire sows (LY, parity 4.06 ± 0.24) at 90 days of gestation were randomly assigned to two groups based on parity and body condition, with 30 replicates for each group and one sow per replicate ($n = 30$). The control group (CON) received basal diet, whereas the treatment group (MA) received the basal diet supplemented with 2.0 g MA/kg. The nutritional requirements for the basal diet of sows during gestation and lactation were based on the National Research Council (2012) (NRC, United States), and the compositions and nutritional levels of the diets were detailed in Supplementary Table S1. The sow trial extended from d 90 of gestation to d 24 of lactation.

At weaning, a total of 80 Duroc × Landrace × Yorkshire (DLY) piglets were selected from each group based on their litter (2–3 piglets per litter) and body weight (BW, the average weight per litter). The 80 piglets from each group of sows were then randomly assigned to receive either a basal diet or a basal diet supplemented with 2.0 g MA/kg. The two factors, maternal diet and post-weaning diet, were arranged in a 2 × 2 factorial design, resulting in four treatment groups, with 10 pens for each group ($n = 10$) and 4 piglets per pen, as follows: CON. CON (basal diet for both sows and piglets), CON. MA (basal diet for sows and MA diet for piglets), MA. CON (MA diet for sows and basal diet for piglets) and MA. MA (MA diet for both sows and piglets). The basal diet was formulated according to the nutritional requirements of 5–7 kg and 7–11 kg piglets, as outlined by the NRC (2012), and the ingredients and nutrient composition were provided in Supplementary Table S1. After 3 days of pre feeding, the piglets trial lasted 21 days until 24 d post-weaning.

The feeding, management, and immunization procedures were conducted according to the farm's standards for gestating and lactating sows and weaned piglets. During gestation, all the sows were housed in individual stalls and fed 3 kg/d of gestation-period diet twice a day (08:00 and 15:00), with access to water *ad libitum* throughout the study. On d 110 of gestation, the sows were moved to the farrowing room and the day of parturition was defined as d 0 of lactation. The sows were no feed on the day of farrowing, 2 kg/d of diet on the following 3 days, and then feed freely twice a day (8:00, 16:00). Feed allocation and refusals were recorded daily during lactation. At farrowing, the numbers of live, stillborn, and mummified piglets, along with their birth weight, were recorded. Cross-fostering occurred within 48 h post-farrowing and the litters were adjusted to consist of 11–12 piglets within the same

treatment. The numbers and weights of piglets were recorded after standardizing the litters and again at weaning. Two sows in the CON were eliminated due to fever and illness before production. Additionally, the piglets had free access to water throughout lactation but did not have access to creep feed. Weaned piglets were fed *ad libitum* four times a day (08:00, 12:00, 16:00, and 20:00). During the trial, weaned piglets had free access to water; pens were regularly cleaned, disinfected, and dewormed, with stable air circulation and temperature maintained. Fecal scores of pigs were recorded daily following observations of the individual piglet and signs of stool consistency in the pen. The calculation of fecal scoring criteria and diarrhea index was performed as previously described by our group (24). On days 0, 7, 14, and 21 of formal trial, all piglets were weighed and feed intake was recorded. The average daily gain (ADG), average daily feed intake (ADFI) and feed to gain ratio (F/G) were calculated for each pen.

2.3 Samples collection

Colostrum samples (15 mL) were collected from 8 sows per group ($n = 8$) before any piglets sucked, and milk samples (15 mL) were obtained from the same sows ($n = 8$) on d 10 of lactation. The milk samples were collected using 2 mL of oxytocin via the ear vein, as previously described (11). All samples were stored at −20°C for subsequent analysis.

Blood samples were collected in sodium heparin-anticoagulated vacuum tubes. Fasting blood samples (10 mL) were collected from marginal ear vein of 8 sows per group ($n = 8$) at 1 h post-farrowing and on d 24 of lactation, before the morning meal. Fasting blood samples (5 mL) were collected from the anterior vena cava of 8 piglets (one per pen, $n = 8$) per group on days 0 and 21 of formal trial. The blood was maintained at room temperature for 30 min, then centrifuged at 3,000 × g and 4°C for 10 min. The supernatant plasma was collected and stored at −20°C for later analysis.

Eight healthy sows ($n = 8$) were randomly selected from each group, and fresh fecal samples were collected immediately after defecation (one sample per pig). The samples were collected in sterile centrifuge tubes 1 h after the first piglet was born and on d 24 of lactation, placed in liquid nitrogen, and then stored at −80°C for microbial analysis. At the end of the weaned piglet trial, 8 pigs per group (one per pen, $n = 8$) were selected and anaesthetized with a lethal injection of sodium pentobarbital (200 mg/kg BW) following an overnight fast. The abdomen was then immediately opened, the jejunum was removed, emptied, and washed with normal saline. The middle section of the jejunum (1.5 cm) was collected and fixed in a 4% paraformaldehyde solution for histological analysis. Additionally, colonic chyme from the mid-region of the colon was collected in sterile centrifuge tubes, quickly placed in liquid nitrogen, and then stored at −80°C for microbial analysis.

2.4 Milk and plasma indicators analysis

The contents of fat, protein, urea nitrogen and lactose were determined using a multifunctional dairy analyzer (MilkoScan FT+, FOSS, Sweden). The dry matter (DM) was measured using method (930.15) of AOAC (2019) (25).

Alanine aminotransferase (ALT), aspartate aminotransferase (AST) and gamma glutamyl transferase (γ-GGT) in sow plasma were measured using reagent kits (CH0101201, CH0101202, CH0101204; Maccura, Chengdu, China) on an automatic biochemistry analyzer (3,100, HITACHI, Tokyo, Japan). The activities of catalase (CAT), total antioxidant capacity (T-AOC), and total superoxide dismutase (T-SOD), along with the concentrations of malondialdehyde (MDA), protein carbonyl (PCO) and inhibition and produce superoxide anion (O_2^-), were quantified using corresponding assay kits (No. A007-1-1, A015-1-2, A001-1-2, A003-1-2, A087-1-2 and A052-1-1; Jiancheng Bioengineering, Nanjing, China), following the manufacturer's instructions. The concentration of protein was determined using the bicinchoninic acid (BCA) method with a BCA protein assay kit (No. A045-3-2, Jiancheng Bioengineering, Nanjing, China).

In piglet plasma, IgG, IgM and complement 3 (C3) were measured using reagent kits (CH0105306, CH0105308, CH0105309; Maccura, Chengdu, China) on an automatic biochemistry analyzer (3,100, HITACHI, Tokyo, Japan). The levels of interleukin-10 (IL-10), tumor necrosis factor (TNF-α) and interleukin-1β (IL-1β) were measured using ELISA kits (No. P1000, PTA00 and PLB00B; R&D Systems China Co., Ltd., Shanghai, China), following the manufacturer's instructions.

2.5 Histomorphology measurements

Jejunum samples of the piglets, preserved in 4% paraformaldehyde solution, were dehydrated and infiltrated with paraffin wax. This procedure was performed as previously described by our group (24). The samples were sectioned to a thickness of 5 μm using a microtome (HistoCore MULTICUT, Leica, Wetzlar, Germany) and stained with periodic acid-Schiff. Crypt depth (CD) and villus height (VH) of each sample were measured using Image-Pro Plus 6.0 software (Media Cybernetics, Maryland, United States) with a digital trinocular microscope camera (BX43F, Olympus, Tokyo, Japan). At least 10 well-oriented villi and crypt columns were counted for each sample, and the ratio of villus height to crypt depth (VH/CD) was calculated. Simultaneously, goblet cells and the corresponding villus area were measured, and the density of goblet cells was calculated.

2.6 Short-chain fatty acids analysis

Sow feces were prepared using methods previously described by our group (24). Briefly, approximately 2 g of feces was mixed with ultra-pure water (1:1, w/v) and then centrifuged for 10 min at 12,000 × g and 4°C. The supernatant was mixed with 25% metaphosphoric acid and crotonic acid, incubated and recentrifuged. Subsequently, the supernatant was mixed with methanol and recentrifuged again. Finally, the supernatant was filtered through a 0.22 μm filter membrane, and the short-chain fatty acids (SCFAs) concentrations were determined using a gas chromatograph system (CP-3800, Varian, Palo Alto, United States).

2.7 Microbial analysis

The microflora structure in sow feces and piglet colonic chyme was analyzed using 16S rRNA amplicon sequencing technology. This procedure was performed as previously described by our group (24). Total DNA was extracted from the samples using the Fecal DNA Isolation Kit (No. D4015-01, Omega, Norcross, United States) according

to the manufacturer's instructions. DNA purity and concentration were analyzed using 1.5% agarose gel electrophoresis and a spectrophotometer (Nanodrop 2000, Thermo Fisher Scientific, Waltham, United States). The qualified DNA samples were sent to Novogene Bioinformatics Technology (Beijing, China) for sequence analysis. The V4 hypervariable regions of the 16S rRNA gene were amplified with primers 515F (5′-GTGCCAGCMGCCGCGGTAA-3′) and 806R (5′-GGAC TACHVGGGTWTCTAAT-3′). Pyrosequencing of bacterial 16S rDNA was performed using the Illumina NovaSeq platform to generate 250 bp paired-end reads. The paired-end reads were merged using FLASH (V1.2.11), quality filtering was performed, and chimera sequences were removed to obtain effective tags. The effective tags were assigned to operational taxonomic units (OTUs) using the Uparse software (V7.0.1001) with 97% sequence similarity. Subsequent analysis of a-diversity and b-diversity was based on these normalized output data. Species annotation and α-diversity analysis were performed using QIIME software (V1.7.0). The LEfSe analysis was performed using LEfSe software (V1.0).

2.8 Statistical analysis

The data were normally distributed after testing homogeneity of variances and normal distribution using the Shapiro–Wilk method in SAS 9.4 (SAS Institute Inc., Cary, NC). The measurement data from sows were analyzed using the *T*-test procedure in SAS 9.4. Reproductive performance was measured in litters, while other indicators of sows were assessed in duplicate individuals. The data from weaned piglets were analyzed using the MIXED procedure in SAS 9.4 for split-plot analysis of variance and significance testing. The statistical models included the effects of sow diet MA levels (0 or 2.0 g/kg), piglet diet MA levels (0 or 2.0 g/kg), and their interaction. Piglet weaning BW was treated as a random effect, and the Tukey method was used for multiple comparisons. Except for growth performance and the diarrhea index, measured in pens, all other indicators were assessed in individual subjects. Data were presented as means ± SE, and differences were considered statistically significant at $p < 0.05$, while results with $0.05 \leq p < 0.1$ were defined as trends.

3 Results

3.1 Reproductive performance

As shown in Table 1, maternal MA supplementation did not significantly alter the number of piglets born alive, stillborn piglets and total born piglets, the litter weight of total born and born alive piglets, or the duration of farrowing. However, the litter size at weaning was significantly increased in the MA group compared to the CON group ($p < 0.05$).

3.2 Composition of colostrum and milk

Maternal MA supplementation tended to increase the contents of dry matter ($p = 0.08$) and fat ($p = 0.09$) in the milk compared to the CON group (Table 2).

TABLE 1 Effect of maternal MA supplementation on reproductive performance of sows and growth performance of suckling piglets[1].

Item	CON	MA	*p*-value
Birth, piglets/litter			
Litter size (total born), no	14.46 ± 0.60	14.46 ± 0.61	0.99
Born alive, no	12.57 ± 0.79	13.14 ± 0.58	0.56
Stillborn piglets, no	1.64 ± 0.64	1.10 ± 0.24	0.44
Litter weight of total born, kg	19.17 ± 1.05	19.46 ± 0.68	0.76
Total born weight per piglet, kg	1.34 ± 0.06	1.33 ± 0.04	0.91
Litter weight of born alive, kg	17.27 ± 1.28	18.19 ± 0.68	0.51
Born alive weight per piglet, kg	1.37 ± 0.06	1.38 ± 0.03	0.80
Duration of farrowing, min	215 ± 27	186 ± 14	0.35
After cross-fostering			
Litter size by cross-fostering, no	11.75 ± 0.18	11.97 ± 0.20	0.28
Litter size at weaning, no	10.74 ± 0.20[b]	11.53 ± 0.55[a]	0.02
Litter weight by cross-fostering, kg	20.11 ± 0.64	19.95 ± 0.67	0.87
Litter weight at weaning, kg	62.30 ± 2.26	62.30 ± 2.02	0.99
Feed intake during lactation, kg/d	5.86 ± 0.17	5.79 ± 0.11	0.74

CON, control; MA, microbe-derived antioxidants. [a,b]Mean values within a row with different letter superscripts are significantly different ($P < 0.05$). [1]Values are means ± standard error; $n = 28$, $n = 30$ for the CON and MA groups, respectively.

3.3 Plasma metabolites and antioxidant enzyme activities

Maternal MA supplementation significantly reduced the levels of ALT ($p = 0.01$), AST ($p = 0.03$), MDA ($p < 0.01$), and CAT activity ($p = 0.01$) in the plasma of sows at weaning, as well as the level of MDA in the plasma at farrowing ($p = 0.01$). Additionally, it tended to increase T-AOC activity in the plasma of sows at farrowing ($p = 0.08$) (Table 3).

3.4 Short-chain fatty acids

Maternal MA supplementation significantly increased ($p < 0.05$) butyrate levels and tended to increase ($p = 0.09$) propionate levels in sow feces at weaning (Figure 1B), but had no significant effect on acetate, propionate, or butyrate levels in feces at farrowing (Figure 1A).

3.5 Microbiota composition

A total of 1800 and 1933 OTUs were assigned at farrowing (Figure 2A) and 2,230 and 2,182 OTUs were assigned at weaning (Figure 2C) in the CON and MA groups, respectively. Compared to the CON group, which had 500 unique OTUs, the MA group had more unique OTUs (633) at farrowing (Figure 2A). However, at d 24 of lactation, the MA group had 645 unique OTUs, which was 48 fewer than the 693 unique OTUs in the CON group (Figure 2C). Maternal MA supplementation had no effect on the α-diversity indices (Chao1, Shannon, and Simpson) at both farrowing and weaning (Supplementary Table S2).

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TABLE 2 Effect of maternal MA supplementation on the composition of colostrum and milk of sows[1].

Item	CON	MA	*P*-value
Colostrum			
Dry matter, %	25.17 ± 0.70	26.20 ± 0.55	0.26
Protein, %	15.05 ± 0.60	16.29 ± 0.60	0.11
Fat, %	3.78 ± 0.30	3.61 ± 0.18	0.64
Lactose, %	3.05 ± 0.08	2.98 ± 0.08	0.58
Urea nitrogen, mg/dL	62.58 ± 2.11	66.04 ± 2.80	0.34
Milk			
Dry matter, %	20.17 ± 0.34	22.04 ± 0.95	0.08
Protein, %	5.05 ± 0.14	5.21 ± 0.08	0.34
Fat, %	7.09 ± 0.35	8.82 ± 0.89	0.09
Lactose, %	5.93 ± 0.09	5.84 ± 0.10	0.53
Urea nitrogen, mg/dL	55.91 ± 1.07	58.87 ± 2.15	0.23

CON, control; MA, microbe-derived antioxidants. [1]Values are means ± standard error; $n = 8$ for each group.

LEfSe analysis (LDA Score > 2.5) identified 4 species (p_Desulfobacterota, f_Bacteroidales_RF16_group, *g_UBA1819*, and *g_Bacteroidales_RF16_group*) and 5 species (*g_Acidaminococcus*, *g_Lachnospiraceae_NK4B4_group 7*, *g_Cellulosilyticum*, *s_bacterium_PS* and *s_Ruminococcus_champanellensis*) enriched in the sows of CON and MA groups at farrowing, respectively (Figure 2B). In contrast, at d 24 of lactation, 9 enriched species were identified in the MA group compared to 4 species (p_Firmicutes, c_Fibrobacteria, *g_Frisingicoccus*, and *g_Pyramidobacter*) in the CON group (LDA Score > 3.0) (Figure 2D). Among the 9 species, 4 belonged to p_Bacteroidota (p_Bacteroidota, c_Bacteroidia, o_Bacteroidales, and *g_Alloprevotella*), 2 belonged to o_Propionibacteriales (f_Nocardioidaceae and *g_Nocardioides*), 2 belonged to p_Firmicutes (*g_Veillonella* and *s_Clostridium_baratii*), and *g_horsej_a03* belonged to f_Oligosphaeraceae.

3.6 Growth performance of piglets

As shown in Table 4, maternal MA supplementation increased the BW of piglets on d 7 ($p = 0.05$) and d 21 ($p = 0.08$) post-weaning, increased the ADG on days 1–7 ($p < 0.05$) and for the entire period (1–21 d, $p = 0.07$), also increased the ADFI on days 8–14, 15–21 and across the entire period ($p < 0.05$).

3.7 Immunological and inflammatory parameters

As shown in Table 5, maternal MA supplementation tended to increase the levels of C3 ($p = 0.07$) and IL-10 ($p = 0.06$) and reduce the level of TNF-α ($p = 0.07$) in the plasma of piglets at weaning. Regardless of post-weaning MA supplementation, maternal MA supplementation significantly increased ($p < 0.05$) the levels of IgM and IL-10 in the plasma of piglets on d 24 post-weaning. Compared to the CON. CON group, the levels of IgM and IL-10 were markedly increased by 54.5 and 188.5% in the MA. MA group, respectively.

TABLE 3 Effect of maternal MA supplementation on metabolic and redox status-related parameters in plasma of sows[1].

