frontiers | Research Topics

Nutricines and derivatives of nutrients in animal health and disease prevention

Edited by
Tao Wang and HongGu Lee

Published in
Frontiers in Veterinary Science

ISSN 1664-8714
ISBN 978-2-8325-4657-4
DOI 10.3389/978-2-8325-4657-4

About Frontiers

Frontiers is more than just an open access publisher of scholarly articles: it is a pioneering approach to the world of academia, radically improving the way scholarly research is managed. The grand vision of Frontiers is a world where all people have an equal opportunity to seek, share and generate knowledge. Frontiers provides immediate and permanent online open access to all its publications, but this alone is not enough to realize our grand goals.

Frontiers journal series

The Frontiers journal series is a multi-tier and interdisciplinary set of open-access, online journals, promising a paradigm shift from the current review, selection and dissemination processes in academic publishing. All Frontiers journals are driven by researchers for researchers; therefore, they constitute a service to the scholarly community. At the same time, the *Frontiers journal series* operates on a revolutionary invention, the tiered publishing system, initially addressing specific communities of scholars, and gradually climbing up to broader public understanding, thus serving the interests of the lay society, too.

Dedication to quality

Each Frontiers article is a landmark of the highest quality, thanks to genuinely collaborative interactions between authors and review editors, who include some of the world's best academicians. Research must be certified by peers before entering a stream of knowledge that may eventually reach the public - and shape society; therefore, Frontiers only applies the most rigorous and unbiased reviews. Frontiers revolutionizes research publishing by freely delivering the most outstanding research, evaluated with no bias from both the academic and social point of view. By applying the most advanced information technologies, Frontiers is catapulting scholarly publishing into a new generation.

What are Frontiers Research Topics?

Frontiers Research Topics are very popular trademarks of the *Frontiers journals series*: they are collections of at least ten articles, all centered on a particular subject. With their unique mix of varied contributions from Original Research to Review Articles, Frontiers Research Topics unify the most influential researchers, the latest key findings and historical advances in a hot research area.

Find out more on how to host your own Frontiers Research Topic or contribute to one as an author by contacting the Frontiers editorial office: frontiersin.org/about/contact

Nutricines and derivatives of nutrients in animal health and disease prevention

Topic editors

Tao Wang — Jilin Agricultural University, China

HongGu Lee — Konkuk University, Republic of Korea

Citation

Wang, T., Lee, H., eds. (2024). *Nutricines and derivatives of nutrients in animal health and disease prevention*. Lausanne: Frontiers Media SA.

doi: 10.3389/978-2-8325-4657-4

Table of contents

frontiers | Frontiers in Veterinary Science

TYPE Editorial
PUBLISHED 12 March 2024
DOI 10.3389/fvets.2024.1391563

OPEN ACCESS

EDITED AND REVIEWED BY
Adronie Verbrugghe,
University of Guelph, Canada

*CORRESPONDENCE
Tao Wang
✉ cagewang@163.com

RECEIVED 26 February 2024
ACCEPTED 01 March 2024
PUBLISHED 12 March 2024

CITATION
Wang T and Lee H-G (2024) Editorial: Nutricines and derivatives of nutrients in animal health and disease prevention. *Front. Vet. Sci.* 11:1391563. doi: 10.3389/fvets.2024.1391563

Editorial: Nutricines and derivatives of nutrients in animal health and disease prevention

Tao Wang[1]* and Hong-Gu Lee[2]

[1]Key Laboratory of Animal Nutrition and Feed Science of Jilin Province, Key Laboratory of Animal Production Product Quality and Security Ministry of Education, JLAU-Borui Dairy Science and Technology R&D Center, College of Animal Science and Technology, Jilin Agricultural University, Changchun, China, [2]Laboratory of Animal Nutrition, Physiology and Proteomics, Department of Animal Science and Technology, Konkuk University, Seoul, Republic of Korea

KEYWORDS

animal production, bioactive potential, nutrition-based health, self-defense system, underlying mechanisms

Editorial on the Research Topic
Nutricines and derivatives of nutrients in animal health and disease prevention

Good animal health is of great importance for the efficient production of animal-derived foods at a low cost. The demand for solutions for animal health maintenance and disease prevention is a major global challenge in animal production, food safety, and public health (1). The animal body has a complete self-defense system, including oxidation and anti-oxidation balance, immune activation and suppression balance, pro- and anti-inflammatory balance, which can help the host against various factors that endanger normal life activities (2). Nowadays, the strategy of nutrition-based health has become an increasingly important solution for animal health maintenance and disease prevention (3). Nutrients (carbohydrates, fats, proteins, minerals, vitamins, et al.) and nutricines (carotenoids, enzymes, fatty acids, flavors, oligosaccharides, organic acids, phospholipids, polyphenols, et al.) are two major categories of components in feeds (4, 5).

This Research Topic attracts considerable studies dealing with the underlying mechanisms of nutricines and derivatives of nutrients in the animal self-defense system. And researches on the latest information about novel techniques and theories to identify the bioactive activity of nutricines and derivatives of nutrients are also received. After peer review, a collection of 14 of those studies are published, 11 are original research and three are review.

Interestingly, three articles addressed the potential benefits of yeast (culture) in animal health and disease prevention. Maturana et al., provided the reader with a comprehensive review on the effects of yeast and its derivatives in pets and the possible mechanisms that confer their functional properties. Aschalew et al. reported that yeast culture and oxalic acid have a great potential to buffer and create a conducive rumen environment and improve rumen fermentation efficiency and hemicellulose digestion. Yin et al. proved that inositol, a potentially potent metabolite in yeast culture, can improve rumen function, affect rumen microorganisms and rumen and blood metabolites and may reduce inflammation, improving animal health.

Moreover, two articles reported the roles of polyphenol-rich botanical stuffs in animal nutrition. In the review of Ferlisi et al., the authors gave an update on the use of olive co-products and their phenolic extracts in monogastric animal (swine, poultry and rabbit) diets and suggested that these stuffs may improve animal health, productive performances and meat quality characteristics, reduce the adverse effect of lipid peroxidation and improve the antioxidant status. Wang J. et al. found that dark tea can mitigate oxidative stress-induced damage by promoting the clearance of free radicals and suggested that dark tea is worth further exploration as a potential dietary supplement for the maintenance of animal health and the prevention of related diseases.

In addition, the bioactive potential of two different molecules in the animals' performance were conducted. Wang C. et al. concluded that β-hydroxybutyrate administration might alleviate the liver injury and inflammation, and improve hepatic energy metabolism by regulating glucose and lipid metabolism, thereby improving the growth performance of postnatal growth retardation piglets. Li et al. presented that supplemental β-alanine can improve the antioxidant status of speed-racing Yili horses reduce post-exercise injuries and bolster their post-race recovery ability.

Overall, this Research Topic contributed to improving the current knowledge of nutricines and derivatives of nutrients in animal health and disease prevention, providing significant contribution to this research area.

Author contributions

TW: Writing – original draft, Writing – review & editing. H-GL: Writing – review & editing.

Funding

The author(s) declare financial support was received for the research, authorship, and/or publication of this article. This work was supported by the Scientific and Technological Developing Scheme of Jilin Province (grant number 20210202037NC).

Conflict of interest

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

Publisher's note

References

1. Adams CA. *Nutrition-Based Health: Nutricines and Nutrients, Health Maintenance and Disease Avoidance in Animals*. Nottingham: Nottingham University Press. (2007).

2. Lu DX. The systemic concept on a nutritional strategy for healthy animal production (in Chinese). *Feed Industry*. (2019) 40:1–5. doi: 10.13302/j.cnki.fi.2019.02.001

3. Adams CA. Nutrition-based health in animal production. *Nutr Res Rev*. (2006) 19:79–89. doi: 10.1079/NRR2005115

4. Adams CA. *Total Nutrition-Feeding Animals for Health and Growth*. Nottingham: Nottingham University Press. (2001).

5. Lu DX. Feed nutricines: a research area that is worth to examine closely (in Chinese). *Feed Industry*. (2020) 41:1–5. doi: 10.13302/j.cnki.fi.2020.03.001

frontiers | Frontiers in Veterinary Science

TYPE Mini Review
PUBLISHED 20 September 2023
DOI 10.3389/fvets.2023.1265689

OPEN ACCESS

EDITED BY
HongGu Lee,
Konkuk University, Republic of Korea

REVIEWED BY
Jingui Li,
Yangzhou University, China

*CORRESPONDENCE
Hyeun Bum Kim
✉ hbkim@dankook.ac.kr

†These authors have contributed equally to this work

RECEIVED 23 July 2023
ACCEPTED 06 September 2023
PUBLISHED 20 September 2023

CITATION
Pandey S, Kim ES, Cho JH, Song M, Doo H, Kim S, Keum GB, Kwak J, Ryu S, Choi Y, Kang J, Choe J and Kim HB (2023) Cutting-edge knowledge on the roles of phytobiotics and their proposed modes of action in swine. *Front. Vet. Sci.* 10:1265689. doi: 10.3389/fvets.2023.1265689

Cutting-edge knowledge on the roles of phytobiotics and their proposed modes of action in swine

Sriniwas Pandey[1†], Eun Sol Kim[1†], Jin Ho Cho[2†], Minho Song[3†], Hyunok Doo[1], Sheena Kim[1], Gi Beom Keum[1], Jinok Kwak[1], Sumin Ryu[1], Yejin Choi[1], Juyoun Kang[1], Jeehwan Choe[4] and Hyeun Bum Kim[1*]

[1]Department of Animal Resources Science, Dankook University, Cheonan, Republic of Korea, [2]Division of Food and Animal Science, Chungbuk National University, Cheongju, Republic of Korea, [3]Division of Animal and Dairy Science, Chungnam National University, Daejeon, Republic of Korea, [4]Major of Beef Science, Department of Livestock, Korea National University of Agriculture and Fisheries, Jeonju, Republic of Korea

With the ban on antibiotics in the swine industry, the exploration of alternative options has highlighted phytobiotics as a promising substitute for antibiotic growth promoters, aiming to foster a more sustainable swine industry. Phytobiotics are non-nutritive natural bioactive components derived from plants that offer numerous health benefits. They exhibit antioxidative, antimicrobial, and anti-inflammatory effects. Phytobiotics can be utilized in various forms, including solid, dried, ground, or as extracts, either in crude or concentrated form. They are characterized by low residual levels, a lack of resistance development, and minimal adverse effects. These qualities make phytobiotics an attractive choice for enhancing health and productivity in swine, presenting them as a viable alternative to antibiotics. While there is a general understanding of the effects of phytobiotics, there is still a need for detailed information regarding their effectiveness and mechanisms of action in practical settings. Therefore, the purpose of this mini review was to summarize the current knowledge supporting the roles of phytobiotics and their proposed modes of action, with a specific focus on swine.

KEYWORDS

phytobiotics, swine, health, growth performance, modes of action

Introduction

The growing concerns over the use of antibiotics as growth promoters in livestock feed have led to a search for better alternatives that can provide similar effects and performance without causing severe negative impacts. In response to this, numerous substances have been studied and found to possess good qualities that aid and improve the health and overall growth of livestock. These substances play major roles in maintaining normal physiological functions and animal health, as well as protecting animals from infectious diseases. One such substance that has been identified is termed "nutraceutical" (1). Nutraceuticals refer to substances that are produced in a purified or extracted form and administered to animals with the purpose of improving their health and well-being (2). This group includes various types of substances such as enzymes, synbiotics, organic acids, polyunsaturated fatty acids, and phytobiotics (3, 4).

Phytobiotics, a specific type of nutraceuticals, are non-nutritive plant-derived natural bioactive components that can be used as feed additives (5–8). These phytobiotics have been extensively studied for their ability to improve the overall growth performance and health of animals. Notably, phytobiotics possess several desirable attributes, including low residue levels, absence of resistance development, and minimal side effects (9–11). These qualities make them a promising option for promoting animal health and productivity in a sustainable and responsible manner (7, 12–14). To date, over 5,000 different dietary phytobiotics have been discovered from a variety of sources including fruits, vegetables, legumes, whole grains, herbs, and essential oils. It is widely accepted that phytobiotics can be used in various forms, such as solid, dried, and ground or as extracts, either in crude or concentrated form, in which the accumulation of biologically active substances is greatest (15, 16). In general, phytobiotics contain essential nutrients, such as carbohydrates, along with other secondary components, including essential oils and phenolic compounds. Because phytobiotics do not significantly contribute to the intake of primary nutrients in animals, the main focus is on the secondary plant components as the main ingredients of interest in phytobiotics, even though certain polysaccharides can also act as phytobiotics (6, 17, 18). Although there are no definitive classification criteria, phytobiotics can be classified into 4 different categories based on their origin and processing characteristics: (1) herbs (blooming, nonwoody, and nonpersistent plants); (2) spices (plants with a strong odor or flavor); (3) essential oils (volatile lipophilic components); and (4) oleoresins (extracts derived from non-aqueous solutions) (Table 1) (13, 25).