Items	CON	MA	*P*-value
At parturition			
ALT, U/L	25.13 ± 1.50	25.73 ± 2.28	0.83
AST, U/L	29.52 ± 3.75	30.49 ± 5.73	0.89
γ-GGT, U/L	32.48 ± 3.95	29.17 ± 3.57	0.54
MDA, nmol/mL	2.41 ± 0.17[a]	1.89 ± 0.09[b]	0.01
O_2^-, U/L	205.21 ± 10.64	216.22 ± 10.64	0.47
PCO, nmol/mL	247.82 ± 7.58	247.46 ± 18.34	0.98
T-SOD, U/mL	94.97 ± 1.05	95.16 ± 1.21	0.90
T-AOC, U/mL	1.69 ± 0.03	1.78 ± 0.05	0.08
CAT, U/mL	5.52 ± 0.88	4.73 ± 0.32	0.41
At day 24 of lactation			
ALT, U/L	47.59 ± 4.31[a]	33.01 ± 2.83[b]	0.01
AST, U/L	41.73 ± 6.64[a]	25.26 ± 2.29[b]	0.03
γ-GGT, U/L	37.58 ± 4.83	29.06 ± 3.05	0.15
MDA, nmol/mL	2.76 ± 0.10[a]	2.10 ± 0.12[b]	<0.01
O_2^-, U/L	105.40 ± 4.45	111.08 ± 3.88	0.34
PCO, nmol/mL	472.65 ± 23.51	469.05 ± 24.38	0.91
T-SOD, U/mL	1.69 ± 0.03	1.74 ± 0.02	0.22
T-AOC, U/mL	79.43 ± 1.02	79.99 ± 1.23	0.73
CAT, U/mL	10.91 ± 1.98[a]	4.54 ± 0.99[b]	0.01

CON, control; MA, microbe-derived antioxidants; ALT, alanine aminotransferase; AST, aspartate amino transferase; γ-GGT, gamma-glutamyl transpeptidase; MDA, malondialdehyde; PCO, protein carbonyl; T-SOD, total peroxide dismutase; T-AOC, total antioxidant capacity; CAT, catalase. [a,b]Mean values within a row with different letter superscripts are significantly different ($P < 0.05$). [1]Values are means ± standard error, $n = 8$ for each group.

3.8 Intestinal morphology

Maternal or post-weaning MA supplementation had no significant effect on the VH, CD, VH/CD ratio, or the number of goblet cells in the jejunum of the weaned piglets (Supplementary Table S3).

3.9 Microbial composition of colonic chyme of piglets

A total of 1,204, 1,519, 1,244, and 1,270 OTUs were assigned to the weaned offspring in the CON. CON, CON. MA, MA. CON and MA. MA groups, respectively. The four groups shared 772 OTUs, with the groups of CON. CON, CON. MA, MA. CON and MA. MA having 144, 406, 127, and 158 unique OTUs, respectively (Figure 3A). There were no significant differences in microbial α-diversity indices (Chao1, Shannon, and Simpson) of colonic chyme among the four groups (Supplementary Table S4).

LEfSe analysis (LDA Score > 3.5) identified 3 species (o_Aeromonadales, f_Succinivibrionaceae, and *g_Succinivibrio*), 3 species (o_Pseudomonadales, f_Pseudomonadaceae, and *g_Pseudomonas*), 5 species (o_RF39, f_RF39, f_Streptococcaceae, *g_RF39*, and *g_Streptococcus*), and 8 species enriched in the CON. CON, CON. MA, MA. CON and MA. MA groups, respectively (Figure 3B). Among the 8

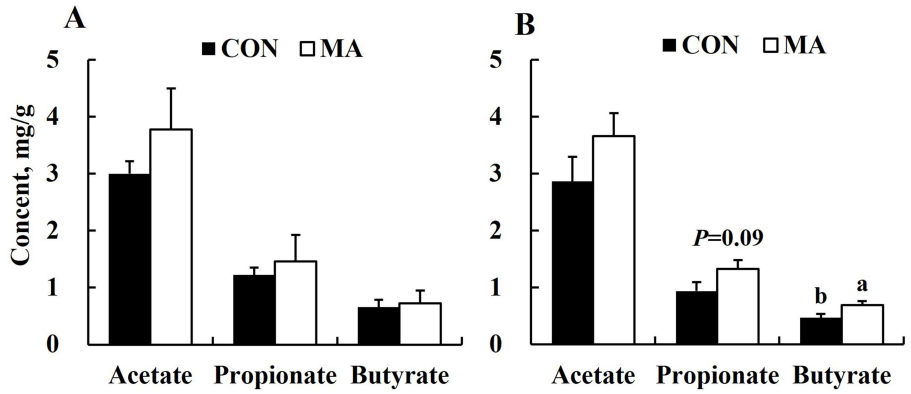

FIGURE 1
Effects of maternal MA supplementation on short-chain fatty acids of feces at farrowing and weaning. **(A)** At farrowing. **(B)** At weaning.

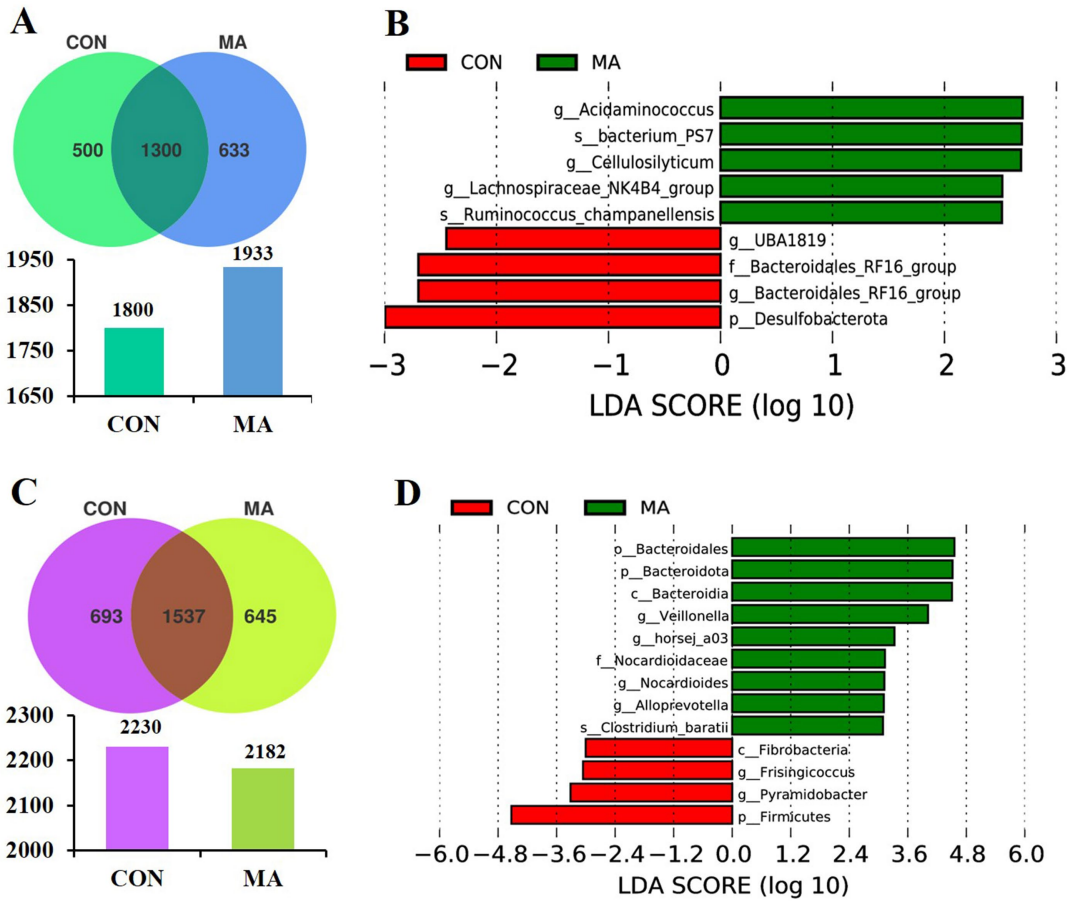

FIGURE 2
Effects of maternal MA supplementation on fecal microbial characteristics of sows at farrowing and weaning (n = 8). **(A)** The unique and shared OTUs in each group at farrowing. **(B)** LDA scores show the significant bacterial differences between the groups at farrowing ($p < 0.05$, LDA Score > 2.5). **(C)** The unique and shared OTUs in each group at weaning. **(D)** LDA scores show the significant bacterial differences between the groups at weaning ($p < 0.05$, LDA Score > 3.0). LDA, linear discriminant analysis; MA, microbe-derived antioxidants; OTUs, operational taxonomic units.

species, 7 belonged to c_Negativicutes (c_Negativicutes, o_Veillonellales_Selenomonadales, o_Acidaminococcales, f_Selenomonadaceae, f_Acidaminococcaceae, *g_Anaerovibrio*, and *g_Phascolarctobacterium*), while *g_Alloprevotella* belonged to f_Prevotellaceae.

4 Discussion

In an intensive feeding system, hyperprolific sows experience metabolic burdens during late gestation and lactation, which may lead

TABLE 4 Effects of maternal and post-weaning supplementation with MA on the growth performance and diarrhea index in piglets (n = 10).

Sow	CON		MA		P-value		
Piglet	CON	MA	CON	MA	Sow	Piglet	Sow×Piglet
BW, kg							
0 d	6.74 ± 0.23	6.74 ± 0.23	6.72 ± 0.22	6.73 ± 0.21	0.55	–	–
7d	6.85 ± 0.25	6.87 ± 0.25	7.00 ± 0.23	7.02 ± 0.23	0.05	0.82	0.96
14d	8.03 ± 0.28	8.06 ± 0.32	8.08 ± 0.25	8.34 ± 0.29	0.14	0.20	0.30
21 d	10.00 ± 0.40	10.18 ± 0.41	10.31 ± 0.26	10.58 ± 0.39	0.08	0.26	0.82
ADG, g/d							
1–7d	16 ± 12	19 ± 11	40 ± 10	42 ± 9	0.03	0.83	0.99
8–14 d	169 ± 16	170 ± 19	155 ± 11	189 ± 15	0.87	0.24	0.29
15–21 d	280 ± 20	303 ± 19	318 ± 16	319 ± 21	0.13	0.49	0.54
1–21 d	155 ± 10	164 ± 12	171 ± 8	183 ± 13	0.07	0.26	0.84
ADFI, g/d							
1–7 d	130 ± 8	126 ± 6	141 ± 8	129 ± 16	0.60	0.36	0.68
8–14 d	271 ± 14	275 ± 17	286 ± 8	310 ± 18	0.04	0.25	0.38
15–21 d	458 ± 29	470 ± 28	509 ± 23	532 ± 28	0.01	0.45	0.80
1–21 d	290 ± 16	290 ± 16	312 ± 12	329 ± 16	0.01	0.48	0.47
F/G, g/g							
8–14 d	1.74 ± 0.17	1.77 ± 0.07	1.92 ± 0.11	1.69 ± 0.07	0.72	0.45	0.34
15–21 d	1.65 ± 0.06	1.55 ± 0.03	1.61 ± 0.05	1.68 ± 0.04	0.23	0.69	0.36
1–21 d	1.89 ± 0.07	1.79 ± 0.05	1.84 ± 0.04	1.83 ± 0.05	0.79	0.25	0.40
Diarrhea index							
1–7 d	0.17 ± 0.03	0.17 ± 0.03	0.14 ± 0.03	0.18 ± 0.03	0.80	0.60	0.46
8–14 d	0.21 ± 0.04	0.12 ± 0.05	0.20 ± 0.05	0.19 ± 0.05	0.59	0.29	0.47
15–21 d	0.08 ± 0.03	0.11 ± 0.05	0.17 ± 0.07	0.12 ± 0.06	0.34	0.82	0.40
1–21 d	0.15 ± 0.02	0.13 ± 0.04	0.17 ± 0.05	0.16 ± 0.04	0.53	0.65	0.90

CON, control; MA, microbe-derived antioxidants; BW, body weight; ADG, average daily gain; ADFI, average daily feed intake; F/G, feed/gain.

TABLE 5 Effects of maternal and post-weaning supplementation with MA on inflammatory parameters in plasma of piglets (n = 8).

Sow	CON		MA		P-value		
Piglet	CON	MA	CON	MA	Sow	Piglet	Sow×Piglet
0 d							
C3, g/dL	0.71 ± 0.04	–	0.83 ± 0.05	–	0.07	–	–
IgG, mg/mL	0.92 ± 0.07	–	1.02 ± 0.06	–	0.24	–	–
IgM, mg/mL	0.09 ± 0.01	–	0.11 ± 0.01	–	0.15	–	–
IL-1β, ng/mL	0.81 ± 0.15	–	0.89 ± 0.11	–	0.68	–	–
IL-10, ng/mL	2.21 ± 0.39	–	4.19 ± 0.91	–	0.06	–	–
TNF-α, pg./mL	41.15 ± 9.11	–	22.32 ± 3.54	–	0.07	–	–
21 d							
C3, g/dL	0.97 ± 0.08	1.04 ± 0.10	1.15 ± 0.06	0.97 ± 0.08	0.47	0.50	0.13
IgG, mg/mL	0.65 ± 0.05	0.66 ± 0.03	0.64 ± 0.05	0.73 ± 0.06	0.60	0.31	0.43
IgM, mg/mL	0.11 ± 0.02	0.13 ± 0.02	0.16 ± 0.01	0.17 ± 0.02	0.03	0.49	0.81
IL-1β, ng/mL	0.93 ± 0.28	0.82 ± 0.18	1.15 ± 0.21	0.81 ± 0.12	0.61	0.26	0.56
IL-10, ng/mL	4.16 ± 1.40	3.93 ± 1.44	6.76 ± 2.23	12.00 ± 3.41	0.01	0.22	0.18
TNF-α, pg./mL	49.33 ± 15.47	36.70 ± 12.51	47.20 ± 22.14	27.17 ± 8.66	0.68	0.25	0.79

CON, control; MA, microbe-derived antioxidants; C3, complement 3; IgG, immunoglobulin G; IgM, immunoglobulin M; IL-1β, interleukin-1β; IL-10, interleukin-10; TNF-α, tumor necrosis factor α.

to oxidative stress and pro-inflammatory responses (13, 26). The oxidative stress is recognized as imbalanced redox status, an imbalance between reactive oxygen species (ROS) production and their elimination by antioxidant system, can lead to chronic inflammation (26). It has been reported that sows exhibited imbalanced redox status during late gestation and lactation, which is associated with lower levels of antioxidant components and total antioxidant capacity (5). To eliminate excessive ROS, the body produces antioxidant enzymes to maintain redox balance, such as T-SOD, CAT, GSH-Px, and nonenzymatic antioxidants. As a mixture of *Rosa roxburghii* and *Sea buckthorn* fermented by probiotics, MA contains bioactive ingredients with antioxidant properties, such as polyphenols, isoflavones, and flavones (19, 20). In this study, MA decreased MDA concentrations in the plasma of sows at farrowing and weaning, while tended to increase T-AOC at farrowing. As a byproduct of lipid peroxidation caused by ROS attacking cell membrane lipids, MDA increases with elevated oxidative stress (27). When the antioxidant status is imbalanced, a higher T-AOC indicates a stronger ability to combat oxidative stress by neutralizing excess ROS. Therefore, the higher levels of antioxidant indicators in sows receiving MA diet suggest these sows had greater antioxidant system to resist oxidative damage. Studies have shown that microbial antioxidants can enhance antioxidant capacity of sows during late pregnancy and/or lactation (11, 28). Similarly, maternal supplementation with antioxidant polyphenols (200–300 mg/kg), a class of compounds with multiple phenolic hydroxyl groups (such as flavonoids, phenolic acids, stilbenes, etc.), reduced MDA concentrations and increased the serum activities of GSH-Px and T-AOC (13).

In this study, we found that MA supplementation during late gestation and lactation significantly improved the litter size at weaning, which may be attributed to its beneficial effects on the redox status of sows. The better redox status by MA diet could be one of the factors contributing to the better milk quality, as indicated by the increasing the contents of dry matter and fat of milk from MA-supplemented sows. Similar results have been found that maternal supplementation with polyphenols, a typical antioxidant component, can increase the levels of colostrum immunoglobulins and milk composition, thereby improving pre-weaning survivability (13). Additionally, serum biochemical parameters are important indicators for evaluating the physical status of animals. ALT and AST are enzymes involved in amino acid metabolism and indicators of liver function and nutritional recovery, and their elevation in serum usually indicates liver cell damage, which is often associated with oxidative stress (29). In this study, maternal MA supplementation reduced the activities of ALT and AST in the plasma of sows at weaning, indicating a potential improvement of MA diet on liver function of sows.

The complex community of microbiota, serving as the microbial barrier of the gastrointestinal tract, plays a crucial role in the intestinal functions of pigs, including nutrient absorption, maintenance of dynamic balance of the intestinal barrier, immune regulation, and resistance to pathogen attacks (30). The composition of intestinal microbiota is influenced by multiple factors, such as diet, physiological stage, and health status. During gestation and lactation, changes in the immune system and metabolism of sows are related to the compositional dynamics of microbiota (31). Postbiotics, including enzymes, EPS, bacterial lysates, and cell wall fragments, exert beneficial effects on gut microorganisms (32, 33). In this study, maternal MA supplementation improved the composition of beneficial bacteria in the intestines of sows, such as significantly increasing the abundances of p_Bacteroidota, c_Bacteroidia, o_Bacteroidales, f_Nocardioidaceae, *g_Alloprevotella*, *g_Nocardioides*, *g_horsej_a03*, *g_Veillonella* and s_*Clostridium_baratii* at weaning, but had no effect on the α-diversity indices. Among these, Bacteroidota contributes to intestinal metabolism and homeostasis (34). *Bacteroidetes* is rich in carbohydrate metabolites and polysaccharide-degrading enzymes that breakdown complex nutrients into simpler forms for absorption by the animal's intestine (35). *Alloprevotella*, a branch of *Prevotella*, can break down complex carbohydrates and dietary fibers, producing SCFAs such as acetate,

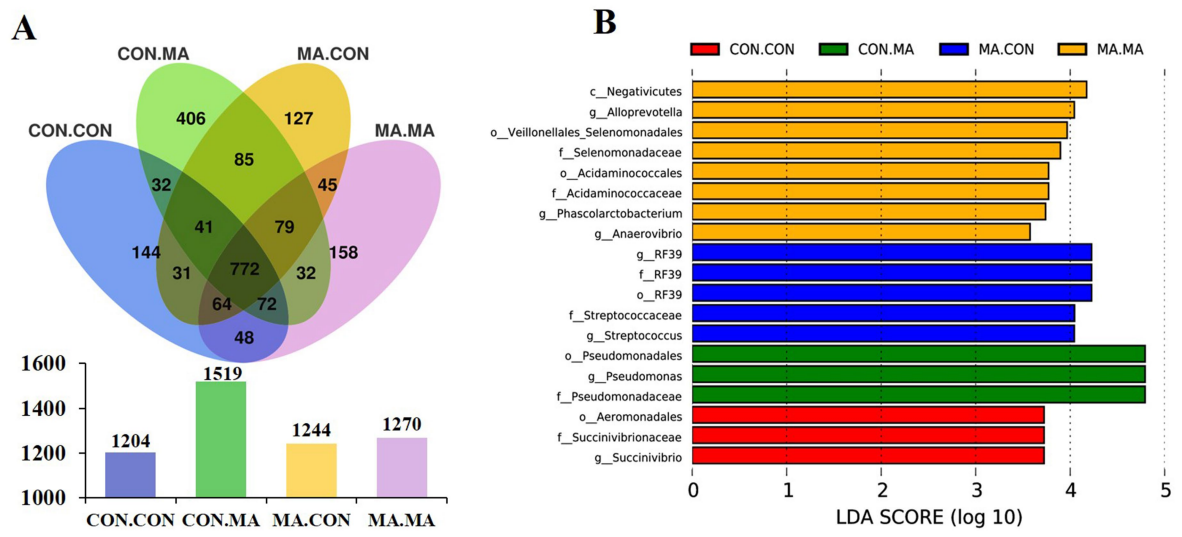

FIGURE 3
Effects of maternal and post-weaning MA supplementation on colonic chyme microbial characteristics of piglets on day 21 post-weaning (n = 8). **(A)** The unique and shared OTUs in each group. **(B)** LDA scores show the significant bacterial differences among the groups (p < 0.05, LDA Score > 3.5). LDA, linear discriminant analysis; MA, microbe-derived antioxidants; OTUs, operational taxonomic units. CON. CON, basal diet for both sows and piglets; MA. CON, 2.0 g MA/kg diet for sows and basal diet for piglets; CON. MA, basal diet for sows and 2.0 g MA/kg diet for piglets; MA. MA, 2.0 g MA/kg diet for both sows and piglets.

propionate, and butyrate (36). In the weaned piglets, the combination of maternal and post-weaning MA supplementation significantly increased the abundances of c_Negativicutes, o_Veillonellales_Selenomonadales, o_Acidaminococcales, f_Selenomonadaceae, f_Acidaminococcaceae, *g_Anaerovibrio*, *g_Phascolarctobacterium*, and *g_Alloprevotella*. As a gram-negative genus producing SCFAs, *Phascolarctobacterium* plays a positive role in maintaining intestinal health (37). Some studies have found that a strong correlation between the enrichment of *Anaerovibrio* and the enhancement of host amino acid metabolism and nucleosides/nucleotides biosynthesis (38).

The intestinal microbiome provides an extensive repertoire of molecules and metabolites that influence host health. Among these molecules, SCFAs, derived from bacteria-dependent hydrolysis of fibers, have attracted considerable attention because of their role in host health (39). As products of the fermentation of plant polysaccharides by intestinal microbiota, SCFAs serve as important fuels for intestinal epithelial cells, enhancing gut barrier functions as well as host metabolism (40). In this study, maternal MA supplementation increased the concentrations of butyrate and propionate in the sows on d 24 of lactation. Butyrate modulates the immune response by inhibiting pro-inflammatory cytokines, while supports intestinal epithelial cell proliferation as the primary energy source (40, 41). As the primary substrate for liver gluconeogenesis, propionate influences carbohydrate metabolism, exhibits a statin-like effect by inhibiting cholesterol synthesis pathways, and demonstrates anti-inflammatory activity comparable to butyrate (42).

Weaning stress often accompanies with serious diarrhea, infection and poor growth performance due to the immature immune system during early life of pigs (43). Previous studies have shown that supplementing with inactivated (heat-killed) *Lactobacillus* can improve growth performance and feed efficiency, reduce the occurrence of diarrhea, and positively influence gut microbiota (44–46). In this study, post-weaning MA supplementation did not significantly improve diarrhea index of piglets, which is consistent with the results of intestinal morphology. However, weaned piglets from sows receiving MA diet exhibited relatively faster growth rate, which could be perhaps ascribed to the optimal redox status and maternal transfer of probiotic bacteria to their offspring. In this study, we found that the enriched *g_Alloprevotella* in sow feces at weaning was also enriched in the colonic chyme of piglets at d 24 post-weaning. Likewise, previous studies have shown that maternal supplementation with yeast-derived postbiotics positively affects the gut microbiome and immunological parameters of offspring (16, 47).

The positive effect of maternal MA supplementation on offspring piglets is also reflected by its beneficial effect on the immunity-related parameters of offspring. In this study, maternal MA supplementation tended to increase the levels of C3 and IL-10, reduce TNF-α at weaning, and increase IgM and IL-10 at d 24 post-weaning. It has been reported that C3 participates in the clearance of microorganisms and mediates the occurrence of inflammation (48). As an anti-inflammatory cytokine produced by inflammatory cells, IL-10 is known to prevent excessive inflammatory responses at the maternal-fetal interface (49). As a pro-inflammatory cytokine, TNF-α had been found to be decreased in MA-supplemented mouse (22). In RAW264.7 cells, MA also significantly down-regulated the level of TNF-α together with the lower accumulation of ROS (23). Similarly, several heat-killed *Lactobacillus* probiotics have shown immunomodulatory abilities by enhancing anti-inflammatory cytokines and suppressing oxidative stress in both *in vitro* and *in vivo* experimental models (50). It can be seen that the enhancement of immune function in weaned piglets should be closely related to the maternal supplementation of MA to enhance their own immune function.

5 Conclusion

In summary, the present results demonstrated that maternal MA supplementation during late gestation and lactation increased the number of weaned piglets, which may be associated with the improved redox status and milk composition of sows. Additionally, maternal MA supplementation increased the growth rate of post-weaning piglets, which is likely related to the improved immunological parameters and gut microbiome of both sows and weaned piglets.

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 approved by the Institutional Animal Care and Use Committee of Sichuan Agricultural University. The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

JT: Conceptualization, Data curation, Methodology, Writing – original draft, Writing – review & editing. YW: Data curation, Investigation, Methodology, Writing – original draft. QZ: Data curation, Methodology, Writing – review & editing. ZF: Data curation, Software, Writing – review & editing. YL: Data curation, Software, Writing – review & editing. SX: Data curation, Software, Writing – review & editing. BF: Data curation, Investigation, Software, Writing – review & editing. YZ: Data curation, Software, Writing – review & editing. XJ: Data curation, Writing – review & editing. HZ: Data curation, Writing – review & editing. DW: Data curation, Software, Writing – review & editing. LC: Conceptualization, Data curation, Funding acquisition, Project administration, Supervision, Writing – original draft, Writing – review & editing.

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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.2025.1574259/full#supplementary-material

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Theaflavins enhances laying hen production performance and egg antioxidant capacity via improving antioxidant status and regulating lipid metabolism

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Introduction: Theaflavins (TF), natural compounds extracted from black tea, demonstrate various beneficial functions including antioxidant properties and lipid metabolism regulation. However, their effects on laying hens remained unclear. This study investigated the effects of different TF levels on production performance, egg quality, antioxidant capacity, lipid metabolism in laying hens, and egg antioxidant capacity.

Methods: A total of 512 twenty-nine-week-old Lohmann commercial laying hens were randomly divided into four dietary treatments (0, 250, 500, or 1000 mg/kg TF) in a completely randomized design. Each treatment consisted of eight replicates of 16 birds, and the experiment lasted for 8 weeks. Data were analyzed using one-way analysis of variance (ANOVA) followed by Duncan's multiple range test, with quadratic polynomial contrasts applied to evaluate dose-response relationships.

Results: Results showed that TF supplementation quadratically increased ($p < 0.05$) egg production, egg yolk color, serum total antioxidant capacity (T-AOC), hepatic T-AOC, uterine T-AOC, hepatic superoxide dismutase activity, serum total cholesterol (TC), hepatic TC, hepatic triglycerides, and the expression of antioxidant-related and lipid metabolism-related genes. It also enhanced the 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging capacity, T-AOC, tyrosine, and tryptophan levels in egg yolks. TF supplementation significantly decreased ($p < 0.05$) malondialdehyde (MDA) levels in serum, liver, ovaries, and egg yolks.

Conclusion: These findings suggest that TF improves laying performance by enhancing antioxidant capacity and regulating lipid metabolism, while simultaneously boosting egg antioxidant potential. Based on the quadratic regression analysis, the optimal TF supplementation level was determined to be 500 mg/kg.

KEYWORDS

theaflavins, production performance, egg antioxidant capacity, antioxidant status, lipid metabolism, laying hen

1 Introduction

Theaflavins (TF) are key functional phytochemicals formed through the enzymatic action of polyphenol oxidase during the processing of black tea. They constitute 2–20 g/kg of the dry weight of solids in brewed black tea (1). The main structure of TF features a seven-membered benzotropolone ring, followed by decarboxylation and simultaneous fusion with the epicatechin ring B or epicatechin gallate (2). Over 25 types of TF have been identified, with the major characterized structures being theaflavin (TF1), theaflavin 3-gallate (TF2A), theaflavin-3′-allate (TF2B), and theaflavin-3,3′-digallate (TF3).

TF have been widely reported to resist the oxidative stress and regulated the lipid metabolism. Firstly, TF have excellent antioxidant capacity. Yang et al. report that TF exhibit the highest antioxidant activity in the Fenton reaction system, 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay, and H_2O_2-mediated oxidative damage in HPF-1 cells, compared to that of other oxidized phenolic compounds in black tea (3). The hydroxyl radical-scavenging ability and DPPH scavenging ability follow the order TF3 > TF2 > TF1 > epigallocatechin-3-gallate (EGCG) (4). Research on diabetic mice reveals that TF could counteract the increase and decrease of malondialdehyde (MDA) and glutathione peroxidase (GSH-*Px*) levels in the liver and kidney tissues, respectively (5). The nuclear factor erythroid 2-related factor 2 (*Nrf2*) pathway is a key regulator of oxidative stress (6). TF upregulate the expression of total *Nrf2*, nuclear *Nrf2*, and its downstream targets heme oxygenase 1 (*HO-1*) and NAD(P)H quinone dehydrogenase 1 (*NQO1*), protecting the liver and kidneys of mice from oxidative damage (4). Second, TF regulate lipid metabolism, improving hyperlipidemia and regulating body composition in human studies. TF decrease the serum levels of total cholesterol (TC), triglyceride (TG), and low-density lipoprotein cholesterol in obese rats by 26.5, 50.8, and 71.7%, respectively (7). This effect is mainly attributed to the ability of TF to inhibit lipid synthesis and catabolism. TF decrease the mRNA and protein expression levels of fatty acid synthase (*FAS*), acetyl-CoA carboxylase (*ACC*), and HMG-CoA reductase while increasing the expression levels of carnitine palmitoyl transferase 1 (*CPT1*), thereby inhibiting fatty acid and cholesterol synthesis and decreasing lipid accumulation (8, 9). In our previous study, dietary supplementation with 600 mg/kg tea polyphenols was observed to alleviate oxidative stress induced by aging corn in laying hens. However, the potential for TF to demonstrate superior antioxidant efficacy in this context remains unclear, warranting further comparative investigation (10).

Currently, the research on the physiological functions of TF is already very extensive, and it has been demonstrated that they have the effects of enhancing antioxidant capacity and regulating lipid metabolism. However, it is unclear whether TF can be applied in laying hens, what their effects are on the production performance of laying hens and the antioxidant capacity of eggs, and what the optimal level of addition is for laying hens. Thereby, this study was conducted to investigate the effects of different levels of TF on production performance, egg quality, antioxidant capacity, lipid metabolism in laying hens, and the antioxidant capacity of eggs. We hypothesized that 500 mg/kg TF would increase the performance, egg quality, antioxidant status and egg antioxidant capacity of laying hens.

2 Materials and methods

2.1 Theaflavins

TF were purchased from Dannisi Technology Co., Ltd. (Shan'xi province, China), with 62.9% purity. The composition includes 40.67% TF1, 8.91% TF3, 7.14% TF2A, and 6.18% TF2B.

2.2 Animals and diets

This study was performed on a poultry farm in Yi'bin, China, during the summer (Latitude: 28.7599°N, Longitude: 104.6399°E). All experimental procedures were approved by the Animal Care and Use Committee of Yibin University (No. 202402). Twenty-nine-week-old (n = 512) Lohmann commercial laying hens were randomly allocated to one of the four dietary treatments. Each treatment included eight replicates, with 16 hens per replicate, housed in two cages containing eight birds each. The housing temperature and relative humidity were 20 ± 4°C and 50–65%, respectively. The four treatment groups consisted of a control group (basal diet) and three experimental groups with different levels of TF addition (basal diet + 250, 500, 1,000 mg/kg TF). The diets (Table 1) were based on corn and soybean meal, with nutrient levels aligned with the Agricultural Trade Standardization of China (11). Feed and water were supplied ad libitum, and a 16L:8D photoperiod was used during the 8-week laying hens trial.

2.3 Hen performance

Daily records were kept of egg production, egg weight, and the quantity of dirty and broken eggs. The laying rate, average egg weight, and rates of dirty and broken eggs were then computed. Weekly feed intake (ADFI) was monitored, and the feed conversion rate (FCR) was determined.

2.4 Egg collection

At the end of the trial, a total of 128 eggs for each treatment, with four eggs in each replicate, were gathered and split into two groups. One half of the eggs was utilized for assessing egg quality. While the other half were used for measuring the antioxidant capacity of the egg yolks (two pooled egg yolks per replicate).

2.5 Sample collection

In the final days of the trial, 10 mL blood samples (n = 8) were taken from the brachial vein of one laying hen per replicate and placed in lithium heparinized vacutainers. The serum was separated at 4°C and then stored at −20°C for analysis of antioxidant status. After the blood collection, the chicks were euthanized by cervical dislocation, and the liver and ovaries were promptly extracted, frozen at −80°C, and stored for antioxidant status analysis.

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TABLE 1 Composition and nutrient levels of the experimental diets (fed-basis).

Ingredient	g/kg	Nutrient content	g/kg[2]
Corn	582.1	Metabolizable energy, kcal/kg	2,689.76
Soybean meal	245.0	Crude protein	165.1
Soybean oil	20.0	Calcium	37.1
Distillers dried grains with solubles	17.4	Available phosphorus	3.7
Cottonseed meal	20.0	Lysine	7.4
Sodium chloride	3.0	Methionine	3.4
Calcium carbonate	87.0	Threonine	5.5
Calcium hydrophosphate	14.5	Tryptophan	1.7
Premix[1]	10.0		
DL-Methionine	1.0		

[1]The premix provided the following (per kilogram of diet): vitamin A, 9,750 IU; vitamin D3, 2,200 IU, vitamin E, 5.0 IU; vitamin B1, 2.5 mg; vitamin B2, 6.25 mg; vitamin B6, 3.75 mg; vitamin B12, 0.019 mg; D-pantothenic acid, 12.25 mg; nicotinic acid, 40 mg; biotin, 0.3 mg; iron, 80 mg; copper, 10 mg; manganese, 120 mg; zinc, 80 mg; selenium, 0.3 mg; iodine, 1.0 mg; choline chloride, 450 mg.
[2]Calculated value.

2.6 Egg quality

The Multi-tester (EMT-7300, Robotmation Co., Ltd., Tokyo, Japan) was utilized to measure the albumen height, Haugh unit, and color of the egg yolk. The yolk and eggshell (which had been dried at room temperature for 3 days) were meticulously separated and weighed. The ratios of the eggshell, albumen, and yolk within the egg were then calculated. The thickness of the eggshell (excluding the inner and outer membranes) was measured using Vernier calipers, with the average taken from three measurements at different locations (top, middle, and bottom).