A variety of literature has proven the positive effects of phytobiotics, which include several beneficial outcomes. These effects encompass enhanced growth of beneficial microbes in the gut, as well as antioxidative, antimicrobial, and anti-inflammatory properties (3, 42). These functional activities of phytobiotics have been attributed to various bioactive compounds present in them. These bioactive compounds include terpenoids (mono- and sesquiterpenes, steroids), flavonoids, alkaloids (in the form of alcohols, aldehydes, ketones, esters, and lactones), phenols (tannins), glycosides and glucosinolates (17, 43, 44). However, the mechanism action of phytobiotics have not been clearly elucidated due to the wide variety of bioactive substances present in these plant-derived products. The content and chemical composition of active substances in phytobiotics can vary based on factors such as the plant part used (seeds, leaves, etc.), geographical location, and harvesting season. These variations contribute to the complexity of understanding the precise mechanisms by which phytobiotics exert their effects (13, 25, 45).

Therefore, the purpose of this mini review was to provide a summary of the current knowledge regarding the roles of phytobiotics and their proposed modes of action, particularly in swine. Despite the complexity of understanding the precise mechanisms of action due to the diverse bioactive substances and variations in content and composition, the review aimed to consolidate the existing literature and shed light on the potential benefits of phytobiotics in swine production.

Effects of phytobiotics on the swine gut microbiome

The intestinal health of animals is crucial for their overall health and well-being, and it is associated with several aspects, including gut microbiome and mucosal barrier. Disturbances in these elements can impact animal health. Phytobiotics tend to promote the intestinal health in animals by enhancing the functions of these elements (46).

The impact of phytobiotics on the gut microbiome has been extensively studied because of their significant roles in the health and productivity of livestock. Phytobiotics have been found to not only alter bacterial proliferation but also influence the composition and function of the microbiota (47). Weaned pigs experience stressors associated with changes in their feed and environment when they are moved from the farrowing room to the nursery facility. These changes can hinder the establishment of a stable gut environment. Phytobiotics have the potential to enhance the microbial balance in young pigs, resulting in improved health and feed efficiency. The presence of beneficial gut bacteria during the weaning period is crucial, and plant-based products can serve as effective means to modulate it (9, 19, 35, 45). In a study by Deng et al. (19), polysaccharides derived from cassiae seeds have been found to improve the intestinal microflora of piglets. In another study by Li et al. (48), when weaned pigs were fed a diet supplemented with coix seed, there was a significant increase in the populations of *Lactobacillus* and *Bacteroides* in the gastrointestinal tract. Additionally, there was a reduction in the abundance of *Prevotellaceae*. These findings suggest that the inclusion of coix seed in the feed can positively influence the composition of the gut microbiota in weaned pigs, promoting a healthier microbial balance. Coix seed contains 60% starch, similar to cereals, but the content of oil, polysaccharides and protein is higher than cereals, making it an enriched medium for gut microbiota. Also, proteins and polysaccharides help regulate water transportation, providing the gut microbiota with a suitable environment to grow (48). In addition, several studies have suggested that gut microbes play a role in metabolizing ingested phytobiotics into simpler metabolites. This microbial metabolism increases the bioavailability of the phytobiotics, leading to enhanced health-promoting effects in the intestine. The gut microbiota's ability to metabolize phytobiotics into bioactive compounds highlights the intricate relationship between the host, microbiota, dietary components, and underscores the importance of considering microbial metabolism in understanding the beneficial effects of phytobiotics on intestinal health (49). In a study conducted by Fresno Rueda et al. (45), the effects of phytobiotics containing polyphenols on weaned pigs were investigated. The study found an increase in the abundance of both lactate-producers and lactate-utilizers in the gut of pigs. Lactate is an important metabolite in the gut, as it can suppress the growth of pathogens and can also be utilized by the host in the form of propionate. Furthermore, beyond the weaning period, phytobiotics continue to play a role in modulating the gut microbiota in growing-finishing pigs. For instance, supplementation of 1.5% bamboo vinegar powder was found to increase the abundance of *Firmicutes* and *Bacteroidetes*, two dominant bacterial phyla in the gut. This supplementation also promoted the richness of *Lactobacillus*, a beneficial genus, and *Thalassospira* (34). These findings highlight the potential of phytobiotics in shaping the gut microbiota composition and promoting the growth of beneficial bacteria in weaned and growing-finishing pigs. For sows with garlic supplementation, a study by Satora et al. (35) observed that the diversity and richness of the microbial community changed across the different taxonomic levels of identification. The study found an increase in species-level diversity and richness, while the trend was

TABLE 1 Classification of phytobiotics and their functional examples addressed in this mini review.

Classification	Types	Functions	References
Herbs	Bloomin Nonwoody Nonpersistent plants	Improve intestinal microflora	Deng et al. (19) Li et al. (9)
		Antimicrobial effects	Wang et al. (20)
		Antioxidative and anti-inflammatory effects	Weber et al. (21)
			Esatbeyoglu et al. (22)
			Spanier et al. (23)
			Wei and Shibamoto (24)
			Gheisar and Kim (25)
			Filazi et al. (3)
		Growth enhancement	Davila-Ramirez et al. (26)
Spices	Plants with a strong odor Flavor that are commonly added to foods	Growth enhancement	Janz et al. (27) Al-Kassie (28)
Essential oils	Volatile lipophilic components	Enhance intestinal barrier functions	Su et al. (29) Liu et al. (30)
		Antimicrobial effects	Ahmed et al. (31)
		Growth enhancement	Li et al. (32) Manzanil et al. (33)
Oleoresins	Extracts derived from non-aqueous solutions	Improve intestinal microflora	Qu et al. (34) Satora et al. (35)
		Antimicrobial effects	Vasconcelos et al. (36)
			Girard et al. (37)
			Fu et al. (38)
		Growth enhancement	Yang et al. (39)
			Davila-Ramirez et al. (26)
			Yan et al. (40)
			Marcin et al. (41)

opposite at the family and genus levels. These findings support the idea that phytobiotics may play a role in shaping the gut microbiome (Figure 1).

Effects of phytobiotics on the swine intestinal barrier function

Phytobiotics also play a role in maintaining intestinal barrier function (Figure 1). The integrity of the intestinal barrier depends on the interplay between various components, including the adhesive mucous gel layer, immunoglobulin A, antibacterial peptides, and intercellular tight junctions. Among these components, tight junctions are the primary factors that influence the integrity of the intestinal barrier. Tight junctions consist of transmembrane proteins, such as claudin, occludin, tricellulin, junctional adhesion molecule-A (JAM-A), as well as intracellular plaque proteins like zonula occludens (ZO) (50). The addition of resveratrol has been documented to effectively restore the expression of ZO-2, occludin, JAM-A, and claudin, while also alleviating the elevation of plasma lipopolysaccharide (LPS)-binding protein levels. Elevated levels of LPS-binding protein serve as an indicator of compromised intestinal barrier function in the animal (51). In addition, naringenin, a flavanone found in citrus fruits, has been shown to increase the expression of occludin, JAM-A, and claudin-3 (52). Also, it has been known that plant extraction oil (PEO) enhances intestinal mucosa growth in weaned pigs, attributing to the mediation of improved intestinal integrity and functions (29). In another study by Liu et al. (30), administering capsicum oleoresin (CAP) and garlic botanical (GAR) was found to increase the expression of genes related to membrane integrity in infected weaning pigs. This administration also promoted gut mucosa health, leading to improvements in diarrhea and clinical immune responses in infected pigs that were given the plant extract. Altogether, it has been shown that phytobiotics tend to promote the intestinal health of the animal by maintaining the intestinal barrier function.

Antimicrobial effects of phytobiotics in swine

The antimicrobial effects of phytobiotics have been the subject of numerous studies and have consequently been extensively investigated (53–55).The majority of these studies have shown that phenolic

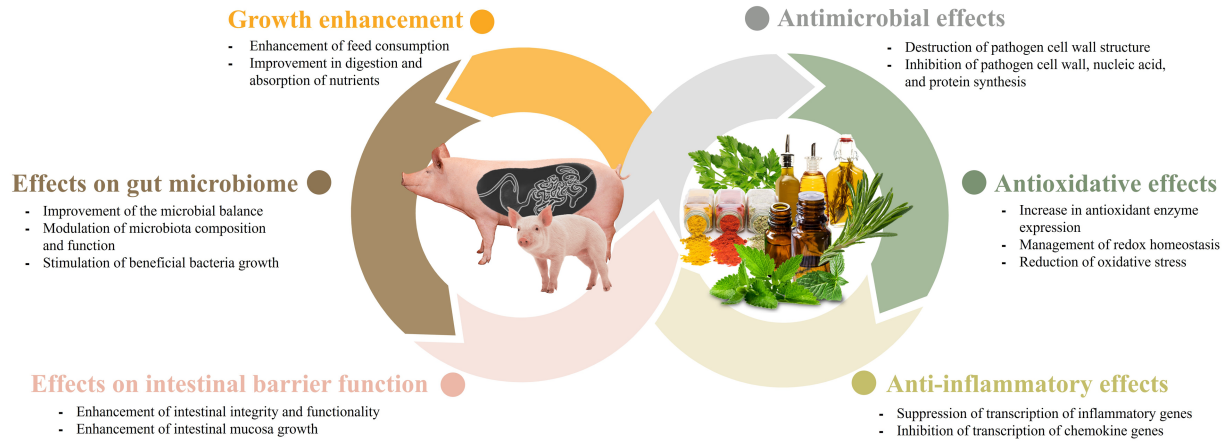


FIGURE 1
The main effects of phytobiotics in swine production systems.

components, such as thymol, carvacrol, limonene, geraniol, phenylpropane, and citronellal, are among the most potent antimicrobials (3). When it comes to the mechanisms of action, the variation in the composition of phytobiotic components has been found to significantly impact the way they act (56, 57). Nevertheless, the antimicrobial effects of phytochemical feed additives, in general, can be attributed to four different modes of action that are: (1) destruction of cell wall structure; (2) inhibition of cell wall synthesis; (3) inhibition of nucleic acid synthesis, and (4) interfering with protein synthesis (58). According to Wang et al. (20), an overview of antimicrobial mechanisms of ginseng revealed several modes of action. These include disrupting biofilm formation, destroying mature biofilms, altering lipid bilayers, and creating pores in the bacterial cell wall membrane. In addition to the mentioned antimicrobial mechanisms of ginseng, it has also been found to inhibit the efflux of antibiotics, which reduces the likelihood of drug resistance development in microbes. Furthermore, cinnamon extracts have been documented to exhibit antimicrobial activity through various mechanisms, including the disruption of cell membrane, inhibition of ATPases, interference with cell division, and inhibition of biofilm formation (36). Several studies also have reported that thymol possesses the ability to integrate into the polar-head group region of the lipid bilayer. This integration leads to the modification of the structural integrity and fluidity of the membrane through hydrogen bonding and hydrophobic interactions (59, 60).