2.7 Antioxidant status of laying hens

Antioxidant enzyme activity. Liver and ovarian tissue samples ($n = 8$) were homogenized in PBS and centrifuged at 1,699×*g* for 10 min. The supernatant was then collected for the determination of correlation indices. The activities of SOD, total antioxidant capacity (T-AOC), malondialdehyde (MDA) levels in the liver, ovarian tissue, and serum, as well as TC and TG in the liver and serum, were analyzed using reagent kits from Jiancheng Bioengineering Institute in Jiangsu, China. SOD activity was assessed using the hydroxylamine method, where superoxide anions generated from the xanthine–xanthine oxidase system oxidized hydroxylamine to produce nitrite, which was measured at 550 nm using a colorimetric approach. The production of nitrite was inhibited by SOD, indicating the activity of superoxide anions. One unit of SOD is defined as the amount of sample that inhibits 50% of the activity under the assay conditions, and SOD activity was reported as units per gram of tissue. T-AOC was evaluated using the ferric reducing-antioxidant power assay, conducted within a 5-min timeframe at 37°C and measured at 593 nm. MDA levels were determined using the thiobarbituric acid method, where 1 mL of the homogenate was treated with thiobarbituric acid, mixed quickly, and heated in a boiling water bath for 40 min. The samples were then centrifuged at 10,621×*g* for 10 min, and the supernatants were placed in a 96-well plate and measured at 532 nm.

Antioxidant-related gene expression. Total RNA was extracted from liver tissue using Trizol reagent (Takara, Dalian, China), according to the protocol of the manufacturer. The absorbance ratio at 260–280 nm was calculated for each sample. cDNA synthesis was conducted using the primeScript RT reagent kit (Takara), and real-time PCR was performed using the SYBR Premix Ex Tap (Takara). The PCRs were conducted on an Applied Biosystems 7900HT Real-Time PCR system (Applied Biosystems, Foster City, CA, USA). The amplification cycles included an initial denaturation at 95°C for 35 s, followed by 40 cycles of denaturation at 95°C for 5 s, annealing at 60°C for 34 s, and extension at 95°C for 15 s, and a final elongation step at 60°C for 15 s. Table 2 presents the primer sequences for antioxidant-related genes.

TABLE 2 Gene-specific primers for real-time quantitative reverse transcription PCR.

Genes	Primers (5′–3′)
Nrf2	Forward: TGTGTGTGATTCAACCCGACT
	Reverse: TTAATGGAAGCCGCACCACT
NQO1	Forward: GTTCAATGCCGTGCTCTCAC
	Reverse: CCGCTTCAATCTTCTTCTGC
HO-1	Forward: TTGGCAAGAAGCATCCAGA
	Reverse: TCCATCTCAAGGGCATTCA
FAS	Forward: ACTGTGGGCTCCAAATCTTCA
	Reverse: CAAGGAGCCATCGTGTAAAGC
ACC	Forward: TGTGGCTGATGTGAGCTTTC
	Reverse: ACTGTCGGGTCACCTTCAAC
CPT1	Forward: GAGAAGAGTGCAGTGAGAAGAG
	Reverse: CCAGCCACAGAAGTAGAGTAAG

Nrf2 = nuclear factor erythroid 2-related 2; NQO1 = NAD(P)H: quinone dehydrogenase 1; HO-1 = heme oxygenase-1; FAS = fatty acid synthase; ACC = dacetyl-CoA carboxylase; CPT1 = carnitine palmitoyl transferase 1.

2.8 Lipid metabolism index of laying hens

TG and TC. TC and TG ($n = 8$) in liver and serum were analyzed using reagent kits (Jiancheng Bioengineering Institute, Jiangsu,

China), and the supernatant was used for antioxidant status analysis. TG and TC were determined using the glycerol-3-phosphate oxidase peroxidase method and measured at 500 nm.

Lipid metabolism-related gene expression. The liver FAS, ACC, HMGCR, and CPT1 were measured according to the methods described for antioxidant-related gene expression analysis. Table 2 shows the primer sequences for lipid metabolism-related genes.

2.9 Antioxidant capacity of eggs

Free radical-scavenging activity on 1,1-diphenyl-2-picrylhydrazyl (DPPH). The scavenging effect of the DPPH free radical ($n = 8$) was measured using a previously described method (12). A sample solution (1 mL, containing 10 mg of egg yolk per mL; with ethanol as a control) was combined with an equal volume of a DPPH (0.1 mM) solution, mixed thoroughly, and left to incubate for 30 min at room temperature. The absorbance was measured at 517 nm. The scavenging effect was calculated using this formula: DPPH scavenging activity (%) = [(Blank absorbance − Sample absorbance) /Blank absorbance] × 100.

MDA. The egg yolk samples ($n = 8$) were homogenized in PBS and centrifuged at 1,699×*g* for 10 min. The resulting supernatant was collected for MDA determination. MDA content in egg yolk was measured as described in the tissue antioxidant status analysis.

SOD and T-AOC. SOD activity and T-AOC ($n = 8$) in egg yolks were measured as described in the tissue analysis methods.

2.10 Egg composition

Free amino acid. Free amino acids were determined using the method described by Nimalaratne et al. (13) with slight modifications ($n = 8$). About 100 mg of freeze-dried egg yolk was mixed with 1 mL of a 10% sulfosalicylic acid solution, vortexed, and then centrifuged at 15,294×*g* for 10 min using a Centrifuge 5430/R from Eppendorf in Hamburg, Germany. The resulting supernatant was passed through a 0.22-μm nylon syringe filter prior to analysis with an automated AA analyzer (L-8900, Hitachi, Tokyo, Japan) equipped with a lithium high-performance column.

Fatty acid profile. The analysis of the egg yolk ($n = 8$) was measured according to the national standard method (14). Fatty acid methyl esters were analyzed using a gas chromatograph (7890A, Agilent Technologies, Santa Clara, CA, USA) that featured a flame ionization detector and a silica capillary column (Supelco SP-2560; 100 × 0.25 mm i.d., 0.20 μm film thickness). Nitrogen was used as the carrier gas at a pressure of 0.5 kg/cm^2. The injector and detector temperatures were kept at 250°C. The findings were reported as percentages (%) of the total fatty acids.

2.11 Statistical analysis

The results were statistically analyzed using one-way ANOVA followed by Duncan's multiple range tests of SPSS 27.0 (SPSS Inc., Chicago, IL, USA). Regression analysis was subsequently performed to evaluate linear and quadratic effects. Differences among treatments were considered significant at $p < 0.05$, and results were reported as mean and standard error mean (SEM).

3 Results

3.1 Hen performance

Dietary TF levels had no significant effect on hen performance, including egg weight, ADFI, FCR, as well as dirty and broken egg rate (Table 3). Increasing TF levels quadratically ($p < 0.05$) increased egg production throughout the entire laying period.

3.2 Egg quality

Dietary TF levels had no significant effect on egg quality, including eggshell color, egg strength, eggshell thickness, albumen height, and Haugh units (Table 4). However, increasing TF levels quadratically ($p < 0.05$) increased the egg yolk color.

3.3 Antioxidant status of laying hens

Antioxidant enzyme activity assay. TF significantly enhanced the antioxidant capacity of laying hens (Table 5). Increasing TF levels quadratically ($p < 0.05$) increased the serum T-AOC, liver T-AOC, uterus T-AOC, and liver SOD, while it quadratically ($p < 0.05$) decreased the MDA levels in the serum, liver, and ovary of laying hens.

TABLE 3 Production performance of laying hens at different theaflavin levels ($n = 8$)[1,2].

Items	CON	TF250	TF500	TF1000	SEM	*p*-value	Linear	Quadratic
Egg production (%)	95.48^{b}	95.76^{ab}	97.68^{a}	94.44^{b}	0.40	<0.05	0.377	<0.05
Egg weight (g)	60.67	60.77	61.29	61.45	0.25	0.632	0.213	0.454
ADFI (g)	117.21	117.68	120.95	117.98	1.42	0.801	0.787	0.715
FCR	2.02	2.02	2.02	2.03	0.02	0.997	0.843	0.978
Dirty egg (%)	6.95	7.46	7.50	7.24	0.39	0.961	0.859	0.867
Broken egg (%)	0.90	0.77	0.66	0.70	0.07	0.711	0.350	0.500

^{a,b}Different superscripts in a row indicate a significance difference ($p < 0.05$).
[1]Each value represents the mean value of 8 replicates per treatment.
[2]CON = control group; TF250 = CON + 250 mg/kg theaflavins; TF500 = CON + 500 mg/kg theaflavins; TF1000 = CON + 1,000 mg/kg theaflavins; ADFI = average daily feed intake; FCR = feed conversion rate; SEM = standard error of the mean.

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TABLE 4 Production performance of laying hens at different theaflavin levels (n = 8)[1,2].

Items		CON	TF250	TF500	TF1000	SEM	p-value	Linear	Quadratic
Eggshell strength (N)		49.25	46.23	49.75	47.19	0.86	0.422	0.661	0.904
Eggshell thickness (mm)		0.35	0.35	0.36	0.35	0.00	0.764	0.758	0.953
Albumen height		7.90	7.91	7.88	8.14	0.10	0.808	0.401	0.624
Haugh unit		87.17	87.65	87.50	89.59	0.59	0.468	0.139	0.295
Yolk color		9.31^{b}	10.19^{ab}	10.56^{a}	9.44^{b}	0.13	<0.05	0.564	<0.05
Albumen height (%)		62.24	62.90	62.88	62.06	0.19	0.268	0.511	0.144
Yolk weight (%)		27.46	27.22	27.42	27.35	0.18	0.186	0.591	0.092
Eggshell weight (%)		10.17	9.97	10.09	10.28	0.07	0.950	0.667	0.845
Eggshell color	L*	77.95	77.23	76.66	77.00	0.41	0.733	0.433	0.527
	a*	7.07	7.84	7.30	7.25	0.25	0.728	0.934	0.809
	b*	15.27	16.48	15.51	14.99	0.34	0.452	0.468	0.488

[a, b]Different superscripts in a row indicate a significance difference ($p < 0.05$).
[1]Each value represents the mean value of 16 replicates per treatment.
[2]CON = control group; TF250 = CON + 250 mg/kg theaflavins; TF500 = CON + 500 mg/kg theaflavins; TF1000 = CON + 1,000 mg/kg theaflavins; L* = lightness; a* = redness; b* = yellowness. SEM = standard error of the mean.

TABLE 5 Antioxidant status of laying hens at different theaflavin levels (n = 8)[1,2].

Items	CON	TF250	TF500	TF1000	SEM	p-value	Linear	Quadratic
Serum								
T-AOC	0.79^{b}	1.18^{ab}	1.39^{a}	1.07^{ab}	0.07	<0.05	0.24	<0.05
SOD	51.81	52.35	53.16	50.73	1.17	0.914	0.73	0.78
MDA	1.91^{b}	1.65^{b}	1.27^{a}	1.63^{b}	0.07	<0.05	0.13	<0.05
Liver								
T-AOC	1.45^{b}	1.82^{ab}	2.64^{a}	1.63^{b}	0.17	<0.05	0.68	<0.05
SOD	238.56^{b}	261.60^{ab}	305.98^{a}	237.73^{b}	9.54	<0.05	0.97	<0.05
MDA	12.96^{a}	12.87^{a}	10.02^{b}	11.59^{ab}	0.41	<0.05	0.12	0.07
Ovary								
T-AOC	1.38	1.49	1.54	1.36	0.05	0.535	0.77	0.33
SOD	77.72	73.42	72.07	70.02	2.47	0.748	0.30	0.54
MDA	1.11^{a}	0.83^{ab}	0.60^{b}	1.08^{a}	0.07	<0.05	0.99	<0.05
Uterus								
T-AOC	3.11^{b}	3.88^{ab}	4.75^{a}	2.79^{b}	0.27	<0.05	0.59	<0.05
SOD	142.45	161.77	153.64	140.96	6.00	0.590	0.68	0.48
MDA	37.70	35.66	30.60	32.30	1.21	0.151	0.08	0.10

[a, b]Different superscripts in a row indicate a significance difference ($p < 0.05$).
[1]Each value represents the mean value of 8 replicates per treatment.
[2]CON = control group; TF250 = CON + 250 mg/kg theaflavins; TF500 = CON + 500 mg/kg theaflavins; TF1000 = CON + 1,000 mg/kg theaflavins; T-AOC = total antioxidant capacity; SOD = superoxide dismutase; MDA = malondialdehyde; SEM = standard error of the mean.

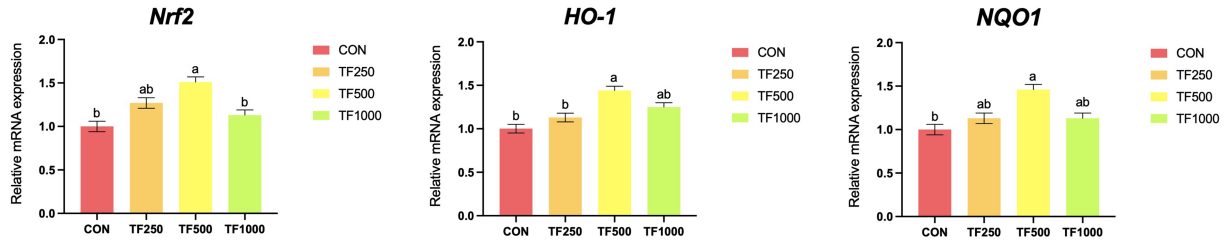

FIGURE 1
Antioxidant-related gene expression of laying hens at different theaflavin levels. [a,b]Different superscripts indicate a significance difference ($p < 0.05$). Nrf2 = nuclear factor erythroid 2-related 2; NQO1 = NAD(P)H: quinone dehydrogenase 1; HO-1 = heme oxygenase-1. [1]Values are means ± SEM. Each value represents the mean value of 8 replicates per treatment. [2]All the data were acquired using real-time PCR.

TABLE 6 Lipid metabolism indexes of laying hens at different theaflavin levels ($n = 8$)[1,2].

Items	CON	TF250	TF500	TF1000	SEM	*p*-value	Linear	Quadratic
Serum								
TG	14.99	14.01	14.10	14.57	0.34	0.738	0.833	0.573
TC	4.57[a]	3.78[ab]	2.95[b]	3.65[ab]	0.20	<0.05	0.104	<0.05
Liver								
TG	1.14[a]	1.00[ab]	0.62[b]	1.12[a]	0.08	<0.05	0.887	<0.05
TC	1.12[a]	0.64[b]	0.60[b]	0.69[b]	0.08	0.041	0.094	<0.05

[a,b] Different superscripts in a row indicate a significance difference ($p < 0.05$).
[1]Each value represents the mean value of 8 replicates per treatment.
[2]CON = control group; TF250 = CON + 250 mg/kg theaflavins; TF500 = CON + 500 mg/kg theaflavins; TF1000 = CON + 1,000 mg/kg theaflavins; TG = triglyceride; TC = total cholesterol; SEM = standard error of the mean.

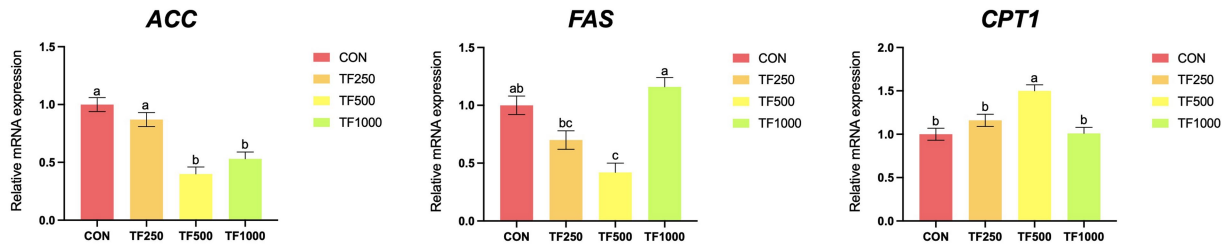

FIGURE 2
Lipid metabolism-related gene expression of laying hens at different theaflavin levels. [a,b]Different superscripts indicate a significance difference ($p < 0.05$). ACC = acetyl-CoA carboxylase; FAS = fatty acid synthase; CPT1 = carnitine palmitoyl transferase 1. [1]Values are means ± SEM. Each value represents the mean value of 8 replicates per treatment. [2]All the data were acquired using real-time PCR.

Antioxidant-related gene expression. Figure 1 illustrates the expression of antioxidant-related genes. Increasing TF levels quadratically ($p < 0.05$) increased the expression of *Nrf2*, *HO-1*, and *NQO1* in the liver of laying hens.

3.4 Lipid metabolism indexes of laying hen

TG and TC. Table 6 shows the lipid metabolism indices in serum and liver. Increasing TF levels quadratically ($p < 0.05$) decreased the serum TC, as well as the liver TC and TG content in laying hens.

Lipid metabolism-related gene expression. Figure 2 depicts the expression of lipid metabolism-related genes. Increasing TF levels quadratically ($p < 0.05$) decreased the expression of *FAS* and *ACC* while increasing the expression of *CPT1* ($p < 0.05$) in the liver of laying hens.

3.5 Antioxidant capacity of eggs

Table 7 shows the antioxidant capacity of eggs. Increasing TF levels quadratically ($p < 0.05$) increased the DPPH value of egg yolks. TF (500 mg/kg) significantly ($p < 0.05$) decreased the egg yolk MDA content compared to those of the other groups and significantly ($p < 0.05$) increased the egg yolk T-AOC compared to that of the control group.

3.6 Egg composition

Free amino acids. Table 8 shows the free amino acids in egg yolks. Increasing TF levels quadratically ($p < 0.05$) increased the tyrosine and tryptophan content in egg yolks.