When phytobiotics are used in swine, their antimicrobial effects have been proven to yield positive results. Essential oils have demonstrated effective antimicrobial activities when utilized in conjunction with organic acids (61). Zhou et al. (61) reported that the combination of essential oils (such as carvacrol or thymol) with acetic acid or citric acid exhibited better efficacy against *Salmonella typhimurium* compared with using individual essential oils or organic acids alone. Another study by Ahmed et al. (31) reported a decrease in the count of fecal *Salmonella* and *Escherichia coli* (*E. coli*), as well as a reduction in diarrhea scores, when benzoic acid and thymol were fed to the swine. The mechanism behind this synergism is still unclear. However, it is well-documented that phenols present in essential oil can alter the structure and functions of the bacterial cell membrane. This alteration leads to swelling, resulting in increased membrane permeability, and consequently, heightened susceptibility of bacteria to organic acids (62). Enterotoxigenic *E. coli* is considered as one of the primary causes of diarrhea in weaning piglets. A study by Girard et al. (37) showed promising result on supplementation of 2% chestnut extract (CE) immediately after weaning instead of antibiotics, improving growth performance, and reducing the occurrence of post-weaning diarrhea (PWD) caused by Enterotoxigenic *E. coli* (ETEC F4). In another study, Chang et al. (63) conducted a study to explore the effects of different combinations of phytogenic feed additives (PFA) on pigs infected with *E. coli*. Among the numerous combinations studied, the mixture of bitter citrus extract, thymol, and carvacrol demonstrated the most effective results in terms of enhancing immune response, intestinal morphology, and tight junction expression. In a study by Fu et al. (38), administration of baicalin-aluminum complex (BBA) altered the composition of the gut microbiome, leading to a significant reduction in the frequency of diarrhea in piglets. Additionally, supplementation with *Scutellaria baicalenis* extracts (SBE) has been demonstrated to effectively reduce the incidence of diarrhea in weaning piglets and decrease the expression of inflammatory cytokines by inhibiting the NF-kB and P38 signaling pathways (64). Overall, these studies demonstrate that phytobiotics can be utilized as potential alternatives to antimicrobial agents in swine (Figure 1).

Antioxidative and anti-inflammatory effects of phytobiotics in swine

The antioxidant and anti-inflammatory properties of phytobiotics are indeed noteworthy and have garnered significant interest (Figure 1). The swine industry experiences substantial economic losses each year due to various types of oxidative stress. Phytobiotics can help mitigate these losses by providing antioxidant support and reducing inflammation in swine (65). Oxidative stress refers to a significant increase in the production of free radicals, such as reactive oxygen species (ROS) and reactive nitrogen radicals (RNS), within the bodies of animals. It can also occur when the capacity to effectively eliminate free radicals is reduced, disrupting the balance between antioxidation and oxidation processes in the body (66). Studies have

identified five primary factors that can trigger oxidative stress in swine. These factors include birth, weaning stress, feeding environment, mycotoxin presence in feed, and social factors (66). Given the detrimental effects of oxidative stress, it is crucial for the swine industry to combat this condition. The unregulated production of free radicals, such as ROS and RNS, associated with oxidative stress, can even trigger inflammation (67, 68).

The antioxidant and anti-inflammatory mechanisms exhibited by phytobiotics are largely attributed to their regulation of signaling pathways, as highlighted by Li et al. (9). The antioxidant responsive element (Nrf2-ARE) signaling pathway plays a vital role in cellular response to oxidative stress (69). The transcription factor Nrf-2 binds with the antioxidant response element (ARE) and initiates the activation of a diverse range of genes, including antioxidant enzymes and proteins, that provides cellular protection against oxidative stress (70). Phytobiotics, thus help activate Nrf-2 pathway, upregulating antioxidant enzymes and several protective proteins. A number of *in vitro* and *in vivo* experiments have indicated that oxidative stress can also trigger activation of Nuclear Factor Kappa B (NF-κB) pathway. Increased expression level of NF-κB can facilitate transcription of multitude of inflammatory genes (71). This signaling pathway serves as a principal regulator of inflammation (72). NF-κB is a transcriptional factor that plays an important role in many critical physiological responses, including the inflammatory response (73). This pathway is responsible for expression of genes encoding many pro-inflammatory cytokines and chemokines (74). NF-κB activation leads to tissue alternations indicative of inflammation (9). In relation to the above mentioned information, the findings of Wang et al. (72) showed that traditional Chinese medicine (TCM) I and II, comprised of several kinds of plant derivatives, can improve antioxidative and anti-inflammatory capabilities in liver of piglets *via* activation of the Nrf2 pathway. In addition, curcumin, a compound presented in turmeric, has been shown to have anti-inflammatory and antioxidant activities, capable of scavenging free radicals and facilitating antioxidant functions through the Nrf2 signaling pathway (21, 22). Another compound, resveratrol, obtained from grapes and wine, was also found to lessen inflammation, and regulate redox homeostasis (23). In cells, redox homeostasis is the balance between generation and elimination of ROS/RNS (75). A study by Cao et al. (76) showed that resveratrol was effective in improving the redox status, decreasing mitochondrial damage, and promoting mitophagy in piglets injected with diquat. Plant species such as coriander, ginger, curcuma as well as those rich in flavonoids (in green tea) and anthocyanins (in various fruits) have antioxidant activities (24, 25). Studies showed black pepper, red pepper and chilli also possess antioxidant properties, however, most of these plants are restricted from being added specially in swine feed because of the pungent smell and taste of the active substances of these plants (3).

Growth enhancement by phytobiotics in swine

With the ban of antibiotic growth promoters (AGPs), phytobiotics have emerged as promising alternative feed additives, as noted by Valenzuela-Grijalva et al. (58). Various studies have demonstrated the potential growth-promoting effects of phytobiotics, although the precise mechanisms underlying their role as growth enhancers have not been fully elucidated (Figure 1). However, it has been suggested by Valenzuela-Grijalva et al. (58) that phytobiotics may promote growth through several different ways. These includes: (1) through improvement of feed status and feed consumption by increasing flavor and palatability; (2) enhancement in nutrition digestion and absorption; and (3) promotes anabolic activity comparable to that of anabolic substances.

Some phytobiotics, when added to feeds, have been shown to enhance flavor and palatability, thereby increasing feed intake in swine. This improvement in feed intake can ultimately lead to improved production performance (28, 77). Janz et al. (27) discovered that pigs demonstrated a preference for feed supplemented with garlic or rosemary compared to feed supplemented with oregano or ginger. However, other studies have indicated that the palatability of feed was negatively affected when pigs were fed essential oils extracted from fennel and caraway, or from thyme or oregano (78–80).

Phytobiotics improve nutrient digestion and absorption by stimulating the secretion of various digestive secretions, including saliva, mucus, digestive enzymes, and bile. These enhanced secretions contribute to the breakdown and assimilation of nutrients from the diet. Furthermore, phytobiotics have been shown to exhibit prebiotic activity, promoting the growth and activity of beneficial gut bacteria, which further supports efficient nutrient digestion and absorption (7, 81, 82). Janz et al. (27) and Manzanil et al. (33) conducted studies where they observed a stimulating effect on the pancreatic enzymes, specifically amylase and trypsin activity, in pigs after the administration of cinnamaldehyde and an essential oil blend. These findings suggest that these phytobiotics can enhance the activity of pancreatic enzymes involved in nutrient digestion, contributing to improved nutrient utilization in pigs (25, 33, 83, 84). The increase in activity of digestive enzymes facilitates an increment in the gastric retention time of ingested feed. This prolonged retention time allows for improved digestibility and availability of nutrients. As a result, the enhanced enzymatic activity induced by phytobiotics contributes to more efficient nutrient digestion and utilization in animals (81). Li et al. (32) conducted a study on pigs fed diets supplemented with essential oils and observed significant improvements in weight gain, as well as digestibility of dry matter and crude protein. They proposed that the enhancing intestinal morphology resulting from essential oil supplementation contributed to improved nutritional digestibility, ultimately leading to better performance in pigs. Similarly, in a study by Yang et al. (39), piglets supplemented with rosemary extract (RE) exhibited increased villus height and villus height/crypt depth ratio in both the jejunum and ileum. These changes in intestinal morphology have the potential to positively impact growth performance, nutrient digestibility, and overall intestinal health in weaned piglets (32, 39).

In a study by Davila-Ramirez et al. (26), the addition of plant extracts (artichoke, beet, celery, garlic, avocado, spinach, oats, and parsley) to the diet of pigs resulted in improved average daily gain (ADG), average daily feed intake (ADFI), and final live weight (LW) under heat stress conditions during the growing to finishing period. Similarly, in a study by Yan et al. (40), supplementation with an herbal extract mixture (HEM) containing buckwheat, thyme, curcuma, black pepper, and ginger resulted in improved growth performance, specifically increased ADG and ADFI, in growing pigs compared to a non-supplemented control (NC) treatment. However, no significant changes in feed conversion ratio (FCR) were observed. Marcin et al.

(41) observed a significant improvement in ADG in piglets that were administered a diet supplemented with extracts of sage and oregano. On the other hand, Ahmed et al. (85) found no significant changes in live body weight and ADG in growing-finishing pigs fed a diet supplemented with medicinal plants such as pomegranate, *ginkgo biloba*, and licorice, compared with a control group. These studies highlight the variable effects of different plant extracts and herbal supplementation on growth performance in pigs, underscoring the importance of considering various factors when evaluating the efficacy of phytobiotics in swine nutrition. These factors include: species variation (different plant species have varying compositions of bioactive compounds, which can result in different effects on growth performance), plant parts and inherited characteristics (the specific plant parts used, such as leaves, stems, and roots), their inherent characteristics (chemical composition and nutrient content), age of plants (the age of plants at the time of harvest can affect the concentration and composition of bioactive compounds, and potentially influencing their impact on growth performance), timing of harvest (the timing of harvest in relation to the plant's growth stage can influence the concentration and potency of bioactive compounds, which may affect their efficacy in promoting growth), and dosage variations (different dosages of herbs or their extracts can have varying effects on growth performance). Taking these factors into account is important in understanding the diverse outcomes observed in studies investigating the effects of herbs on animal growth performance (86–88).

Conclusion

In recent times, the use of phytobiotics as natural growth promoters in the swine industry has gained increasing popularity. Various studies have demonstrated the potential of phytobiotics to exert anti-oxidative, antimicrobial, and anti-inflammatory effects. These findings suggest that phytochemicals could serve as a promising alternative to antibiotics in swine production, enhancing growth performance and health. While there is a general understanding of the effects of phytobiotics, there is still a lack of detailed information regarding their specific mechanisms of action, and the effectiveness of their implementation in practical settings. Therefore, further studies are necessary to investigate the modes of action of each type and dose of active compound in phytobiotics, as well as their potential interactions with other feed constituents. It is also important to assess the effects of phytobiotics throughout all phases of pig production to draw concrete conclusions regarding their use. By conducting more comprehensive research, we can gain a deeper understanding of phytobiotics and their potential benefits, allowing for their optimal utilization in swine nutrition and production. This will ultimately contribute to improved animal health and performance, as well as more sustainable and efficient swine farming practices.

Author contributions

SP: Writing – review & editing, Writing – original draft, Resources. EK: Visualization, Writing – original draft, Writing – review & editing, Resources. JiC: Conceptualization, Writing – review & editing. MS: Conceptualization, Writing – review & editing. HD: Writing – original draft, Resources. SK: Conceptualization, Writing – review & editing, Resources. GK: Resources, Writing – original draft. JiK: Resources, Writing – original draft. SR: Resources, Writing – original draft. YC: Resources, Writing – original draft. JuK: Resources, Writing – original draft. JeC: Writing – review & editing, Conceptualization. HK: Conceptualization, Supervision, Validation, Writing – review & editing, Writing – original draft.

Funding

The author(s) declare financial support was received for the research, authorship, and/or publication of this article. This work was supported by the National Research Foundation of Korea (NRF) funded by the Ministry of Education: (NRF-2021R1I1A3059910 and NRF-2019M3A9F3065227).

Conflict of interest

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

Publisher's note

References

1. Das L, Bhaumik E, Raychaudhuri U, Chakraborty R. Role of nutraceuticals in human health. *J Food Sci Technol*. (2012) 49:173–83. doi: 10.1007/s13197-011-0269-4

2. Gupta RC, Srivastava A, Lall R. *Nutraceuticals in veterinary medicine*. Cham: Springer (2019).

3. Filazi A, Yurdakok-Dikmen B. Nutraceuticals in poultry health and disease In: R Gupta, A Srivastava and R Lall, editors. *Nutraceuticals in veterinary medicine*. Cham: Springer (2019). 661–72.