TABLE 7 Antioxidant capacity of egg yolks at different theaflavin levels (n = 8)[1,2].

Items	CON	TF250	TF500	TF1000	SEM	p-value	Linear	Quadratic
MDA	118.88[a]	106.96[a]	89.45[b]	106.54[a]	4.57	0.150	0.341	0.089
DPPH	22.74[b]	23.06[b]	25.14[a]	23.21[b]	0.29	<0.05	0.409	<0.05
T-AOC	3.86[b]	4.08[ab]	5.36[a]	4.17[ab]	0.24	0.097	0.522	0.121
SOD	272.92	266.74	282.69	284.92	14.25	0.970	0.693	0.926

[a,b]Different superscripts in a row indicate a significance difference ($p < 0.05$).
[1]Each value represents the mean value of 8 replicates per treatment.
[2]CON = control group; TF250 = CON + 250 mg/kg theaflavins; TF500 = CON + 500 mg/kg theaflavins; TF1000 = CON + 1,000 mg/kg theaflavins; MDA = malondialdehyde; DPPH = free radical-scavenging activity on 1,1-diphenyl-2-picrylhydrazyl; T-AOC = total antioxidant capacity; SOD = superoxide dismutase; SEM = standard error of the mean.

TABLE 8 Free amino acids of egg yolks at different theaflavin levels (n = 8)[1,2].

Items	CON	TF250	TF500	TF1000	SEM	p-value	Linear	Quadratic
Aspartic	246.75	265.44	214.07	234.08	10.37	0.355	0.414	0.636
Threonine	366.51	358.15	354.93	358.61	7.32	0.961	0.764	0.861
Serine	410.66	400.95	412.05	429.14	7.28	0.593	0.247	0.420
Glutamic	1,087.14	1,084.46	1,098.56	1,107.33	14.67	0.947	0.569	0.851
Glycine	127.43	120.87	123.14	131.67	3.78	0.765	0.532	0.580
Alanine	144.31	148.84	158.99	149.62	3.52	0.539	0.597	0.407
Valine	321.69	327.92	327.64	314.21	3.89	0.569	0.368	0.359
Methionine	173.01	173.74	178.11	171.49	1.97	0.681	0.798	0.569
Isoleucine	278.09	282.85	282.55	266.49	3.13	0.203	0.112	0.096
Leucine	649.48	663.53	662.29	634.58	6.12	0.297	0.239	0.155
Tyrosine	419.16[b]	436.61[b]	468.37[a]	431.25[b]	4.74	<0.05	0.424	<0.05
Phenylalanine	474.72	511.75	492.03	466.79	7.99	0.191	0.342	0.173
Histidine	105.01	101.60	108.48	90.28	3.31	0.230	0.127	0.174
Lysine	578.10	634.91	648.15	628.48	12.11	0.214	0.263	0.109
Arginine	441.87	474.10	522.12	503.98	13.66	0.188	0.107	0.104
Tryptophan	155.96[c]	178.34[ab]	184.40[a]	160.99[bc]	3.60	<0.05	0.943	<0.05

[a,b,c] Different superscripts in a row indicate a significance difference ($p < 0.05$).
[1]Each value represents the mean value of 8 replicates per treatment.
[2]CON = control group; TF250 = CON + 250 mg/kg theaflavins; TF500 = CON + 500 mg/kg theaflavins; TF1000 = CON + 1,000 mg/kg theaflavins; SEM = standard error of the mean.

Fatty acid profile. Table 9 shows the fatty acid profile in egg yolks. TF (250 mg/kg) significantly ($p < 0.05$) increased the linoleic acid content compared to that of the 500 and 1,000 mg/kg TF group and increased the eicosenoic acid content compared to that of the 1,000 mg/kg TF group of egg yolks. Additionally, TF linearly ($p < 0.05$) decreased the arachidic acid content in egg yolks.

4 Discussion

TF, present in black, red, and oolong teas, exhibit various biological activities and health benefits (15). In this study, we observed that 500 mg/kg TF significantly enhanced the production performance of laying hens. Although reports on the application of TF in laying hens are unknown, other tea extracts, such as theabrownins (TB) and EGCG, have been widely investigated. Studies show that 200–400 mg/kg TB and EGCG enhance the production performance of laying hens (16–18). Furthermore, tea polyphenols (600 mg/kg) could counteract the adverse effects of aging corn and heavy metals on the production performance and egg albumen quality of laying hens (10, 19, 20). Both TF and TBs are key pigments in black tea, derived from the oxidation of catechins and their gallates during the fermentation process. Structurally, TF are dimeric polyphenols with a benzotropolone core ($C_{17}H_{16}O_6$), enabling stronger electron delocalization and antioxidant capacity than the polymeric TBs (irregular catechol-quinone frameworks) or monomeric EGCG ($C_{22}H_{18}O_{11}$) (21). Therefore, TF exhibit higher antioxidant activity than TBs and hold greater potential for application in the egg production industry (4).

Egg yolk color plays a significant role in the purchasing decisions of consumers. The typical yellow color results from the accumulation of lutein and carotenoids in the diet (22). Tea leaves contain chlorophyll, lutein, carotenoids, and other pigments (22). The TF obtained in this experiment may contain residual lutein and carotenoids that were present during the extraction process of TF, which could increase their deposition in eggs. In China, consumers prefer eggs with a more intense yolk color. The increased yolk color indicates that TF-fed layers may be more preferred.

TF is the main antioxidant active components of black tea and have been shown to effectively improve the antioxidant status in different cell cultures and animal models (23). Our results showed that TF quadratically increased the activities of antioxidant enzymes and decreased the MDA content in laying hens. Ai et al. report that TF

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TABLE 9 Fatty acid profiles of egg yolks at different theaflavin levels (n = 8)[1,2].

Items	CON	TF250	TF500	TF1000	SEM	p-value	Linear	Quadratic
Myristic acid (C14:0)	0.36	0.36	0.36	0.36	0.00	0.949	0.596	0.850
Palmitic acid (C16:0)	28.46	28.84	28.16	27.97	0.18	0.343	0.170	0.371
Stearic acid (C18:0)	10.45	10.83	10.66	10.89	0.10	0.416	0.206	0.418
Oleic acid (C18:1)	38.60	39.35	39.34	39.23	0.36	0.875	0.635	0.733
Linoleic acid (C18:2)	17.64^{ab}	18.51^{a}	17.14^{b}	17.07^{b}	0.23	0.104	0.141	0.318
Arachidic acid (C20:0)	0.14^{a}	0.13^{ab}	0.11^{b}	0.10^{b}	0.01	0.051	<0.05	<0.05
Eicosenoic acid (C20:1)	0.57^{ab}	0.61^{a}	0.55^{ab}	0.48b	0.02	0.153	0.054	0.101
Erucic acid (C22:1)	2.51	2.62	2.49	2.61	0.03	0.165	0.407	0.687
Nervonic acid (C24:1)	0.26	0.21	0.20	0.27	0.02	0.571	0.704	0.360
Docosahexaenoic acid (C22:6)	1.01	1.05	1.00	1.03	0.01	0.581	0.802	0.931
Saturated fatty acid	39.41	40.17	39.29	39.31	0.23	0.498	0.554	0.767
Unsaturated fatty acid	60.59	62.35	60.71	60.69	0.43	0.429	0.684	0.729

^{a,b,c} Different superscripts in a row indicate a significance difference ($p < 0.05$).
[1]Each value represents the mean value of 8 replicates per treatment.
[2]CON = control group; TF250 = CON + 250 mg/kg theaflavins; TF500 = CON + 500 mg/kg theaflavins; TF1000 = CON + 1,000 mg/kg theaflavins; SEM = standard error of the mean.

could increase the activities of SOD and decrease the MDA levels in serum, thereby alleviating oxidative stress caused by ovariectomy in mice (24). Zeng et al. report that TF administration significantly decreases MDA levels, increases SOD activity, and alleviates oxidative damage in the aorta caused by high-fat diet exposure in mice. Our findings align with that of a previous study demonstrating that TF intake enhances antioxidant capacity in laying hens (5).

Nrf2 is a key transcription factor that regulates cellular antioxidant defenses against oxidative stress by controlling the expression of various antioxidant and detoxifying enzymes. *NQO1* and *HO-1*, downstream genes of *Nrf2*, are widely recognized for their strong antioxidant properties. Reactive oxygen species (ROS) are highly unstable oxygen-free radicals. TF could suppress receptor activators of nuclear factor-kappa B ligand-induced ROS generation by upregulating the expression of antioxidant genes, such as *Nrf2*, *HO-1*, and catalase in osteoclasts (5, 25, 26). In this study, TF levels quadratically ($p < 0.05$) increased the expression of *Nrf2*, *HO-1*, and *NQO1*. These findings indicate that TF may exert antioxidant effects by upregulating the expression of *HO-1* and *NQO1* genes, with the Nrf2 signaling pathway likely serving as an underlying mechanism.

The role of TF in preventing diabetes has been extensively investigated. TF could regulate energy metabolism, enhance fatty acid oxidation, and reduce *de novo* lipogenesis through the AMP-activated protein kinase (AMPK) signaling pathway (27). In this study, TF quadratically decreased the serum TC, liver TC, and TG content. In obese mice, TF reduce the adiposity index by 24.5% and significantly decrease serum levels of TC and TG by 26.5 and 50.8%, respectively (28). Clinical trials show that oral administration of TF reduces serum levels of TC, TG, and LDL in patients with hypercholesterolemia. TF also improve body composition by proportionally decreasing total and subcutaneous fat while increasing skeletal muscle (29–31). Our finding was consistent with that of a previous study showing that the intake of TF by laying hens regulates energy metabolism and reduces TG and TC content in the serum and liver. By reducing lipid deposition, TF may redirect metabolic energy toward egg synthesis rather than adipose storage, thereby improving feed efficiency and egg production (32). Additionally, enhanced fatty acid oxidation via AMPK activation could maintain hepatic health, ensuring sufficient yolk precursor (VLDL) synthesis for egg formation (33). This dual modulation of energy partitioning and reproductive resource allocation may explain the improved laying performance observed in TF-supplemented hens.

AMPK serves as a key regulator of lipid metabolism, controlling whole-body metabolism by downregulating the *ACC* and *CPT-1* pathways, which regulate beta-oxidation (34). Our results showed that TF quadratically decreased the expression of *FAS* and *ACC* while increasing *CPT1* expression. TF reduced the gene expression of *ACC* and *FAS* while increasing *CPT1* expression in mice liver. This regulation inhibits fatty acids and cholesterol synthesis, decreases lipid accumulation, and promotes fatty acid oxidation (8, 9, 35). Our findings align with this, indicating that TF regulate lipid metabolism in laying hens through the modulation of *ACC*, *FAS*, and *CPT1*, with AMPK serving as a potential signaling pathway.

The antioxidant activity of eggs was determined using the DPPH, T-AOC and MDA contents. The DPPH radical scavenging model and T-AOC are widely used to evaluate the free radical scavenging ability of natural compounds (36). In our study, TF (500 mg/kg) increased the DPPH value and T-AOC while decreasing the MDA content in egg yolks. Studies show that tea extracts increase the antioxidant capacity of eggs. Zhou et al. and Wang et al. report that 600 mg/kg tea polyphenols increased the DPPH value and most free amino acids (isoleucine, leucine, tyrosine, phenylalanine, tryptophan, lysine, threonine, serine, glutamic acid, valine, and methionine) in egg yolks (10, 16). Wang et al. report that 400 mg/kg TBs improved the DPPH value, reducing power value, T-AOC, and levels of tryptophan, cysteine, methionine, and histidine in egg yolks while decreasing the MDA content (17). Our findings are consistent with those of previous studies, which show that antioxidative compounds such as egg-derived peptides, tryptophan, tyrosine, vitamin E, and carotenoids contribute to the T-AOC of egg yolks (13). Our results showed that 500 mg/kg of TF increased the tyrosine and tryptophan content in egg yolks. This indicates that TF enhance the antioxidant capacity of eggs by increasing the content of tyrosine and tryptophan in egg yolks. However, the mechanism by which TF increase the content of tyrosine and tryptophan in egg yolks remains unclear and requires further research.

Based on our findings, TF (250 mg/kg) significantly increased the linoleic acid content compared to that of the 500 and 1,000 mg/kg TF group. Studies show that high antioxidant capacity in the yolk preserves lipid quality by preventing oxidation and inhibiting the degradation of certain unsaturated fatty acids (37, 38). This indicates that TF can inhibit the degradation of unsaturated fatty acids by enhancing the antioxidant capacity of eggs.

5 Conclusion

TF improved the laying rate of laying hens by enhancing their antioxidant capacity and regulating lipid metabolism. TF also enhanced the antioxidant capacity of eggs by increasing the content of tyrosine and tryptophan levels in egg yolks. The optimal supplementation level of TF in laying hens is 500 mg/kg.

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 studies were approved by the Animal Care and Use Committee of Yibin University. The studies were conducted in accordance with the local legislation and institutional requirements. Written informed consent was obtained from the owners for the participation of their animals in this study.

Author contributions

LZ: Conceptualization, Investigation, Methodology, Software, Writing – original draft, Writing – review & editing. PZ: Conceptualization, Supervision, Writing – review & editing. JinZ: Conceptualization, Data curation, Investigation, Writing – review & editing. JieZ: Conceptualization, Formal analysis, Investigation, Writing – review & editing. YR: Investigation, Resources, Software, Writing – review & editing. JT: Data curation, Formal analysis, Project administration, Writing – review & editing. LJ: Conceptualization, Funding acquisition, Writing – review & editing. FC: Funding acquisition, Project administration, Writing – review & editing. LC: Funding acquisition, Project administration, Writing – review & editing. KZ: Funding acquisition, Project administration, Supervision, Writing – review & editing. OW: Writing – review & editing, Supervision. JP: Writing – review & editing.

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Conflict of interest

LJ, FC, and LC were employed by YibinShangougou Agricultural Science and Technology Co., Ltd.

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Effects of diet supplemented with water extracts of *Artemisia annua* L. on small intestinal immune and antioxidative indexes in lambs

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Introduction: *Artemisia annua* L., an herbaceous plant, belong to the Artemisia genus within the Asteraceae family. Due to its significant medicinal properties, it has emerged as a focal point of research in the field of animal production. In the present study, the responses of intestinal immune and antioxidative indexes, and the related gene expression to water extracts of *Artemisia annua* L. (WEAA) supplementation in diet were profiled in lambs.

Methods: In total, 32 female lambs (Dorper × Han), with eight replicates per group, were randomly assigned to four treatment groups. These groups were created by supplementing 0, 500, 1,000, and 1,500 mg/kg WEAA to the basal diet, respectively.

Results: The results showed that WEAA addition increased sIgA, IgG, IL-1β, IL-2 and IL-4 levels in the duodenal and jejunal mucosa in a manner that was dependent on the dosage ($p < 0.05$). Moreover, WEAA promoted the expression of factors (*TLR4, MyD88, IKKβ, IκBα, NF-κB p50, NF-κB p65, IL-1β* and *IL-4*) related with the TLR4/NF-κB pathway, thus improving small intestinal immune function, thereby showing peak effects in the 1,000 mg/kg WEAA group. Additionally, WEAA supplementation also enhanced antioxidative function through the Nrf2/Keap1 pathway in the small intestinal mucosa, particularly by increasing GSH-Px and CAT concentrations and decreasing MDA content in a manner that was dependent on the dosage ($p < 0.05$), with maximal effects observed in the 1,000 mg/kg group. Furthermore, expressions levels of *Nrf2*, *GSH-Px* and *HO-1* in the small intestine increased quadratically ($p < 0.05$), while *Keap1* expression levels exhibited a downward quadratic trend ($p < 0.10$).

Conclusion: In summary, the optimal dietary addition of 1,000 mg/kg WEAA significantly enhanced intestinal immune function, antioxidant capacity, and the expression of related genes in the intestinal mucosa of lambs.

KEYWORDS

water extracts of *Artemisia annua* L., lamb, small intestine, immune index, antioxidative index

1 Introduction

In recent decades, the global sheep industry has undergone a significant transformation from traditional extensive grazing systems to modern intensive production models. Due to increased demand for lamb and its by-products, land degradation problem is increasingly serious along with the pursuit of industry to improve the market competitiveness of livestock products and other issues (1, 2). In contemporary sheep production systems, this shift has brought about substantial changes to the natural habitat and innate behavior of sheep, which are likely to have negative effects on their metabolism, immune function and health (3, 4). These changes are different from the natural conditions to which sheep have been adapted for a long time (5). In addition, sheep health and productivity are heavily influenced by their diet composition, which holds a key position in growth performance, body development, and immune function, but the nutritional requirements of sheep are often easily overlooked in intensive production systems (6). Therefore, there has been a growing interest in improving diet nutrition strategies to enhance lamb health and performance. Among these strategies, natural plant-derived feed additives have potential to be a suitable alternative to synthetic growth promoters and alternatives to antibiotics in animal production in recent years (7–9).

The natural additives have been shown to have safe and reliable positive effects in livestock and poultry production, to have high consumer acceptance, and have the potential to promote animal growth and health without the developing resistance (10). *Artemisia annua* L. (*A. annua*), a member of the family Asteraceae which is considered native to Asia with its origin in China. *A. annua* is commonly known as sweet wormwood (11). This plant has a long history of being used as a traditional herbal remedy for malaria in China. Extensive research has been conducted on the *A. annua* and its extracts owing to their rich phytochemical profile and diverse biological activities, including polysaccharides, flavonoids, sesquiterpenes (artemisinin), phenols, essential oils, etc. (10, 12). Artemisinin is globally recognized for its effectiveness against malaria. In addition to its anti-malaria properties, *A. annua* also exhibits various other significant biological activities, including antibacterial, anti-inflammatory, anti-tumor, and antioxidant effects (13, 14).