4. Alagawany M, Elnesr SS, Farag MR, Abd El-Hack ME, Barkat RA, Gabr AA, et al. Potential role of important nutraceuticals in poultry performance and health-a comprehensive review. *Res Vet Sci*. (2021) 137:9–29. doi: 10.1016/j.rvsc.2021.04.009

5. Kim S, Fan M, Applegate T. Nonruminant nutrition symposium on natural Phytobiotics for health of young animals and poultry: mechanisms and application. *J Anim Sci*. (2008) 86:E138–9. doi: 10.2527/jas.2007-0769

6. Grashorn M. Use of phytobiotics in broiler nutrition–an alternative to infeed antibiotics. *J Anim Feed Sci*. (2010) 19:338–47. doi: 10.22358/jafs/66297/2010

7. Kikusato M. Phytobiotics to improve health and production of broiler chickens: functions beyond the antioxidant activity. *Anim Biosci*. (2021) 34:345. doi: 10.5713/ab.20.0842

8. Serge Cyrille Houketchang N, Herve KM. The use of plants as phytobiotics: a new challenge In: S-H Marcos, A-H Eva and P-T Mariana, editors. *Phytochemicals in agriculture and food*. Rijeka: IntechOpen (2023). Ch. 9.

9. Li L, Sun X, Zhao D, Dai H. Pharmacological applications and action mechanisms of phytochemicals as alternatives to antibiotics in pig production. *Front Immunol*. (2021) 12:798553. doi: 10.3389/fimmu.2021.798553

10. Mandey JS, Sompie FN. Phytogenic feed additives as an alternative to antibiotic growth promoters in poultry nutrition In: L Babinszky, J Oliveira and E Mauro Santos, editors. *Advanced studies in the 21st century animal nutrition*. London: IntechOpen Limited (2021). 19.

11. Dialoke N, Onimisi P, Afolayan M. Performance, blood parameters and economic indices of broiler chickens fed graded levels of chestnut (Castenea sativa) phytobiotics as replacement for antibiotics growth promoters. *Niger J Anim Prod*. (2020) 47:161–70. doi: 10.51791/njap.v47i2.123

12. Suryanarayana M, Durga S. Role of phytogenic feed additives in swine production-a review. *Int. J. Environ. Agric. Biotech.* (2018) 3:264375. doi: 10.22161/ijeab/3.3.46

13. Windisch W, Schedle K, Plitzner C, Kroismayr A. Use of phytogenic products as feed additives for swine and poultry. *J Anim Sci*. (2008) 86:E140–8. doi: 10.2527/jas.2007-0459

14. Lin ZN, Ye L, Li ZW, Huang XS, Lu Z, Yang YQ, et al. Chinese herb feed additives improved the growth performance, meat quality, and nutrient digestibility parameters of pigs. *Anim Model Exp Med*. (2020) 3:47–54. doi: 10.1002/ame2.12104

15. Krauze M. Phytobiotics, a natural growth promoter for poultry In: L Babinszky, J Oliveira and E Mauro Santos, editors. *Advanced studies in the 21st century animal nutrition*. London: IntechOpen Limited (2021)

16. Lillehoj H, Liu Y, Calsamiglia S, Fernandez-Miyakawa ME, Chi F, Cravens RL, et al. Phytochemicals as antibiotic alternatives to promote growth and enhance host health. *Vet Res*. (2018) 49:76. doi: 10.1186/s13567-018-0562-6

17. Pashtetsky V, Ostapchuk P, Kuevda T, Zubochenko D, Yemelianov S, Uppe V, editors. Use of phytobiotics in animal husbandry and poultry. E3S Web of Conferences; (2020). EDP Sciences.

18. Vidanarachchi JK, Mikkelsen L, Sims I, Iji P, Choct M. Phytobiotics: alternatives to antibiotic growth promoters in monogastric animal feeds. Recent Advances in Animal Nutrition in Australia, Vol. 15, pp. 131–144 (2005). Available at: https://hdl.handle.net/1959.11/4186

19. Deng Z-Y, Zhang J-W, Li J, Fan Y-W, Cao S-W, Huang R-l, et al. Effect of polysaccharides of cassiae seeds on the intestinal microflora of piglets. *Asia Pac J Clin Nutr*. (2007) 16:143–7.

20. Wang L, Huang Y, Yin G, Wang J, Wang P, Chen ZY, et al. Antimicrobial activities of Asian ginseng, American ginseng, and notoginseng. *Phytother Res*. (2020) 34:1226–36. doi: 10.1002/ptr.6605

21. Weber WM, Hunsaker LA, Abcouwer SF, Deck LM, Vander Jagt DL. Anti-oxidant activities of curcumin and related enones. *Bioorg Med Chem*. (2005) 13:3811–20. doi: 10.1016/j.bmc.2005.03.035

22. Esatbeyoglu T, Huebbe P, Ernst IM, Chin D, Wagner AE, Rimbach G. Curcumin—from molecule to biological function. *Angew Chem Int Ed*. (2012) 51:5308–32. doi: 10.1002/anie.201107724

23. Spanier G, Xu H, Xia N, Tobias S, Deng S, Wojnowski L, et al. Resveratrol reduces endothelial oxidative stress by modulating the gene expression of superoxide dismutase 1 (Sod1), glutathione peroxidase 1 (Gpx1) and Nadph oxidase subunit (Nox4). *J Physiol Pharmacol*. (2009) 60:111–6.

24. Wei A, Shibamoto T. Antioxidant activities and volatile constituents of various essential oils. *J Agric Food Chem*. (2007) 55:1737–42. doi: 10.1021/jf062959x

25. Mohammadi Gheisar M, Kim IH. Phytobiotics in poultry and swine nutrition–a review. *Ital J Anim Sci*. (2018) 17:92–9. doi: 10.1080/1828051X.2017.1350120

26. Dávila-Ramírez JL, Munguía-Acosta LL, Morales-Coronado JG, García-Salinas AD, González-Ríos H, Celaya-Michel H, et al. Addition of a mixture of plant extracts to diets for growing-finishing pigs on growth performance, blood metabolites, carcass traits, organ weight as a percentage of live weight, quality and sensorial analysis of meat. *Animals*. (2020) 10:1229. doi: 10.3390/ani10071229

27. Janz J, Morel P, Wilkinson B, Purchas R. Preliminary investigation of the effects of low-level dietary inclusion of fragrant essential oils and oleoresins on pig performance and pork quality. *Meat Sci*. (2007) 75:350–5. doi: 10.1016/j.meatsci.2006.06.027

28. Al-Kassie GA. Influence of two plant extracts derived from thyme and cinnamon on broiler performance. *Pak Vet J*. (2009) 29:169–73.

29. Su G, Zhou X, Wang Y, Chen D, Chen G, Li Y, et al. Dietary supplementation of plant essential oil improves growth performance, intestinal morphology and health in weaned pigs. *J Anim Physiol Anim Nutr*. (2020) 104:579–89. doi: 10.1111/jpn.13271

30. Liu Y, Song M, Che T, Lee J, Bravo D, Maddox C, et al. Dietary plant extracts modulate gene expression profiles in Ileal mucosa of weaned pigs after an *Escherichia Coli* infection. *J Anim Sci*. (2014) 92:2050–62. doi: 10.2527/jas.2013-6422

31. Ahmed S, Hossain M, Kim G, Hwang J, Ji H, Yang C. Effects of resveratrol and essential oils on growth performance, immunity, digestibility and fecal microbial shedding in challenged piglets. *Asian Australas J Anim Sci*. (2013) 26:683. doi: 10.5713/ajas.2012.12683

32. Li P, Piao X, Ru Y, Han X, Xue L, Zhang H. Effects of adding essential oil to the diet of weaned pigs on performance, nutrient utilization, immune response and intestinal health. *Asian Australas J Anim Sci*. (2012) 25:1617. doi: 10.5713/ajas.2012.12292

33. Manzanilla E, Perez J, Martin M, Kamel C, Baucells F, Gasa J. Effect of plant extracts and formic acid on the intestinal equilibrium of early-weaned pigs. *J Anim Sci*. (2004) 82:3210–8. doi: 10.2527/2004.82113210x

34. Qu H, Huang Y, Shi Y, Liu Y, Wu S, Bao W. Effect of bamboo vinegar powder as an antibiotic alternative on the digesta bacteria communities of finishing pigs. *Can J Microbiol*. (2018) 64:732–43. doi: 10.1139/cjm-2018-0058

35. Satora M, Magdziarz M, Rząsa A, Rypuła K, Płoneczka-Janeczko K. Insight into the intestinal microbiome of farrowing sows following the administration of garlic (*Allium Sativum*) extract and probiotic bacteria cultures under farming conditions. *BMC Vet Res*. (2020) 16:442. doi: 10.1186/s12917-020-02659-y

36. Vasconcelos N, Croda J, Simionatto S. Antibacterial mechanisms of cinnamon and its constituents: a review. *Microb Pathog*. (2018) 120:198–203. doi: 10.1016/j.micpath.2018.04.036

37. Girard M, Hu D, Pradervand N, Neuenschwander S, Bee G. Chestnut extract but not sodium salicylate decreases the severity of diarrhea and Enterotoxigenic *Escherichia Coli* F4 shedding in artificially infected piglets. *PLoS One*. (2020) 15:e0214267. doi: 10.1371/journal.pone.0214267

38. Fu S, Zhuang F, Guo L, Qiu Y, Xiong J, Ye C, et al. Effect of Baicalin-aluminum complexes on fecal microbiome in piglets. *Int J Mol Sci*. (2019) 20:2390. doi: 10.3390/ijms20102390

39. Yang M, Yin Y, Wang F, Bao X, Long L, Tan B, et al. Effects of dietary rosemary extract supplementation on growth performance, nutrient digestibility, antioxidant capacity, intestinal morphology, and microbiota of weaning pigs. *J Anim Sci*. (2021) 99:skab237. doi: 10.1093/jas/skab237

40. Yan L, Meng Q, Kim I. The effect of an herb extract mixture on growth performance, nutrient digestibility, blood characteristics and fecal noxious gas content in growing pigs. *Livest Sci*. (2011) 141:143–7. doi: 10.1016/j.livsci.2011.05.011

41. Marcin A, Lauková A, Mati R. Comparison of the effects of enterococcus faecium and aromatic oils from sage and oregano on growth performance and diarrhoeal diseases of weaned pigs. *Biologia*. (2006) 61:789–95. doi: 10.2478/s11756-006-0159-9

42. Dundar E, Olgun EG, Isiksoy S, Kurkcuoglu M, Baser KHC, Bal C. The effects of intra-rectal and intra-peritoneal application of *Origanum Onites* L. essential oil on 2, 4, 6-trinitrobenzenesulfonic acid-induced colitis in the rat. *Exp Toxicol Pathol*. (2008) 59:399–408. doi: 10.1016/j.etp.2007.11.009

43. Ulrikh EV, Khaliullin RS, Ganieva IA, Izhmulkina E, Arzjutov M. The content of biologically active substances in phytobiotics used for agricultural animals and poultry. *Int J Eng Technol (UAE)*. (2018) 7:445–9. doi: 10.14419/ijet.v7i3.14.17040

44. Huyghebaert G, Ducatelle R, Van Immerseel F. An update on alternatives to antimicrobial growth promoters for broilers. *Vet J*. (2011) 187:182–8. doi: 10.1016/j.tvjl.2010.03.003

45. Fresno Rueda A, Samuel R, St-Pierre B. Investigating the effects of a phytobiotics-based product on the fecal bacterial microbiome of weaned pigs. *Animals*. (2021) 11:1950. doi: 10.3390/ani11071950

46. Ducatelle R, Goossens E, De Meyer F, Eeckhaut V, Antonissen G, Haesebrouck F, et al. Biomarkers for monitoring intestinal health in poultry: present status and future perspectives. *Vet Res*. (2018) 49:43. doi: 10.1186/s13567-018-0538-6

47. Castillo M, Martín-Orúe S, Roca M, Manzanilla E, Badiola I, Perez J, et al. The response of gastrointestinal microbiota to Avilamycin, butyrate, and plant extracts in early-weaned pigs. *J Anim Sci*. (2006) 84:2725–34. doi: 10.2527/jas.2004-556

48. Li Z, Lin Z, Lu Z, Feng Z, Chen Q, Deng S, et al. Coix seed improves growth performance and productivity in post-weaning pigs by reducing gut Ph and modulating gut microbiota. *AMB Express*. (2019) 9:115. doi: 10.1186/s13568-019-0828-z

49. Iqbal Y, Cottrell JJ, Suleria HA, Dunshea FR. Gut microbiota-polyphenol interactions in chicken: a review. *Animals*. (2020) 10:1391. doi: 10.3390/ani10081391

50. Suzuki T. Regulation of the intestinal barrier by nutrients: the role of tight junctions. *Anim Sci J*. (2020) 91:e13357. doi: 10.1111/asj.13357

51. Mayangsari Y, Suzuki T. Resveratrol ameliorates intestinal barrier defects and inflammation in colitic mice and intestinal cells. *J Agric Food Chem*. (2018) 66:12666–74. doi: 10.1021/acs.jafc.8b04138

52. Azuma T, Shigeshiro M, Kodama M, Tanabe S, Suzuki T. Supplemental naringenin prevents intestinal barrier defects and inflammation in colitic mice. *J Nutr*. (2013) 143:827–34. doi: 10.3945/jn.113.174508

53. Burt S. Essential oils: their antibacterial properties and potential applications in foods—a review. *Int J Food Microbiol*. (2004) 94:223–53. doi: 10.1016/j.ijfoodmicro.2004.03.022

54. Panghal M, Kaushal V, Yadav JP. In vitro antimicrobial activity of ten medicinal plants against clinical isolates of oral cancer cases. *Ann Clin Microbiol Antimicrob*. (2011) 10:21. doi: 10.1186/1476-0711-10-21

55. Si W, Gong J, Tsao R, Zhou T, Yu H, Poppe C, et al. Antimicrobial activity of essential oils and structurally related synthetic food additives towards selected pathogenic and beneficial gut bacteria. *J Appl Microbiol*. (2006) 100:296–305. doi: 10.1111/j.1365-2672.2005.02789.x

56. Yang C, Chowdhury MK, Hou Y, Gong J. Phytogenic compounds as alternatives to in-feed antibiotics: potentials and challenges in application. *Pathogens*. (2015) 4:137–56. doi: 10.3390/pathogens4010137

57. Salehi B, Mishra AP, Shukla I, Sharifi-Rad M, Contreras MM, Segura-Carretero A, et al. Thymol, thyme, and other plant sources: health and potential uses. *Phytother Res*. (2018) 32:1688–706. doi: 10.1002/ptr.6109

58. Valenzuela-Grijalva NV, Pinelli-Saavedra A, Muhlia-Almazan A, Domínguez-Díaz D, González-Ríos H. Dietary inclusion effects of phytochemicals as growth promoters in animal production. *J Anim Sci Technol*. (2017) 59:8. doi: 10.1186/s40781-017-0133-9

59. Di Pasqua R, Betts G, Hoskins N, Edwards M, Ercolini D, Mauriello G. Membrane toxicity of antimicrobial compounds from essential oils. *J Agric Food Chem*. (2007) 55:4863–70. doi: 10.1021/jf0636465

60. Gill A, Holley R. Inhibition of membrane bound atpases of Escherichia Coli and *Listeria Monocytogenes* by plant oil aromatics. *Int J Food Microbiol*. (2006) 111:170–4. doi: 10.1016/j.ijfoodmicro.2006.04.046

61. Zhou F, Ji B, Zhang H, Jiang H, Yang Z, Li J, et al. Synergistic effect of thymol and carvacrol combined with chelators and organic acids against *Salmonella Typhimurium*. *J Food Prot*. (2007) 70:1704–9. doi: 10.4315/0362-028X-70.7.1704

62. Omonijo FA, Ni L, Gong J, Wang Q, Lahaye L, Yang C. Essential oils as alternatives to antibiotics in swine production. *Anim Nutr*. (2018) 4:126–36. doi: 10.1016/j.aninu.2017.09.001

63. Chang SY, Song MH, Lee JH, Oh HJ, Kim YJ, An JW, et al. Phytogenic feed additives alleviate pathogenic *Escherichia Coli*-induced intestinal damage through improving barrier integrity and inhibiting inflammation in weaned pigs. *J Anim Sci Biotechnol*. (2022) 13:107. doi: 10.1186/s40104-022-00750-y

64. Huang C, Wang Y, He X, Jiao N, Zhang X, Qiu K, et al. The involvement of Nf-Kb/P38 pathways in Scutellaria baicalensis extracts attenuating of *Escherichia Coli* K88-induced acute intestinal injury in weaned piglets. *Br J Nutr*. (2019) 122:152–61. doi: 10.1017/S0007114519000928

65. Cruzen S, Baumgard L, Gabler N, Pearce S, Lonergan S. Temporal proteomic response to acute heat stress in the porcine muscle sarcoplasm. *J Anim Sci*. (2017) 95:3961–71. doi: 10.2527/jas2017.1375

66. Hao Y, Xing M, Gu X. Research progress on oxidative stress and its nutritional regulation strategies in pigs. *Animals*. (2021) 11:1384. doi: 10.3390/ani11051384

67. Tsai W-H, Yang C-C, Li P-C, Chen W-C, Chien C-T. Therapeutic potential of traditional Chinese medicine on inflammatory diseases. *J Tradit Complement Med*. (2013) 3:142–51. doi: 10.4103/2225-4110.114898

68. Shingnaisui K, Dey T, Manna P, Kalita J. Therapeutic potentials of *Houttuynia Cordata* Thunb. against inflammation and oxidative stress: a review. *J Ethnopharmacol*. (2018) 220:35–43. doi: 10.1016/j.jep.2018.03.038

69. Shaw P, Chattopadhyay A. Nrf2–are signaling in cellular protection: mechanism of action and the regulatory mechanisms. *J Cell Physiol*. (2020) 235:3119–30. doi: 10.1002/jcp.29219

70. Niture SK, Kaspar JW, Shen J, Jaiswal AK. Nrf2 signaling and cell survival. *Toxicol Appl Pharmacol*. (2010) 244:37–42. doi: 10.1016/j.taap.2009.06.009

71. Kaltschmidt C, Greiner JF, Kaltschmidt B. The transcription factor Nf-Kb in stem cells and development. *Cells*. (2021) 10:2042. doi: 10.3390/cells10082042

72. Wang X, Wang Y, Mao Y, Hu A, Xu T, Yang Y, et al. The beneficial effects of traditional Chinese medicine on antioxidative status and inflammatory cytokines expression in the liver of piglets. *Front Vet Sci*. (2022) 9:937745. doi: 10.3389/fvets.2022.1063573

73. Park MH, Hong JT. Roles of Nf-Kb in cancer and inflammatory diseases and their therapeutic approaches. *Cells*. (2016) 5:15. doi: 10.3390/cells5020015

74. Liu T, Zhang L, Joo D, Sun S-C. Nf-Kb signaling in inflammation. *Signal Transduct Target Ther*. (2017) 2:1–9. doi: 10.1038/sigtrans.2017.23

75. Trachootham D, Lu W, Ogasawara MA, Valle NR-D, Huang P. Redox regulation of cell survival. *Antioxid Redox Signal*. (2008) 10:1343–74. doi: 10.1089/ars.2007.1957

76. Cao S, Shen Z, Wang C, Zhang Q, Hong Q, He Y, et al. Resveratrol improves intestinal barrier function, alleviates mitochondrial dysfunction and induces mitophagy in diquat challenged piglets 1. *Food Funct*. (2019) 10:344–54. doi: 10.1039/C8FO02091D

77. Bartoš P, Dolan A, Smutný L, Šístková M, Celjak I, Šoch M, et al. Effects of phytogenic feed additives on growth performance and on ammonia and greenhouse gases emissions in growing-finishing pigs. *Anim Feed Sci Technol*. (2016) 212:143–8. doi: 10.1016/j.anifeedsci.2015.11.003

78. Jugl-Chizzola M, Ungerhofer E, Gabler C, Hagmüller W, Chizzola R, Zitterl-Eglseer K, et al. Testing of the palatability of *Thymus Vulgaris* L. and *Origanum Vulgare* L. as flavouring feed additive for weaner pigs on the basis of a choice experiment. *Berl Munch Tierarztl Wochenschr*. (2006) 119:238–43.

79. Schöne F, Vetter A, Hartung H, Bergmann H, Biertümpfel A, Richter G, et al. Effects of essential oils from fennel (Foeniculi Aetheroleum) and caraway (Carvi Aetheroleum) in pigs. *J Anim Physiol Anim Nutr*. (2006) 90:500–10. doi: 10.1111/j.1439-0396.2006.00632.x

80. Zhai H, Liu H, Wang S, Wu J, Kluenter A-M. Potential of essential oils for poultry and pigs. *Anim Nutr*. (2018) 4:179–86. doi: 10.1016/j.aninu.2018.01.005

81. Costa L, Luciano F, Miyada VS, Gois F. Herbal extracts and organic acids as natural feed additives in pig diets. *South Afr J Anim Sci*. (2013) 43:181–93.

82. Jamroz D, Wiliczkiewicz A, Wertelecki T, Orda J, Skorupińska J. Use of active substances of plant origin in chicken diets based on maize and locally grown cereals. *Br Poult Sci*. (2005) 46:485–93. doi: 10.1080/00071660500191056

83. Jang I, Ko Y, Kang S, Lee C. Effect of a commercial essential oil on growth performance, digestive enzyme activity and intestinal microflora population in broiler chickens. *Anim Feed Sci Technol*. (2007) 134:304–15. doi: 10.1016/j.anifeedsci.2006.06.009

84. Diaz-Sanchez S, D'Souza D, Biswas D, Hanning I. Botanical alternatives to antibiotics for use in organic poultry production. *Poult Sci*. (2015) 94:1419–30. doi: 10.3382/ps/pev014

85. Ahmed ST, Mun H-S, Islam MM, Ko S-Y, Yang C-J. Effects of dietary natural and fermented herb combination on growth performance, carcass traits and meat quality in grower-finisher pigs. *Meat Sci*. (2016) 122:7–15. doi: 10.1016/j.meatsci.2016.07.016

86. Hashemi S, Davoodi H. Phytogenics as new class of feed additive in poultry industry. *J Anim Vet Adv*. (2010) 9:2295–304. doi: 10.3923/javaa.2010.2295.2304

87. Zhang G, Lee Y, Wang Z-Y, Wang Y. Synthesis and bioactivities of plant-derived biomolecules. *Front Plant Sci*. (2022) 13:949057. doi: 10.3389/fpls.2022.1077403

88. Wenk C. Herbs and botanicals as feed additives in Monogastric animals. *Asian Australas J Anim Sci*. (2003) 16:282–9. doi: 10.5713/ajas.2003.282

10.3389/fvets.2023.1265689

Glossary

AGPs	Antibiotic growth promoters
ADFI	Average daily feed intake
ADG	Average daily gain
BBA	Baicalin-aluminum complex
CAP	Capsicum oleoresin
FCR	Feed conversion ratio
GAR	Garlic botanical
HEM	Herbal extract mixture
JAM-A	Junctional adhesion molecule-A
LPS	Lipopolysaccharide
LW	Live weight
NC	Non-supplemented control
NF-kB	Nuclear factor-kB
PEO	Plant extraction oil
PWD	Post-weaning diarrhea
RNS	Reactive nitrogen radicals
ROS	Reactive oxygen species
RE	Rosemary extract
TCM	Traditional Chinese medicine
ZO	Zonula occludens

frontiers | Frontiers in Veterinary Science

TYPE Original Research
PUBLISHED 28 September 2023
DOI 10.3389/fvets.2023.1253778

OPEN ACCESS

EDITED BY
Tao Wang,
Jilin Agricultural University, China

REVIEWED BY
Shad Mahfuz,
Sylhet Agricultural University, Bangladesh
Jian Ying Zhang,
Linyi University, China
Cheng-long Jin,
Guangdong Academy of Agricultural Sciences (GDAAS), China

*CORRESPONDENCE
Kai-Min Niu
✉ niulele88@126.com
Xin Wu
✉ wuxin@isa.ac.cn

RECEIVED 06 July 2023
ACCEPTED 05 September 2023
PUBLISHED 28 September 2023

CITATION
Liang X, Zhai Z, Ren F, Jie Y, Kim S-K, Niu K-M and Wu X (2023) Metagenomic characterization of the cecal microbiota community and functions in finishing pigs fed fermented *Boehmeria nivea*.
Front. Vet. Sci. 10:1253778.
doi: 10.3389/fvets.2023.1253778

Metagenomic characterization of the cecal microbiota community and functions in finishing pigs fed fermented *Boehmeria nivea*