In a series of studies involving different species, the multiple benefits of *A. annua* and its extracts on animal nutrition and health have been scientifically validated. Studies using rodents and poultry as models have shown that adding an appropriate amount of *A. annua* extract to the feed can significantly improve growth performance, optimize intestinal health, and enhance antioxidant status. Zhang et al. (15) evaluated immunomodulatory and antioxidant capabilities of polysaccharides derived from *A. annua*. These polysaccharides showed significant immunostimulatory activity and antioxidant effects, promoting the synthesis of TNF-α and IL-6 in mouse macrophages. Cui et al. (16) studied the effect of 1% *A. annua* supplemented to the diet on antioxidant capacity in serum and jejunum of geese. The results showed improved antioxidant enzyme activity (CAT, GSH-Px, SOD, T-AOC) and decreased MDA contents. Guo et al. (17) reported that the supplementing polysaccharides extracted from *A. annua* to the diet could significantly increase the expression of *CAT* and *Nrf2* in the small intestinal mucosa of broilers. This suggest that the enhancement of antioxidant enzyme activities may be through the Nrf2 signaling pathway. Similarly, in the field of aquaculture, *A. annua* leaf extract has been found to increased serum immunoglobulin levels, and activity of CAT and GSH-Px, while decreasing the content of malondialdehyde (MDA) in serum of Common Carp (18). In a study on weaned pigs, Niu et al. (19) found that the enzyme-treated *A. annua* (EA) affected the intestinal immune ability in weaned pigs, and the EA dosage of 2 g/kg decreased levels of IL-1β, IL-6, and TNF-αin the jejunum and increased the concentration of immunoglobulin (Ig) A and IgG in the ileum compared to the CON group. Song et al. (20) demonstrated that EA the intestinal inflammatory response of heat-stressed broilers, and found that EA could significantly increase IL-10 production and decrease *TLR4* mRNA expression in both the jejunum and ileum, suggesting that EA can inhibit TLR4/NF-κB pathway activation in heat-stressed conditions, thereby reducing inflammation. In addition, studies have shown that the regulatory mechanisms of Artemisia plants and their extracts are related to recognition of bio-active compounds by TLR4 on the surfaces of immune cells. This recognition triggers a series of signaling pathways, among which the NF-κB pathway is an extremely important pathway in cellular signaling and plays a key role in regulating the body's immune inflammatory response (21, 22). The results indicate that *A. annua* may have a relatively positive effect in different livestock species, including sheep. The intestinal tissue serves as both a physical and immune barrier against pathogens and toxins, playing a crucial role in nutrient absorption and immune system (23). Enhancing intestinal immunity and antioxidant function through dietary interventions may improve lambs' health and productivity.

The potential advantages of *A. annua* in animal nutrition have been demonstrated across several animal species. However, there is limited research exploring its role in sheep nutrition. Based on the unique physiological and nutritional demands of lambs, as well as the special challenges associated with intensive farming models, it has become increasingly important to study the effectiveness of *A. annua* supplements. Consequently, there is demand for further research into the impact of supplementing with WEAA on the intestinal immunity and antioxidant ability of lambs. These studies will provide valuable insights into the potential of *A. annua* as a natural feed additive in intensive sheep production, and may offer a sustainable and effective approach to improving lamb health and productivity. This research endeavor aims to fill this knowledge gap by evaluating the effects of WEAA supplementation on the intestinal immunity, antioxidant function, and their underlying molecular regulation in lambs, all within the context of conventional feeding practices.

2 Materials and methods

The animals used in the study were from a sheep farm in Hohhot, Inner Mongolia, China. All experimental protocols were approved by the Animal Care and Use Committee of Inner Mongolia Agricultural University (approval no. NND2021097) and followed the ethical guidelines for animal research established by the Laboratory Animal Sciences and Technical Committee (SAC/TC281) of the Standardization Administration of China, in accordance with *Laboratory animal—Guideline for ethical review of animal welfare* (GB/T 35892-2018) (24, 56).

2.1 Animals, diets, and design

In total, 32 female lambs (Dorper × Han) aged 3 months, weighing 24 ± 0.09 kg each, were recruited to the study. These lambs were divided into 4 groups, with 8 lambs in each group. The control group was fed solely a basal diet, whereas the treatment groups were provided with the same basal diet supplemented with different amounts of WEAA: 500, 1,000, and 1,500 mg/kg, respectively. The feeding trial lasted for 75 days (adaptation phase: 15d; formal experimental phase: 60d). The lambs were provided with pelleted feed twice a day (at 08:00 and 16:00 h) with *ad libitum* access to experimental diets and water. Each of the lambs was kept in individual pen, and their feed intake was carefully monitored to make sure that any leftover feed was over 5% of the total supply. The basic diet was designed to fulfill the Nutritional Requirements of Meat-type Sheep by NY/T 816–2021 (57). In Table 1, the feed components and the chemical makeup of the basal diet are outlined. Small intestinal (duodenum, jejunum and ileum) tissue samples were gathered to assess immune and antioxidative factors, along with the expression of related genes.

2.2 Preparation of WEAA

The extract utilized in this research originated from *A. annua* gathered in Hohhot, Inner Mongolia. The WEAA was produced according to the protocol detailed by Guo et al. (25). Briefly, the *A. annua* were shade-dried, cut into small pieces, and then processed through decoction, concentration, and finally, freeze-drying. The extraction process involved incubating the plant material in water (1:25 ratio) at 80°C for 7 h. Subsequently, the mixture underwent vacuum filtration, and the filtrate was concentrated at a temperature of 80°C by a rotary evaporator. The concentrated solution was then freeze-dried using an ALPHA1-2LD plus freeze-drying machine (Christ, Germany) until a powdered extract was obtained. All extracts were stored at 4°C for future use. We used UPLC-Orbitrap-MS system to identify the structures of the active ingredients of WEAA, according to the method used by Xin et al. (26). The results are shown in Table 2.

2.3 Sample collection and preparation of intestinal homogenate

On day 60 of the experiment, all 32 lambs (4 groups × 8 replicates, 1 lamb per replicate) were fasted for 12 h and then slaughtered at a commercial slaughterhouse by professionals, following the National Standard Operating Procedures (GB/T 43562-2023, *Code of Practice for Livestock and Poultry Slaughtering Operation—Sheep*, China). Each lamb was rendered unconscious using an electrical stunning method, exsanguinated via the jugular vein to induce death, and subsequently had their intestinal tissue collected. Samples from the duodenum, jejunum, and ileum were finely minced and homogenized using a hand-held homogenizer was employed in a 0.9% sodium chloride solution, kept ice-cold at 4°C, with a mixture ratio of 10% (w/v). After homogenization, the mixture was centrifuged at 3000 rpm for a duration of 10 min at 4°C. The supernatant obtained was then gathered for further analysis (27).

2.4 Analyses of intestinal immune and antioxidative indicators

Immune and antioxidant markers were evaluated through commercially available assays. Levels of IgA, IgG, IgM, IL-1β, IL-2,

TABLE 1 The composition and nutrient levels of the basal diet (air-dry basis, %).

Ingredients	Content	Nutrients composition[b]	Level
Alfalfa hay	16.25	Digestible energy (MJ/kg)	12.01
Corn straw	14.00	Dry matter	89.89
Oat grass	24.75	Crude protein	15.80
Corn	23.25	Neutral detergent fiber	40.80
Soybean meal	10.95	Acid detergent fiber	26.24
Wheat bran	4.25	Calcium	1.08
Corn germ meal	1.95	Phosphorus	0.40
Soybean oil	1.10		
Premix[a]	0.50		
Limestone	1.10		
Calcium phosphate dibasic	0.70		
Salt	0.40		
Sodium bicarbonate	0.80		
Total	100.00		

[a]The premix provided the following nutrient content for one kilogram of diet: vitamin A, 6000 IU; vitamin D3, 2,500 IU; vita-min E, 12.5 IU; vitamin K3, 31.8 mg; vitamin B1, 0.035; vitamin B2, 8.5 mg; vitamin B6, 0.9 mg; nicotinic acid, 22 mg; D-pantothenic acid, 17 mg; vitamin B12, 0.03 mg; biotin, 0.14 mg, folic acid, 1.5 mg; Fe, 0.04 g; Cu, 0.008 g; Zn,0.05 g; Mn, 0.03 g; I,0.3 mg, Se,0.3 mg; Co, 0.25 mg.
[b]Digestible energy was calculated according to the Nutritional Requirements of Meat Sheep (NY/T 816-2021), while the rest were measured values.

TABLE 2 Compound contents of WEAA (DM basis, %).

Compounds	Contents
Organic acids and derivatives	24.61
Soluble polysaccharide	18.64
Flavonoids	9.80
Prenol lipids	7.75
Organoheterocyclic compounds	7.75
Organooxygen compounds	5.01
Nucleosides, nucleotides, and analogues	5.01
Fatty acyls	4.79
Benzene and substituted derivatives	3.87
Glycerophospholipids	2.28
Coumarins and derivatives	2.05
Cinnamic acids and derivatives	1.82
Phenols	1.60
Others	5.01

IL-4, IL-6, and TNF-α in the duodenum, jejunum, and ileum were determined with ELISA kits (Wuhan Colorful Gene Biological Technology Co., Ltd., Wuhan, China; 96 T per kit each), following the instructions set forth by the manufacturer. Additionally, antioxidant indicators (SOD, CAT, GSH-Px, MDA and T-AOC) were assessed with a V-1000 spectrophotometer (AOE Instruments Shanghai Co., Ltd., Shanghai, China) in the duodenum, jejunum, and ileum. These measurements kits were purchased from Jiancheng Bioengineering Institute, located in Nanjing, China. All procedures were performed in accordance with the manufacturer's guidelines, and samples were assayed in duplicate.

2.5 Reverse transcriptase-quantitative PCR (RT-qPCR)

2.5.1 RNA extraction and cDNA synthesis

Total RNA was isolated from small intestine tissues (duodenum, jejunum, ileum) using TRIzol reagent (TaKaRa Biotechnology, Dalian, China). RNA concentration was quantified using a Pultton P200 + spectrophotometer (Plextech, San Jose, CA, United States), and purity was assessed by A_{260}/A_{280} ratio (1.8–2.1). RNA integrity was verified via 1% agarose gel electrophoresis. Genomic DNA contamination was eliminated by DNase I treatment (TaKaRa). cDNA was synthesized using the PrimeScript RT Master Mix kit (TaKaRa) on a LifeECO thermal cycler (Bori Technology, Hangzhou, China), following the protocol: 37°C for 15 min, 85°C for 5 s.

2.5.2 Quantitative real-time PCR (qRT-PCR)

The target genes and their specific primer sequences can be found in Table 3. Quantitative real-time PCR (qRT-PCR) was performed using the LightCycler® 96 System (Roche Diagnostics, Switzerland) and the TB® Premix Ex Taq™ Kit (TaKaRa). qPCR Cycling Conditions: pre—denaturation at 95°C for 30 s; followed by 45 cycles of denaturation at 95°C for 5 s; annealing at 60°C for 30 s; extension at 72°C for 20 s; followed by melting curve analysis (95°C for 15 s, 60°C for 1 min, 95°C for 15 s). All reactions were performed in strict accordance with the manufacturers' protocols (28).

2.5.3 Data analysis

Melting curves were analyzed to verify single peaks for product specificity. Outliers in triplicate Ct values ($n = 3$) were excluded, and the relative fold difference in mRNA expression levels was calculated using the $2^{-\Delta\Delta Ct}$ method (58).

2.6 Statistical analysis

All data were first processed using Microsoft Excel 2021 and then subjected to quadratic polynomial regression analysis with SAS Version 9.2 (2008, SAS Institute, Cary, NC, United States). Specifically, the analysis evaluated both linear and quadratic effects of dietary WEAA levels on various indices, in order to determine of dose-dependent relationships. Results were reported as mean and standard error of the mean (SEM), with $p < 0.05$ as the threshold for statistical significance. Additionally, a p-value within the range of $0.05 \leq p < 0.10$ was considered as a tendency.

3 Result

3.1 Small intestine inflammatory cytokines and immunoglobulins

The influence of WEAA supplementation on immunoglobulin levels in the small intestine of lambs is shown in Table 4. With increasing WEAA supplementation, the duodenal sIgA level demonstrated a linear upward trend ($p < 0.10$) and a significant quadratic increase ($p < 0.05$), whereas the IgG level exhibited a linear or quadratic increase ($p < 0.05$). The jejunal sIgA level demonstrated a quadratic increasing trend ($p < 0.10$), while IgG levels exhibited a linear upward trend ($p < 0.10$) and a significant quadratic increase ($p < 0.05$), peaking at 1000 mg/kg WEAA group. Additionally, WEAA supplementation did not significantly affect immunoglobulin levels in ileum tissue ($p > 0.10$).

In Table 5, intestinal inflammatory cytokine contents were shown. With increasing WEAA supplementation, IL-1β content of duodenum tissue demonstrated a significant linear increase ($p < 0.05$) and a quadratic trend ($p < 0.10$), while jejunal IL-1β level exhibited a quadratic upward trend ($p < 0.10$). Additionally, the duodenal IL-2 and IL-4 levels exhibited a significant quadratic increase ($p < 0.05$), and the jejunal and ileal IL-2 levels showed a linear or quadratic increase ($p < 0.05$), while jejunal IL-4 level exhibited a significant linear or quadratic increase ($p < 0.05$). Meanwhile, the ileal IL-4 content tended to increase linearly ($p < 0.10$).

3.2 Small intestine antioxidative indexes

The effects of WEAA on the antioxidative indexes in the intestinal tissue of lambs is showed in Table 6. With increased WEAA supplementation, the jejunal T-AOC demonstrated a significant quadratic increase ($p < 0.05$), while the ileal T-AOC exhibited a linear increasing trend ($p < 0.10$). The duodenal GSH-Px concentration

TABLE 3 Primer sequences and parameter.

Gene	Sequence (5′- > 3′)[1]	GenBank no.	Length/bp
β-actin	F- ACAATGTGGCCGAGGACTTT	NM_001009784.3	278
	R- GCCGTGATGGCTGACCATTC		
GAPDH	F- TTATGACCACTGTCCACGCC	NM_001190390.1	216
	R- TCAGATCCACAACGGACACG		
Nrf2	F- TGTGGAGGAGTTCAACGAGC	XM_004004557.1	88
	R- CGCCGCCATCTTGTTCTTG		
Keap1	F- TTCAACAGCGAAAGTCAGGC	XM_027969637.2	157
	R- TGCGTAGCCTCCGATACTCT		
SOD1	F- GGAGACCTGGGCAATGTGAA	NM_001145185	182
	R- CCTCCAGCGTTTCCAGTCTT		
SOD2	F- AAACCGTCAGCCTTACACC	NM_001280703.1	116
	R- ACAAGCCACGCTCAGAAAC		
CAT	F- GAGCCCACCTGCAAAGTTCT	XM_004016396	148
	R- CTCCTACTGGATTACCGGCG		
GSH-Px	F- TGGTCGTACTCGGCTTCCC	XM_004018462.1	163
	R- AGCGGATGCGCCTTCTCG		
HO-1	F- CGATGGGTCCTCACACTCAG	XM_027967703.2	74
	R- CACACTCGCATTCACATGGC		
NQO1	F- CTCTGGCCAATTCAGAGTGG	XM_004015102.5	296
	R- TCCATTGGGATGGACTTGCC		
TLR4	F- CCTTGCGTACAGGTTGTTCC	NM_001135930.1	99
	R- GTCCAGCATCTCGGTTGACA		
MyD88	F- ATTGAGAAGAGGTGCCGTCG	NM_001166183.1	189
	R- ACAGACAGTGATGAAGCGCA		
IKKβ	F- GCCGCCCATTACAAGCTGAA	XM_042241396.1	165
	R- CTGGAAGAACGGGAGGTTCC		
IκB-α	F- TCACCTACCAGGGCTACTCC	NM_001166184.1	153
	R- CTGTGAACTCTGATTCGGTGTC		
NF-κBp50	F- GATGCCACTGCCAACAGAGA	XM_042251202.1	191
	R- GCGTCTGTCATTCGTGCTTC		
NF-κBp65	F- CTCCTCTCGGGGGATGAAGA	XM_027959295.2	123
	R- ATCCCTTGCTAACCCACTGC		
IL-1β	F- CTGTGGCCTTGGGTATCAGG	NM_001009465.2	251
	R- GCCACCTCTAAAACGTCCCA		
IL-4	F- GCTGAACATCCTCACATCGAG	AF1721681	87
	R- TTCTCAGTTGCGTTCTTTGG		
TNF-α	F- AGTCTGGGCAGGTCTACTTTG	NM_001024860	127
	R- GGTAACTGAGGTGGGAGAGG		
iNOS	F- AGACTGAGCCTCTCTAGCCC	XM_042255454.1	96
	R- GGAACCGTCTATAGCTGCCC		
COX-2	F- ACTTTCACGACCACACATTA	NC_001941.1	501
	R- GACGAGTTGACATAAGGGTT		

[1] F: forward primer; R: reverse primer.

TABLE 4 Effects of WEAA on immunoglobulins in the small intestine of lambs.