Xiaoxiao Liang[1], Zhenya Zhai[2,3], Fengyun Ren[4], Yucheng Jie[4], Soo-Ki Kim[5], Kai-Min Niu[2,3]* and Xin Wu[2,3,6]*

[1]College of Animal Science and Technology, Henan Agricultural University, Zhengzhou, China, [2]Jiangxi Functional Feed Additive Engineering Laboratory, Institute of Biological Resource, Jiangxi Academy of Sciences, Nanchang, China, [3]CAS Key Laboratory of Agro-Ecological Processes in Subtropical Region, Institute of Subtropical Agriculture, Chinese Academy of Sciences, National Engineering Laboratory for Pollution Control and Waste Utilization in Livestock and Poultry Production, Changsha, China, [4]College of Animal Science and Technology, Hunan Agricultural University, Changsha, China, [5]Department of Animal Science and Technology, Konkuk University, Seoul, Republic of Korea, [6]Tianjin Institute of Industrial Biotechnology, Chinese Academy of Sciences, Tianjin, China

Ramie (*Boehmeria nivea*, BN) is used as livestock forage through suitable silage fermentation owing to its nutritional value. To date, relatively few studies have investigated the effects of dietary fermented BN (FBN) on gut health in finishing pigs. The aim of the present study was to investigate the effects of dietary supplementation with 20% FBN on intestinal morphology, gene expression, and the functional response of the gut microbiota in finishing pigs. We found that FBN did not significantly affect serum antioxidant enzyme activities, ileal morphology, or the expression of genes encoding antioxidant enzymes, inflammatory cytokines, or tight junction proteins in the liver of the pigs. However, the gene expression levels of aryl hydrocarbon receptor (*AHR*) and interleukin 6 (*IL6*) were significantly downregulated in the ileum. A metagenomic analysis demonstrated that, compared with that seen in the control group, the cecal microbiota of pigs in the FBN treatment group was more closely clustered and contained a greater number of unique microbes. Bacteria were the predominant kingdom in the cecal microbiota, while Firmicutes, Bacteroidetes, and Proteobacteria were the dominant phyla, and *Streptococcus*, *Lactobacillus*, and *Prevotella* were the dominant genera. Dietary FBN significantly increased the abundance of the probiotic bacterium *Roseburia inulinivorans* ($p < 0.05$). Functional analysis of the cecal microbiota showed that ABC transporter levels and glycolysis/gluconeogenesis-associated functions were diminished in FBN-fed pigs. Meanwhile, CAZyme analysis revealed that dietary FBN significantly downregulated the contents of carbohydrate-active enzymes, such as GT2, GH1, GH25, and GH13_31. In addition, cytochrome P450 analysis revealed that the abundance of CYP51 and CYP512 decreased with FBN treatment. An assessment of antibiotic resistance based on the Comprehensive Antibiotic Resistance Database (CARD) annotation indicated that the cecal microbes from pigs in the FBN treatment group had increased resistance to lincosamide, streptogramin, and chloramphenicol and reduced resistance to amikacin, isepamicin, neomycin, lividomycin, gentamicin, paromomycin, ribostamycin, and butirosin. Finally, virulence factor-related analysis showed that putative hemolysin-associated functions were decreased, whereas fibronectin-binding protein, flagella, and alginate-associated functions were increased. Taken together, our data showed that FBN supplementation exerted only minor effects on intestinal morphology and microbial community composition, suggesting that

it is potentially safe for use as a supplement in the diets of finishing pigs. However, more studies are needed to validate its functionality.

KEYWORDS

pigs, metagenomics, *Boehmeria nivea*, fermentation, cecal microbiota

1. Introduction

Over the past few decades, China has imported an enormous quantity of soybean meal (SBM) owing to the rapid scaling-up of the modern swine industry and the limited domestic supply of SBM. Researchers and feed manufacturers have increasingly focused on identifying cost-effective native alternatives to SBM to reduce the reliance on imported SBM and maintain the sustainable development of the Chinese swine industry. Ramie (*Boehmeria nivea*) is an ancient, fast-growing, perennial textile fiber crop widely cultivated in the south of China (1). The leaves and tender tops of ramie are low in fiber but high in protein (20% crude protein content on a dry matter basis) and rich in amino acids (especially lysine), minerals, flavonoids, polyphenols, and vitamins (1). Ramie has been applied in feed for black goats as a substitute for alfalfa (2) and has not impacted the health, meat quality, or gut microbiota diversity of Boer goats, even at an inclusion rate as high as 40% (3). Dietary ramie and rice straw mixed silage has been proven to increase milk fat percentage, milk solid content, and dry matter digestibility in dairy cows (4). Ramie has good palatability and nutritional value as a supplement in ruminant feed but remains underutilized. Ramie has also been used in the diets of finishing pigs to improve carcass traits, muscle chemical composition (5), serum antioxidant enzyme activities, and pork fatty acid composition (6). A low dietary inclusion level of ramie has been reported to increase the average daily gain and feed-to-gain ratio in pigs, although higher inclusion levels adversely affected the growth performance of the animals (6).

Silage is one of the best methods for preserving forage resources in regions with heavy rainfall because it contains high levels of lactic acid bacteria and organic acids and low levels of non-digestible fiber (7). In general, silage is produced through natural bacteria-mediated fermentation, with some additives being included to improve silage quality. *Aspergillus niger* is commonly used as an inoculant to treat a wide range of agricultural and household waste residues for the production of value-added products owing to its multi-enzymatic (mannanase, xylanase, cellulase, glucanase, and lipase) activities (8, 9). *A. niger* is also used for the production of organic acids and the hydrolysis of bound polyphenols using low-value materials (10, 11).

The gut microbiota and feed ingredients can mutually affect the host's nutrition, metabolism, and overall health (12–14). However, it is not possible to predict how the introduction of a new feed ingredient into the diet of pigs will affect the development of the gut microbiota of the animals. The advancements in high-throughput sequencing technology have enabled a more comprehensive analysis of complex gut microbiota (15). Studies have traditionally employed 16S rRNA gene sequencing of fecal samples to analyze the gut microbiota diversity and composition (16); nevertheless, this method cannot achieve a thorough understanding of the effects of diet on the functionality of gut microorganisms. In this study, we prepared ramie silage using an autochthonous *A. niger* strain previously isolated from ramie and explored its effects on finishing pigs. We performed a metagenomic shotgun sequencing analysis of the cecal content of the animals to comprehensively elucidate how a high inclusion level of ramie silage affects their gut microbiota. The effects of dietary ramie silage on ileal morphology and expression of antioxidant and anti-inflammatory genes were also evaluated.

2. Materials and methods

2.1. Animals and experimental diets

All experimental procedures involving animals were approved (2015-8A) by the Animal Care and Use Committee of the Institute of Subtropical Agriculture, Chinese Academy of Sciences. A total of 12 DLY (Duroc × Landrace × Yorkshire) finishing pigs with an average initial body weight of 60.11 ± 0.09 kg were used in the study. A basal diet containing corn, SBM, and rice bran was formulated based on the recommendations of the National Research Council (Supplementary Table S1). The pigs were randomly allocated to two treatment groups, with six replicates per group. Pigs in the control (Con) group were fed a basal diet, and those in the fermented *Boehmeria nivea* (FBN) group were fed a basal diet in which the corn, SBM, and rice bran mixture were replaced by the isocaloric and isonitrogenous inclusion of 20% FBN. The fermented ramie was provided by Hunan Agricultural University. The ramie silage was prepared via the inoculation of 100 mL of 1×10^6 CFU/mL *A. niger* into 1 kg of ramie powder. After packaging and fermenting at 30°C for 60 days, the FBN was formulated and pelleted. The pigs were provided with feed twice a day and had free access to their diets. The feeding trial lasted for 60 days after a 7 day adaptation period.

2.2. Euthanization and sample collection

At the end of the feeding experiment, the pigs were euthanized according to standard commercial procedures. Samples of blood, liver, ileum, and colon contents were collected from each pig. The blood was collected from the jugular vein as previously described (17). Serum samples were prepared by centrifugation of the blood at 3,000 × *g* for 10 min at 4°C and stored at −20°C for analysis of antioxidant enzyme activity. Liver and ileal tissue samples and cecal contents (squeezed out from the cecum) were snap-frozen in liquid nitrogen and stored at

−80°C for gene expression analysis and metagenomic shotgun sequencing.

2.3. Serum antioxidant parameters

Serum total antioxidant capacity (TAC) and superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GSH-Px) activities were determined using commercially available kits obtained from Suzhou Comin Biotechnology Co., Ltd. (Suzhou, Jiangsu, China) following the manufacturer's instructions.

2.4. Histological analysis of ileal morphology

Paraffin-embedded ileal tissue sections (5 μm) were stained with hematoxylin and eosin (H&E). Ileal tissue integrity was evaluated by measuring the villus length (VL) and crypt depth (CD) and determining the VL/CD ratio under a light microscope (Olympus, Japan) fitted with a digital camera.

2.5. Quantitative real-time PCR (qPCR) analysis of gene expression in the ileum and liver

Total RNA extraction, cDNA synthesis, and qPCR were performed using previously described methods (5). Briefly, total RNA was extracted from 0.1 g of liver or ileal tissue using column RNA extraction kits (Magen, Guangzhou, China). RNA concentration and purity were determined using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) and 1% agarose gel electrophoresis, respectively. Approximately 1 μg of the isolated RNA was reverse transcribed into cDNA using a cDNA synthesis kit (CWBIO, Jiangsu, China) following the manufacturer's instructions. qPCR was conducted on an ABI 7900HT Real-Time PCR System (Applied Biosystems, Branchburg, NJ, USA). The primers used in the study were designed with Primer3 and BLAST using default parameters (Premier Biosoft International, Palo Alto, CA, USA) and are described in Supplementary Table S2. The PCR cycling conditions were as follows: 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 60 s, and then 1 cycle of 72°C for 30 s. Relative gene expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method after normalization to *GAPDH* or β-actin (18).

2.6. Microbial DNA extraction and metagenomic sequencing

Genomic DNA was extracted from cecal content using an E.Z.N.A. Soil DNA Kit (Omega Bio-Tek, Norcross, GA, USA) according to the manufacturer's protocol. DNA concentration and quality were assessed using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) and 1% agarose gel electrophoresis, respectively. Metagenomic DNA was paired-end sequenced (400 bp fragments) on the NovaSeq/HiSeq X 10 platform (Illumina Inc., San Diego, CA, USA) at Majorbio Bio-Pharm Technology Co., Ltd. (Shanghai, China) using NovaSeq/HiSeq X 10 reagent kits.

2.7. Analysis of sequencing data

The sequencing data were analyzed online on the Majorbio Cloud Platform.[1] Low-quality reads were removed using fastp[2] (19). Reads were aligned using the Burrows-Wheeler Aligner (BWA)[3] (20). Contigs of at least 300 bp were selected and used for further gene prediction and annotation. Open reading frames (ORFs) from each assembled contig were predicted using MetaGene[4] (21). The predicted ORFs (length ≥ 100 bp) were retrieved and translated into amino acid sequences based on the NCBI translation table.[5] A non-redundant gene catalog was constructed using CD-HIT[6] (22) with 90% sequence identity and 90% coverage. After quality control, the reads were mapped to the non-redundant gene catalog with 95% identity using SOAPaligner[7] (23), and the gene abundance in each sample was calculated.

2.8. Functional annotation

Representative sequences from the non-redundant gene catalog were aligned to the NCBI non-redundant database for taxonomic annotation and Cluster of Orthologous Groups (COG) analysis of proteins. Functional annotation of the representative sequences against the Kyoto Encyclopedia of Genes and Genomes (KEGG)[8] was performed using Diamond[9] (24). In addition, functional annotation of the representative genes was further analyzed in the Carbohydrate-Active enZYme (CAZy) database (v07312018)[10], the Comprehensive Antibiotic Resistance Database (CARD)[11], and the Virulence Factor Database (VFDB)[12] using Diamond (24).

2.9. Statistical analysis

Differences between the Con and FBN treatments were assessed using the *t*-test in SPSS 24.0 (SPSS IBM, NY, USA). The results were expressed as mean ± standard error of the mean (SEM), and a p-value <0.05 was considered significant.