Item	WEAA mg/kg				SEM	*P*-value	
	0	500	1,000	1,500		Linear	Quadratic
sIgA, μg/mg prot.							
Duodenum	2.78	3.10	4.00	3.13	0.22	0.068	0.004
Jejunum	2.24	2.62	2.77	2.54	0.29	0.183	0.080
Ileum	3.04	2.95	3.20	3.42	0.10	0.099	0.181
IgG, μg/mg prot.							
Duodenum	5.78	7.23	8.75	7.19	0.32	0.045	0.005
Jejunum	4.96	6.15	6.86	5.91	0.41	0.056	0.003
Ileum	5.79	5.54	6.13	6.18	0.15	0.180	0.353
IgM, μg/mg prot.							
Duodenum	6.05	6.69	7.45	6.42	0.23	0.367	0.116
Jejunum	4.58	4.42	4.59	4.33	0.38	0.726	0.932
Ileum	4.84	4.96	5.55	5.08	0.20	0.477	0.603

TABLE 5 Effects of WEAA on inflammatory cytokines in the small intestine of lambs.

Item	WEAA mg/kg				SEM	*P*-value	
	0	500	1,000	1,500		Linear	Quadratic
IL-1β, pg./mg prot.							
Duodenum	68.99	72.30	79.67	78.02	3.83	0.032	0.082
Jejunum	74.95	81.76	88.35	80.47	6.95	0.188	0.065
Ileum	86.33	83.08	85.09	89.03	3.09	0.598	0.612
IL-2, pg./mg prot.							
Duodenum	60.64	67.82	82.61	61.77	2.50	0.425	0.010
Jejunum	47.41	58.01	57.34	58.05	4.74	0.043	0.043
Ileum	48.52	49.83	62.60	65.77	2.56	0.003	0.014
IL-4, pg./mg prot.							
Duodenum	23.03	30.39	34.28	26.35	1.11	0.169	<0.001
Jejunum	21.79	33.13	35.26	32.98	2.18	<0.001	<0.001
Ileum	25.37	23.28	29.71	29.15	1.09	0.067	0.181
IL-6, pg./mg prot.							
Duodenum	34.09	33.42	34.25	31.95	0.96	0.349	0.540
Jejunum	33.70	32.20	31.25	29.59	1.96	0.126	0.315
Ileum	30.48	20.63	27.23	25.34	1.26	0.441	0.215
TNF-α, pg./mg prot.							
Duodenum	69.52	70.53	70.67	70.76	2.68	0.801	0.961
Jejunum	73.99	74.15	70.98	73.29	6.06	0.781	0.932
Ileum	79.07	77.38	81.15	81.03	2.28	0.645	0.889

demonstrated a significant quadratic increase ($p < 0.05$), and jejunal GSH-Px concentration tended to increase quadratically ($p < 0.10$). Moreover, with the increase of WEAA dose, the ileal GSH-Px, duodenal and jejunal CAT concentration revealed a significant linear or quadratic increase effect ($p < 0.05$), while the duodenal and jejunal MDA content demonstrated a linear or quadratic decrease effect ($p < 0.05$), in addition, the concentration of MDA in the ileal tended to decline linearly ($p < 0.10$).

3.3 Duodenum TLR4/NF-κB and Nrf2/Keap1 pathway related gene expression

Figure 1 shows the levels of mRNA expressions of immune-related genes in the duodenum. With the supplementation of WEAA increased, the expression levels of *TLR4*, *NF-κBp50* and *IL-1β* demonstrated a significant linear or quadratic increase effect ($p < 0.05$). The expression levels of *MyD88* exhibited a linear

TABLE 6 Effects of WEAA on antioxidative indexes in the small intestine of lambs.

Item	WEAA mg/kg				SEM	*P*-value	
	0	500	1,000	1,500		Linear	Quadratic
T-AOC, μmol/mg prot.							
Duodenum	0.07	0.07	0.08	0.08	0.00	0.147	0.250
Jejunum	0.04	0.05	0.05	0.04	0.00	0.385	0.017
Ileum	0.04	0.04	0.05	0.05	0.00	0.062	0.136
T-SOD, U/mg prot.							
Duodenum	152.88	166.35	169.85	154.60	14.02	0.814	0.200
Jejunum	88.16	91.12	92.42	85.17	3.33	0.528	0.128
Ileum	93.30	96.04	98.11	98.63	2.63	0.214	0.443
GSH-Px, U/mg prot.							
Duodenum	48.30	58.44	72.72	49.87	3.38	0.381	<0.001
Jejunum	23.90	26.38	27.96	20.88	1.75	0.478	0.086
Ileum	20.16	25.21	32.12	35.47	1.72	<0.001	0.001
CAT, U/mg prot.							
Duodenum	12.36	22.12	22.28	19.01	1.62	0.025	<0.001
Jejunum	14.37	21.33	26.43	24.59	1.42	<0.001	<0.001
Ileum	13.52	16.80	13.21	17.69	0.86	0.252	0.494
MDA, nmol/mg prot.							
Duodenum	2.78	2.64	2.45	1.80	0.19	0.010	0.023
Jejunum	1.35	1.02	1.00	1.11	0.14	0.017	<0.001
Ileum	0.78	0.65	0.61	0.64	0.03	0.090	0.108

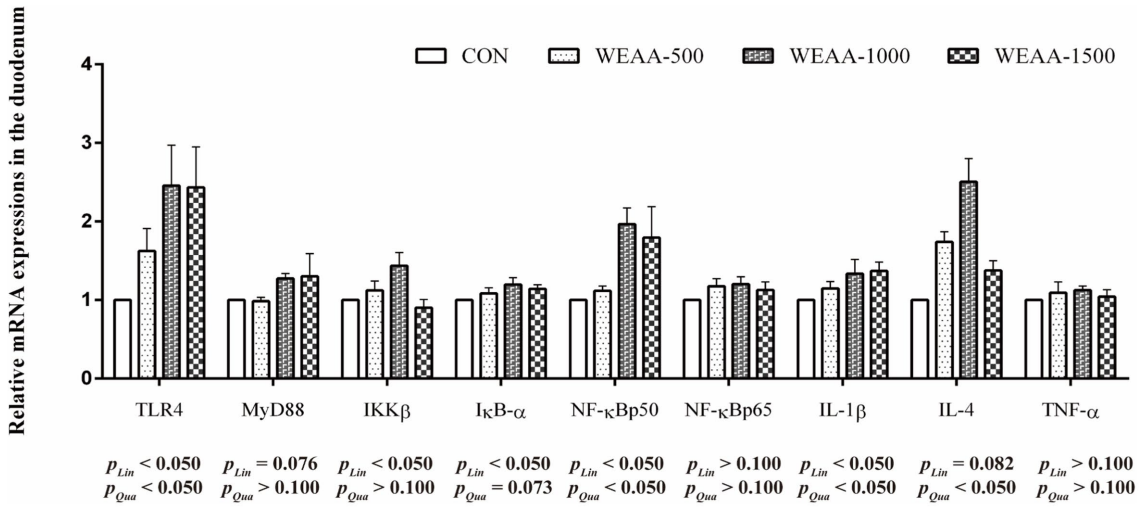

FIGURE 1
Effects of WEAA on expression of immune-related genes in the duodenum of lambs. Dose-dependent effects of WEAA (Lin: Linear; Qua: Quadratic) and the same as below.

upward trend ($p < 0.10$). The expression levels of *IKKβ* demonstrated a linear increase ($p < 0.05$). Meanwhile, the expression levels of *IκB-α* exhibited a linear increase ($p < 0.05$) and a quadratic increasing trend ($p < 0.10$). In addition, the *IL-4* expression exhibited a linear increasing trend ($p < 0.10$), and a significant quadratic increase ($p < 0.05$).

Figure 2 showed the levels of mRNA expression related to antioxidant genes within the duodenum. With the supplementation of WEAA increased, the expression levels of *Nrf2* revealed an effect of increasing quadratically ($p < 0.05$). The expression levels of *Keap1* demonstrated a significant quadratic downward trend ($p < 0.10$), while the expression levels of *CAT* and *GSH-Px*

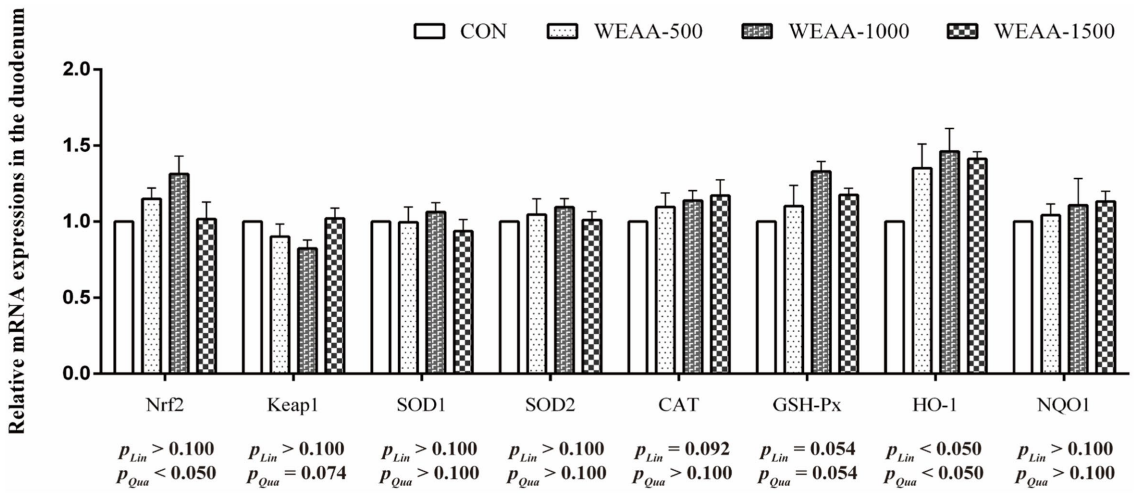

FIGURE 2
Effects of WEAA on expression of antioxidant-related genes in the duodenum of lambs.

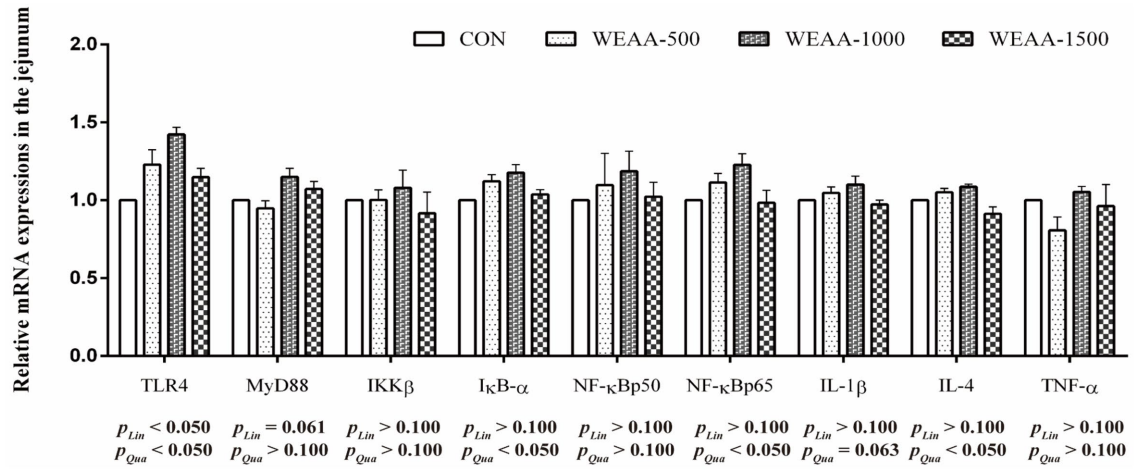

FIGURE 3
Effects of WEAA on expression of immune-related genes in the jejunum of lambs.

revealed a linear upward trend ($p < 0.10$) and *GSH-Px* gene expression revealed a quadratic increasing trend ($p < 0.10$). Moreover, with the supplementation of WEAA increased, the expression level of *HO-1* revealed a significant linear or quadratic increase ($p < 0.05$).

3.4 Jejunum TLR4/NF-κB and Nrf2/Keap1 pathway related gene expression

Figure 3 revealed the relative mRNA expressions levels of immune-related genes in the jejunum. With the supplementation of WEAA increased, the expression of *TLR4* demonstrated a significant linear or quadratic increase ($p < 0.05$). The expression levels of *MyD88* demonstrated a linear increasing trend ($p < 0.10$). Meanwhile, the expression of *IκB-α*, *NF-κBp65* and *IL-4* demonstrated a significant quadratic increase ($p < 0.05$), and the expression of *IL-1β* revealed a quadratic upward trend ($p < 0.10$).

Figure 4 showed the relative mRNA expressions of relative antioxidant genes in the jejunum. With the supplementation of WEAA increased, *Nrf2*, *CAT*, *GSH-Px* and *HO-1* demonstrated a significant quadratic increase ($p < 0.05$). The expression levels of *Keap1* revealed a quadratic downward trend ($p < 0.10$). Meanwhile, the expression of *SOD2* demonstrated a significant linear increase effect ($p < 0.05$).

3.5 Ileum TLR4/NF-κB and Nrf2/Keap1 pathway related gene expression

Figure 5 demonstrated the relative expressions of immune-related genes in the ileum. With the supplementation of WEAA increased, the expression of *MyD88* and *IL-4* revealed a significant linear increase

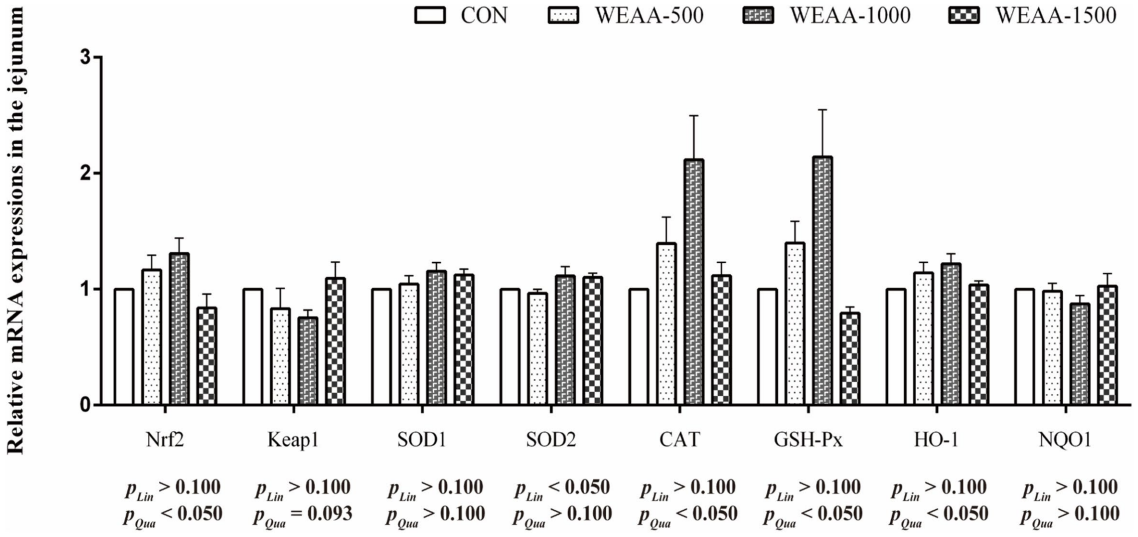

FIGURE 4
Effects of WEAA on expression of antioxidant-related genes in the jejunum of lambs.

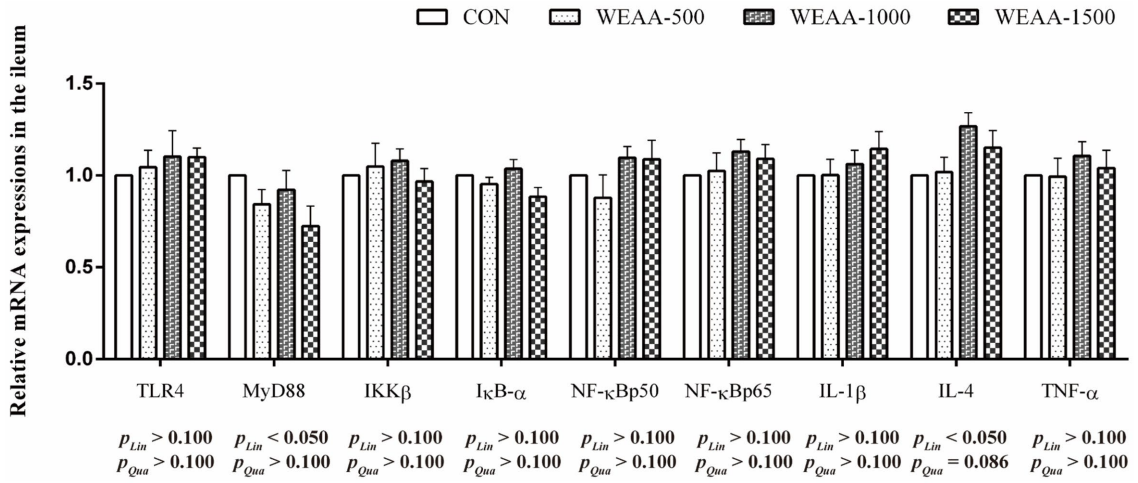

FIGURE 5
Effects of WEAA on expression of immune-related genes in the ileum of lambs.

($p < 0.05$). Meanwhile, the *IL-4* gene expression demonstrated a quadratic increasing trend ($p < 0.10$).

Figure 6 showed the relative expressions levels of relative antioxidant genes in the ileum. With the supplementation of WEAA increased, the expression levels of *Nrf2* and *GSH-Px* revealed a significant quadratic increase ($p < 0.05$). Meanwhile, the *HO-1* expression demonstrated a quadratic upward trend ($p < 0.10$).

4 Discussion

A. annua is a traditional medicinal plant from China, well-known for its significant biological effectiveness and minimal toxicity. It is typically prepared as decoctions to break down the plant cell wall, thus aiding in the release of bioactive constituents (29). Notably, among these constituents are polysaccharides, flavonoids, and sesquiterpenes, which have particular relevance in the field of immunopharmacology (22) *A. annua* contains a variety of active components, such as flavonoids, polysaccharides, and sesquiterpenes (17, 30). In the present study, the identification of active ingredients by structure analysis for WEAA indicated the presence of organic acids, polysaccharide, flavonoids and prenol lipids, which were the dominant substances (Table 2). Previous research indicated that flavonoids from *Artemisia ordosica* that significantly boost antioxidant enzyme activity when added to broiler diets (31). Moreover, extracts from *A. annua* have shown antioxidative properties in mouse models (32). Additionally, polysaccharides obtained from the leaves of *Artemisia argyi* have been found to possess antioxidant and immune-boosting abilities (33, 34).

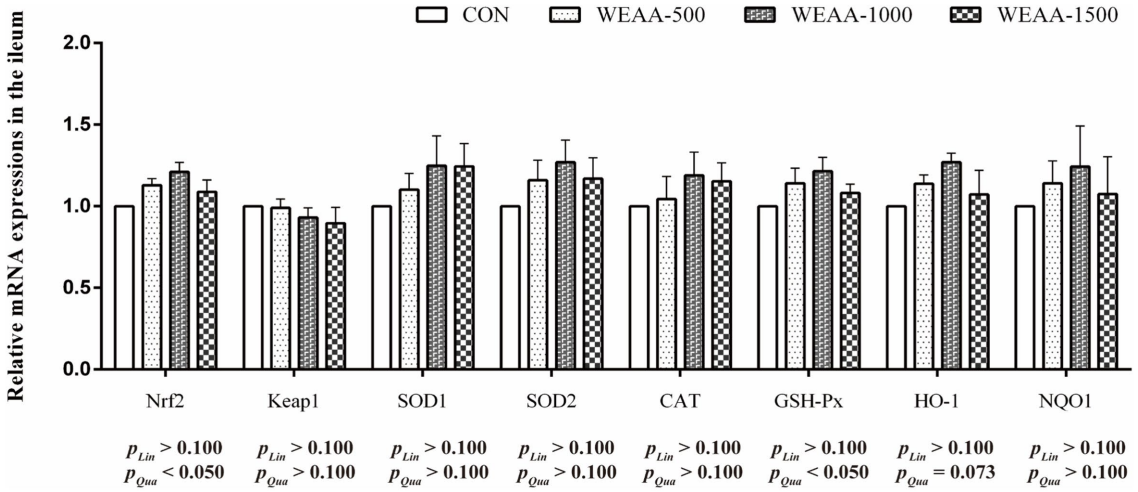

FIGURE 6
Effects of WEAA on expression of antioxidant-related genes in the ileum of lambs.

This study observed elevated sIgA and IgG concentrations in the duodenum and jejunum of lambs as dietary intake of WEAA increased. This result agrees with the findings of Guo et al. (35), who demonstrated that a diet high in WEAA increased the concentrations of sIgA, IgG, and IgM in the small intestine of broiler chickens. Immunoglobulins, which are antigen-specific receptors, are essential for humoral immunity as they bind to specific antigens, such as flavonoids and plant polysaccharides, thereby triggering immune cell signaling that initiates effective adaptive immune responses (36). Specifically, sIgA is essential for the maintenance of intestinal homeostasis, that is, maintaining the integrity of biological barriers and limiting microorganism and antigen exposure. Moreover, the immune response is directly influenced by IgG, which is produced by B cells (37). Supporting these findings, Yang et al. (38) demonstrated that the inclusion of alcohol extracts from *Artemisia argyi* in the diet mitigated the adverse effects induced by LPS challenges. This effect is attributed to an increase in immunoglobulin levels (IgA, IgG, and IgM) in the small intestine of broiler chickens, suggesting an enhanced immune response in the intestinal tract. Moreover, Zhang et al. (39) investigated impact of water extracts from *Artemisia rupestris* L. (AEAR) in both *in vivo* and *in vitro*, showing that AEAR significantly increased IgG titers in ICR mice, partly by promoting dendritic cell maturation via the TLR4 signaling pathway, thereby facilitating immune cell proliferation, differentiation, and autoantibody production.

TNF-α, IL-1β, and IL-6 are crucial inflammatory cytokines in the intestinal immune system, where they activate adaptive immune responses to combat pathogenic bacterial invasion. However, their excessive expression can damage mucosal cells. Song et al. (23) demonstrated that exposure to heat enhanced the *TLR4* and pro-inflammatory cytokines expression in the intestinal of broiler chickens. However, pretreatment with EA mitigated the increase of IL-1β expression levels of *TLR4* and *IL-6* in the intestinal regions under heat stress. Additionally, a dietary supplement of 0.1% *A. annua* extract led to a decrease in both the content and expression of inflammatory cytokines within the small intestine of both neonatal and post-weaning piglets (15). These results suggest that *A. annua* plays a beneficial role in alleviating intestinal inflammation. Yang et al. (40) also demonstrated that AEAR stimulated the content of IL-1β and IL-6 through the activation of TLR2/4 signaling pathways in dendritic cells, without inducing toxicity. Du et al. (41) demonstrated that polysaccharides derived from *Artemisia ordosica* significantly stimulated inflammatory cytokine production in the small intestine with a dose-dependent manner, thereby improved the immune function of broilers under conventional feeding conditions. Similarly, *Artemisia argyi* polysaccharide at an optimal dosage regulated immune functions, elevating cytokine levels (IL-1β, IL-2, and IL-4) and IFN-γ in broiler serum (42). The present study suggested that supplementing the diet with WEAA might enhance the content and gene expression of IL-1β, IL-2, and IL-4 in the duodenum and jejunum of lambs. These results suggested that WEAA could play a significant role in modulating immune responses.

In order to delve deeper into the molecular mechanisms underlying the impact of WEAA addition on intestinal immunoregulation, we detected the expression levels of crucial mRNAs associated with the TLR4/NF-κB signaling cascades. *A. annua*, a well-known medicinal plant, primarily comprises polysaccharides, flavonoids, and artemisinin as its main components which are noted for their structural diversity and enhance their affinity with various pattern recognition receptors (PRRs), thereby initiating signal pathways related to immune response. TLR4, a pattern recognition receptor belonging to the TLR family. This receptor is essential for immune responses and is present in nearly all types of intestinal epithelial cells. Research identified that water-soluble polysaccharide from *Artemisia rupestris* L. interacted with TLR2/4 on dendritic cells, facilitating cytokine production and signal transduction processes. This interaction not only enhanced the phosphorylation levels of IKKβ and NF-κBp65 but also stimulated the degradation of IκB-α in the cytoplasm and promoted the translocation of NF-κBp65 into the nucleus (39). These mechanism insights suggest that the elicitation of NF-κB via TLR2/4 pathways by these polysaccharides is crucial for cytokine synthesis. Further studies by Ando et al. (43) uncovered that safflower polysaccharides could activate TLR4/NF-κB pathway, thereby inducing cytokine production

in macrophages. Additionally, Guo et al. (44) found that *A. annua* polysaccharide alleviated excessive release of intestinal pro-inflammatory factors induced by *E. coli* challenge in broilers via inhibiting TLR4/MyD88/NF-κB signaling pathway, and up-regulated anti-inflammatory factor IL-4 and immunoglobulins (IgA, IgG, IgM) to synergistically enhance immune regulation; moreover, *A. annua* polysaccharide also increased serum IgA in non-challenged broilers, reversed *E. coli*-induced decrease in serum IgM and jejunal IgG/IgM, and promoted IL-4 secretion, furthermore, enhanced anti-inflammatory effect. These results suggest that *A. annua* polysaccharide coordinates immune regulation by modulating TLR4/NF-κB pathway and immune effector molecules. The present study found that dietary supplementation with WEAA could significantly enhance *TLR4* expression in the duodenum and jejunum of lambs, and up-regulated the gene expression levels of its downstream signaling molecules *MyD88*, *IKKβ*, *IκBα*, *NF-κBp50* and *NF-κBp65*. The results suggest that WEAA may recognize extracellular signals through activating TLR4 receptors, thereby initiating the cascade activation of the TLR4/MyD88/NF-κB signaling pathway. It was noteworthy that the significant increase in *IκBα* expression might be due to the transcription and expression of *IκBα* induced by the pathway to form negative feedback regulation to limit the excessive activation of NF-κB (45), thereby avoiding excessive inflammatory response in intestinal tissues. Generally, the activation of the NF-κB signaling pathway promotes the pathway's binding to the κB response element of target genes, and initiates the transcription of pro-inflammatory cytokines such as TNF-α, IL-1β, and IL-6, mediates inflammatory responses and immune responses, and indirectly promotes the production of immunoglobulins (46), regulates the secretion of T cell-related cytokines (IL-2), and affects the secretion of IL-4 by Th2 cells, thereby enhancing the body's immune function from multiple dimensions (47). Some studies found that AEAR significantly increased the gene expression of IL-1β and IL-2 and the protein expression level of NF-κB in dendritic cells, which significantly enhanced the immunomodulatory activity of dendritic cells (40). Furthermore, polysaccharides derived from *Artemisia ordosica* were reported to enhance the contents of immunoglobulins (IgG, IgA, IgM) and cytokines (IL-1β, IL-2, IL-4), also to increase the mRNA expression of the *TLR4*, *MyD88*, *NF-κBp50*, and *IL-1β* in the small intestine of broilers (41). Similarly, the current study revealed that supplementing WEAA into the diet up-regulated the mRNA expression of *IL-1β* in the duodenum and jejunum of lambs, which was consistent with the increase in its content. Additionally, the trend of its change was similar to the expression of *NF-κB*. Meanwhile, the content of IL-4 and its mRNA expression level were also significantly increased, demonstrating the anti-inflammatory activity of WEAA. Based on the above results, it is speculated that under normal physiological conditions, WEAA can synergistically improve the immune regulation function of the body by regulating the dynamic balance of the TLR4/NF-κB signaling pathway. In addition, a relatively significant change in relative expressions of the related factors was noted in the small intestine that is located in its proximal and middle regions of lambs.

Reactive oxygen species (ROS) are produced throughout the normal growth and metabolic processes of mammals. They play a crucial and continuous role in several functions, including intracellular signal transduction and the modulation and activation of immune responses. If these radicals are not promptly scavenged, they accumulate in the body, damaging cellular morphology and function. The peroxidation of unsaturated lipids within biological membranes results in the formation of lipid peroxides and malondialdehyde, which precipitate a cascade of inflammatory changes (48). The antioxidant defense system has a variety of endogenous antioxidant enzymes in cells, which are the first line of defense against oxidative stress. Specifically, Mn-SOD catalyzes the conversion of O^{2-} to e H_2O_2 and plays a significant role in activating associated signaling pathways that promote the activity of SOD1, CAT, GSH-Px, HO-1, thereby facilitating the detoxification of H_2O_2 into H_2O (49). In the current research, dietary WEAA was found to improve the activity of CAT and GSH-Px in the duodenum. Meanwhile, it increased the activity of T-AOC, CAT, and GSH-Px in the jejunum, and the activity of GSH-Px in the ileum of lambs. This finding aligned with a prior study by Zhang et al. (50) on broilers, which stated that dietary *Artemisia argyi* powder could enhance intestinal antioxidative enzyme activities. Moreover, this study found that an increase in the relative gene expressions of CAT and GSH-Px were observed in the small intestine, and the changes in relative gene expression were consistent with the change in enzymatic activity. These results suggest that WEAA may improve small intestinal antioxidant capacity by regulating gene expression and activity of the antioxidant enzymes. By contrast, MDA is a reliable biomarker for the assessment of oxidative stress (51). The current study demonstrated that the dietary supplementation of WEAA to lambs MDA levels were found to decrease in duodenum and jejunum of lambs. The observation of Wan et al. (8) that *A. annua* leaves and EA can enhance antioxidant enzyme activities, while simultaneously reducing MDA levels in both the serum and liver of broilers support our research findings. This finding underscores the potential for *in vivo* antioxidant defense. Furthermore, Song et al. (23) reported that *A. annua* was rich in polysaccharides, phenolic and flavonoids. Their researches demonstrated that dietary addition with EA could alleviate the elevation of MDA concentration in the intestine induced by heat stress and relieve the stress response in broilers following such stress. As we all know, under the normal physiological conditions or the status with low levels of lipid peroxidation, organisms have an inherent capacity to regulate cellular metabolism. This regulatory mechanism via the activation of related signaling pathways to effectively enhance the activity of antioxidant enzymes. Consequently, this leads to an adaptive stress response that helps maintain cellular homeostasis (52). Nrf2 is a key regulatory factor with intracellular antioxidant and anti-inflammatory damage. Nrf2 dissociates from Keap1 into the nucleus and binds to antioxidant response elements (ARE), promoting the transcription of cell protective factors and upregulating the expression of related antioxidant genes. This leads to an increase in intracellular antioxidant enzyme levels, thereby clearing free radicals and reactive oxygen species, and resistance to oxidative damage (53). In addition, the activation of the Nrf2-ARE signaling pathway not only has the effect of anti-oxidative stress, but also enhance the body's immune response (54). Therefore, the Nrf2/Keap1 signaling pathway, as a key protective pathway, has important biological significance for the immune and antioxidant functions of the body. Niu et al. (55) when they fed weaned piglets with EA, it could improve the intestinal antioxidant status. Specifically, the mRNA expression of *Nrf2* and *HO-1* was raised. Xing et al. (46) further suggested that the effect of adding polysaccharides derived from *Artemisia ordosica* to diet on lipopolysaccharide induced oxidative stress in broilers, and found can alleviate oxidative stress in broilers by regulating the Nrf2 signaling pathway and increasing the production of

antioxidant enzymes. The current research demonstrated that WEAA greatly enhanced *Nrf2* and *HO-1* expression levels in intestine, accompanied by a significant decrease in *Keap1* gene expression. These findings showed that WEAA might enhance the antioxidant activity of intestinal tissue by activating Nrf2-mediated pathway related mRNA expressions. Therefore, WEAA emerges as a promising green feed additive capable of enhancing immunomodulatory and antioxidant capabilities within the intestines of lambs.

5 Conclusion

To summarize, this research showed that dietary supplementation of WEAA in lambs effectively modulated the TLR4/NF-κB signaling pathway in the duodenum and jejunum, promoting the production of immunoglobulins (sIgA, IgG) and cytokines (IL-1β, IL-2, IL-4). Concurrently, WEAA significantly upregulated Nrf2/HO-1 expression while downregulating Keap1, thereby activating the antioxidant pathway and enhancing antioxidant enzyme (CAT, GSH-Px) activity. The study determined that the optimal dosage of WEAA in lamb's diet was 1,000 mg per kg of feed. Future studies should explore the molecular mechanisms for WEAA regulating TLR4/NF-κB and Keap1/Nrf2 pathways.

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 approved by Animal Care and Use Committee of Inner Mongolia Agricultural University (approval code: NND2021097). The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

GG: Conceptualization, Formal analysis, Methodology, Visualization, Writing – original draft. RG: Resources, Writing – review & editing. HZ: Investigation, Writing – review & editing. XJ: Data curation, Writing – review & editing. YXi: Validation, Writing – review & editing. LH: Supervision, Writing – review & editing. SY: Conceptualization, Supervision, Writing – review & editing. YXu: Supervision, Validation, Writing – review & editing. BS: Conceptualization, Supervision, Writing – review & editing.

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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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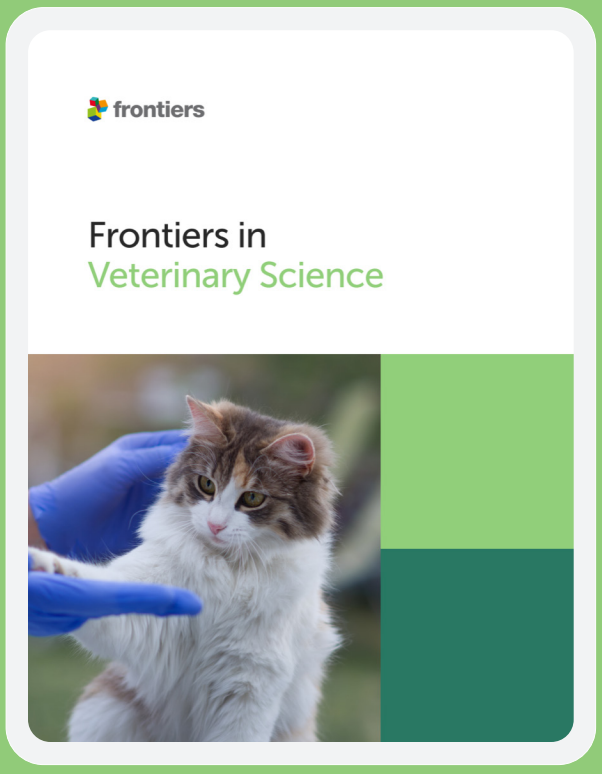

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