1 www.majorbio.com
2 https://github.com/OpenGene/fastp, version 0.20.0
3 http://bio-bwa.sourceforge.net, version 0.7.9a
4 http://metagene.cb.k.u-tokyo.ac.jp/
5 http://www.ncbi.nlm.nih.gov/Taxonomy/taxonomyhome.html/index.cgi?chapter=tgencodes#SG1
6 http://www.bioinformatics.org/cd-hit/, version 4.6.1
7 http://soap.genomics.org.cn/, version 2.21
8 http://www.genome.jp/kegg/
9 http://www.diamondsearch.org/index.php, version 0.8.35
10 http://www.cazy.org/
11 https://card.mcmaster.ca/home
12 http://www.mgc.ac.cn/VFs/

3. Results

3.1. Serum antioxidant parameters

The effects of dietary FBN on serum antioxidant enzyme activities in finishing pigs are shown in Table 1. Serum TAC, SOD, CAT, and GSH-Px activities were not significantly affected by the supplementation of 20% FBN in the diet.

3.2. Ileal morphology

The effects of dietary FBN on ileal histomorphology are shown in Figure 1. No significant differences in VL, CD, or VL/CD ratio were found between the two treatment groups ($p > 0.05$).

TABLE 1 Dietary effect of fermented *Boechmeria nivea* leaves on serum antioxidant enzymes.

Items	Con	FBN	*p* value
TAC (U/mL)	1.82 ± 0.17	1.24 ± 0.10	0.055
SOD (U/mL)	38.09 ± 8.53	37.25 ± 7.01	0.992
CAT (nmol/min/mL)	22.90 ± 1.47	20.36 ± 0.62	0.287
GSH-Px (nmol/min/mL)	15.84 ± 3.84	16.44 ± 3.46	0.992

3.3. Gene expression in the liver and ileum

Compared with the Con treatment, no significant changes in the relative expression levels of genes encoding antioxidants, inflammatory cytokines, and tight junction proteins were detected in the livers of animals administered with FBN (Figure 2A); however, the expression of the *AHR* and *IL6* genes in the ileum was significantly downregulated by dietary FBN supplementation ($p < 0.05$) (Figure 2B).

3.4. Taxonomic composition of the cecal microbiomes of the two groups

Principal component analysis (PCA) indicated that the cecal microorganisms were more closely clustered in the FBN treatment group than in the Con treatment group, and there was a distinct separation between the microbiota of the two groups (Figure 3). A total of 11,692 species were identified using shotgun metagenomic sequencing, of which 10,271 were shared between the two groups and 528 and 893 were unique to the Con and FBN groups, respectively (Figure 4). The taxonomic composition of the cecal microbiome was determined at the domain, kingdom, phylum, and genus levels (Figure 5). Bacteria were the most abundant organisms at the domain and kingdom levels, with other organisms making up only a very small proportion of the total (Figures 5A,B). At the phylum level, Firmicutes, Bacteroidetes, and Proteobacteria were predominant in

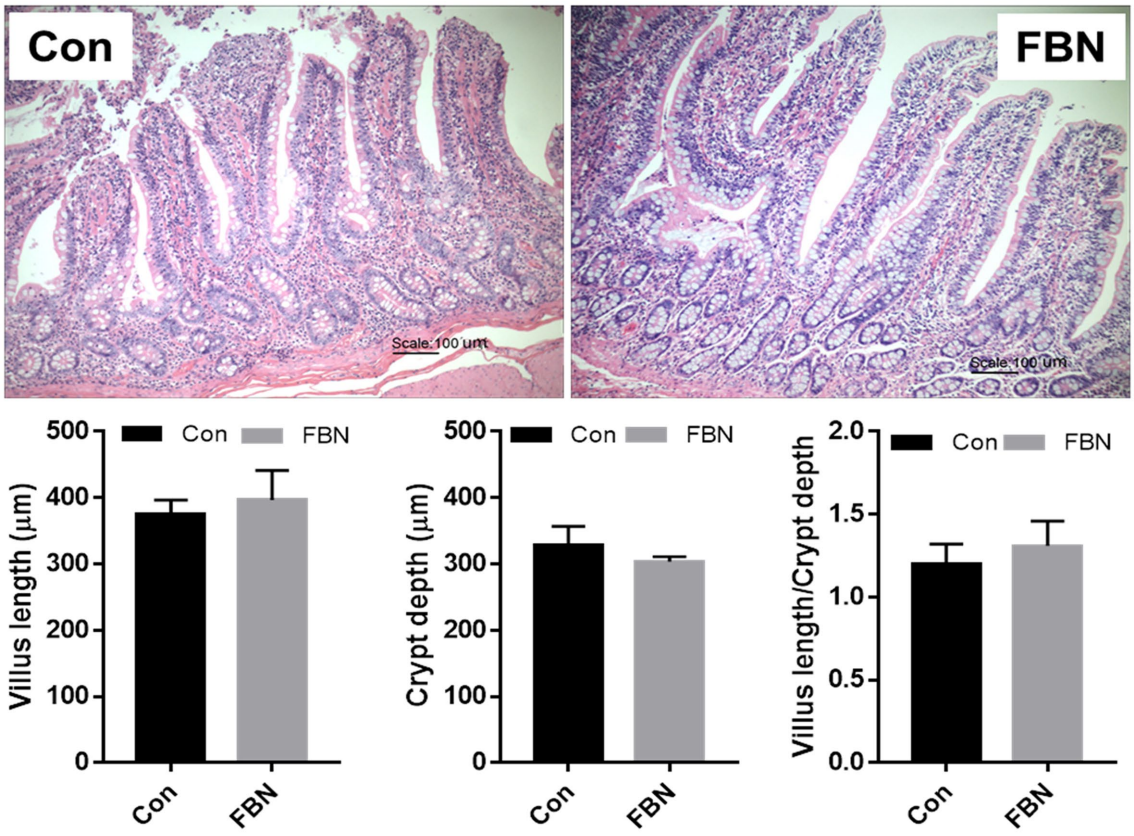


FIGURE 1
Dietary effect of fermented *Boechmeria nivea* on ileal morphology of finishing pigs.

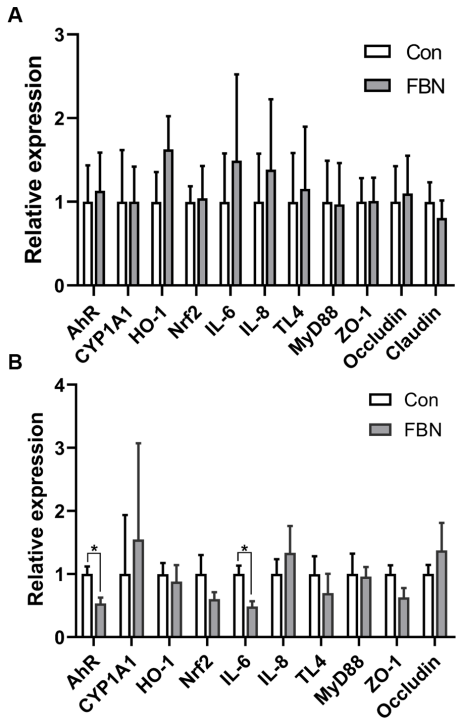


FIGURE 2
Dietary effects of fermented *Boechmeria nivea* on relative gene expression in liver **(A)** and ileum **(B)** of finishing pigs.

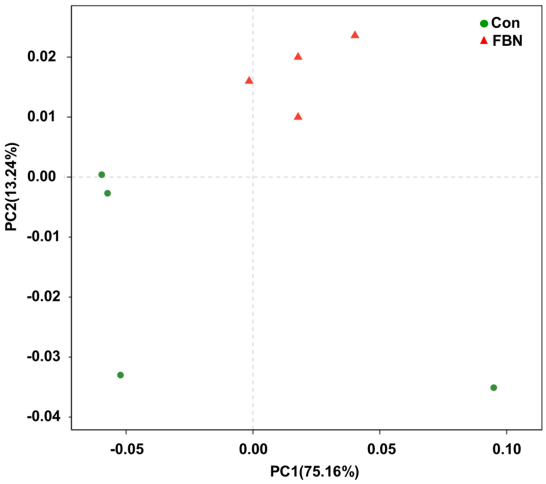


FIGURE 3
Principal component analysis of the similarity of the cecal microbiota.

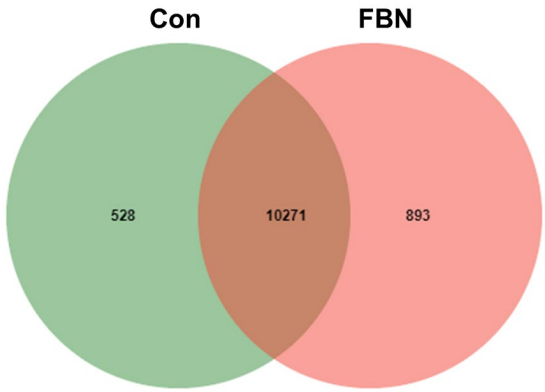


FIGURE 4
Venn diagram showing the unique and shared species in the cecal microbiota.

that order of abundance (Figure 5C). At the genus level, 15 dominant genera were identified in the cecum of the finishing pigs. Dietary FBN supplementation significantly reduced the relative abundance of *Streptococcus* and *Ruminococcus*, and increased that of *Clostridium*, unclassified_f_*Lachnospiraceae*, *Eubacterium*, and unclassified_o_Clostridiales in the cecal contents of the animals (Figure 5D).

3.5. Functional annotation of the microbiome

Changes in the functional profiles of the cecal microbial communities were investigated using Tax4FUN software based on the 16S rRNA gene datasets. To analyze the putative functionality of the microbiome, the genes were annotated based on six functional annotation databases. The sequences in the final gene catalog were aligned and analyzed in the probiotic database. A total of 15 dominant probiotic strains were identified among the sequences of the cecal microbiome of finishing pigs, with *Lactobacillus reuteri* exhibiting the greatest abundance. Additionally, the abundance of *Roseburia inulinivorans* was significantly improved by FBN treatment (Figure 6). The correlation analysis showed that the abundance of *R. inulinivorans* was negatively correlated with the gene expression of AHR and IL6 in the ileum (Figure 7). The gene catalog was then functionally annotated in the KEGG database, and the top 15 functional categories were identified (at level 3). Genes involved in pathways predicted to be related to ABC transporters and glycolysis/gluconeogenesis were significantly underrepresented, whereas those associated with mismatch repair were enriched with dietary FBN treatment (Figure 8). The gene catalogs were further aligned to their protein sequences and analyzed in the CAZy database. The sequences were mainly classified into three enzyme groups, namely, glycosyl transferases (GTs), glycoside hydrolases (GHs), and carbohydrate esterases (CEs). Among them, GT2, GH1, GH25, and GH13_31 were significantly reduced by dietary FBN treatment, coupled with a decline in CE1 abundance (Figure 9). Moreover, the gene sequences were analyzed in the Cytochrome P450 Engineering Database. The results showed that CYP51, CYP505, and CYP125 were the predominant CYP450 families. Among these P450 families, the abundance of CYP51 and CYP512 was significantly decreased with dietary FBN supplementation (Figure 10). We also analyzed the gene catalog in the CARD to identify and compare the abundance of antibiotic resistance genes (ARGs) (Figure 11). The results showed that dietary FBN reduced the abundance of ARGs and increased that of antibiotic susceptive genes. Finally, we analyzed the virulence factors in the cecal microbiome in the VFDB. We found that the abundance of hemolysin-related genes

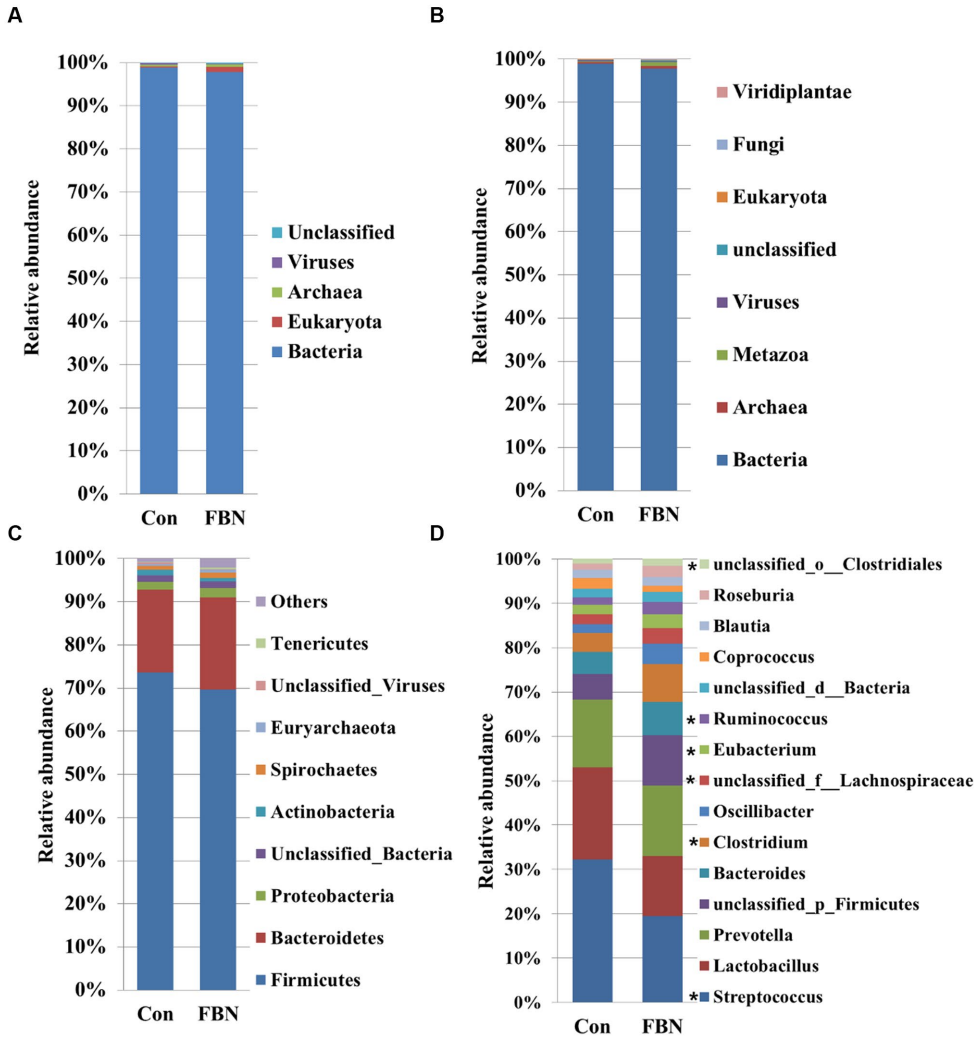


FIGURE 5
The dominant cecal microbiome at the domain **(A)**, kingdom **(B)**, phylum **(C)**, and genus **(D)** levels. *indicates significant differences at the level $P < 0.05$.

was decreased, whereas that of genes related to fibronectin-binding protein, flagella, and alginate was significantly increased in the FBN supplementation group (Figure 12).

4. Discussion

The use of ramie as an unconventional forage source in the diet of pigs has been explored in some areas of southern China with the aim of reducing the region's reliance on imported SBM (5, 6). It has been reported that the inclusion of ramie powder at less than 9% in the diet of finishing pigs partially improved carcass traits and serum antioxidant capacity without impairing growth (5, 6). Similarly, in this study, we did not observe any detrimental effect of FBN on the activities of antioxidant enzymes in serum when supplemented at 20% in the diets of finishing pigs. Antioxidant enzymes such as SOD, CAT, and GSH-Px play important roles in the elimination of ROS and the maintenance of redox homeostasis (6). The fact that serum antioxidant enzyme contents were unaltered suggests that even high inclusion levels of FBN do not induce oxidative stress in finishing pigs.

In goats, high dietary inclusion levels of ramie (up to 40%) have been reported to improve butyrate concentrations in the rumen without altering the diversity of the ruminal microbiota (3). Compared with monogastric animals, ruminants are better at digesting ramie forage. During silage fermentation, the fiber content is typically reduced, which enhances the digestibility of the resulting forage. This implies that even a high inclusion level of fermented ramie may not be detrimental to pigs. Nevertheless, when a new ingredient is introduced into animal feed, the effects on gut health must be carefully assessed. In this study, no changes in intestinal architecture or morphology were observed in the FBN treatment group, indicating that FBN can be safely included in the diets of finishing pigs. Given its detoxifying functions, it is also important to assess the status of the liver when a new feed ingredient is introduced. AhR has been extensively studied for its role in mediating xenobiotic metabolism; however, it is also known to be a critical regulator of immunity and inflammation (25, 26). One study reported that a microbial polyphenol metabolite enhanced gut barrier function

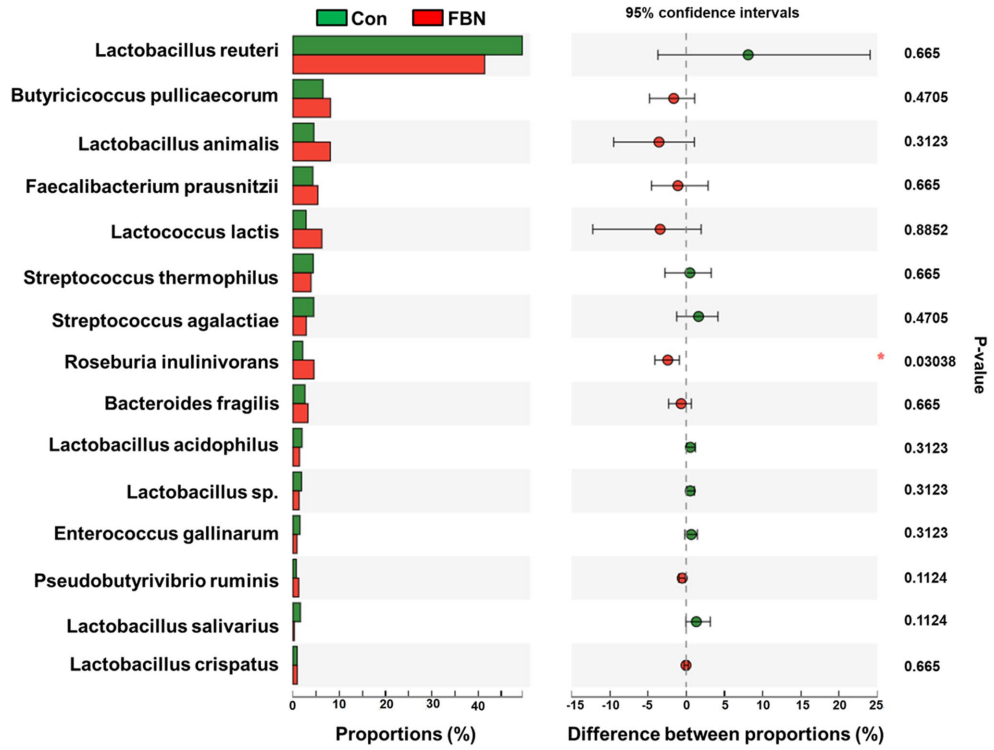


FIGURE 6
Putative probiotic abundance in the cecal microbiota annotated by the probiotic database.

via activation of AhR/nuclear factor erythroid 2-related factor 2 (Nrf2)-dependent pathways (27). Nrf2 is a downstream target of the AhR and plays a vital role in the antioxidant defense system (28). We assessed the expression of genes involved in the AhR-Nrf2 pathway, such as those involved in antioxidant, immune, and barrier functions, in both the liver and the ileum. We found that the expression of both the *AHR* and *IL6* genes was downregulated in the ileum, which may be due to the presence of unknown phytochemicals in the FBN. IL-6 is a pro-inflammatory cytokine and is also involved in immune responses (29). The observed decline in *IL6* gene expression suggests that FBN has anti-inflammatory potential.

The gut microbiota plays a critical role in nutrient digestion and adsorption, gastrointestinal immunity, and epithelial cell renewal (30). Many factors, both intrinsic and extrinsic, can influence gut microbial diversity, composition, function, and metabolic activities. To date, no study has investigated the effects of dietary FBN on the gut microbiota of finishing pigs. Knowledge of the alterations that occur in the composition and relevant functions of the gut microflora in response to dietary changes will help in the development of practical new forage for pigs. In this study, FBN treatment-induced changes in the composition of the cecal microbiota were characterized by a greater number of unique species compared to the control treatment. Dietary FBN did not significantly affect microbial composition at the domain, kingdom, and phylum levels, suggesting that even high dietary inclusion levels of FBN do not alter gut microbial stability. At the genus level, the abundance of *Streptococcus* was reduced, while that of *Clostridium*, Unclassified_f_*Lachnospiraceae*, *Eubacterium*, *Ruminococcus*, Unclassified_o_*Clostridiales*, and the probiotic *Roseburia inulinivorans* was increased when FBN was included in the diet of the pigs. *Streptococcus*, *Lactobacillus*, and *Clostridium* species are prevalent in specific pig species, as revealed by a meta-analysis of 16S rRNA sequences and metagenomics (31).

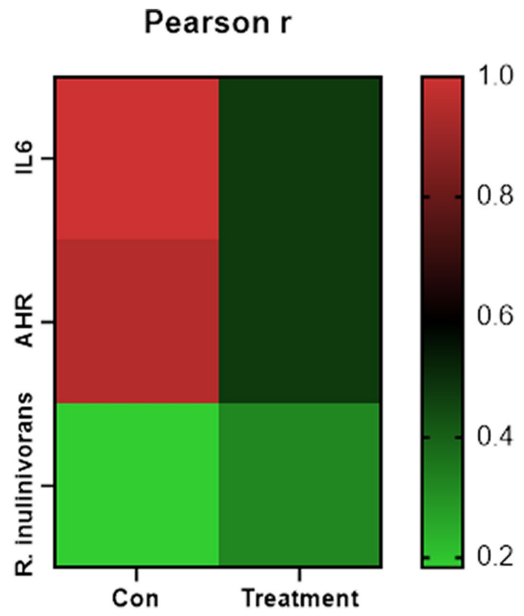


FIGURE 7
Correlation analysis between the differentially altered species and gene expression.

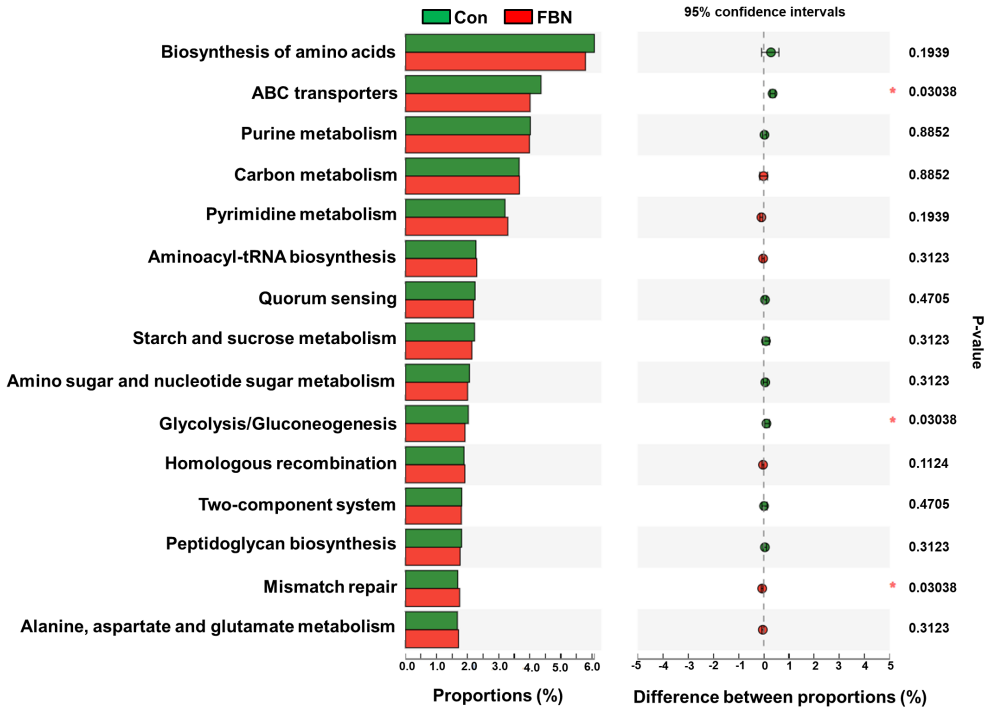


FIGURE 8
Putative functions of the cecal microbiome annotated by the Kyoto Encyclopedia of Genes and Genomes (KEGG) at level 3.

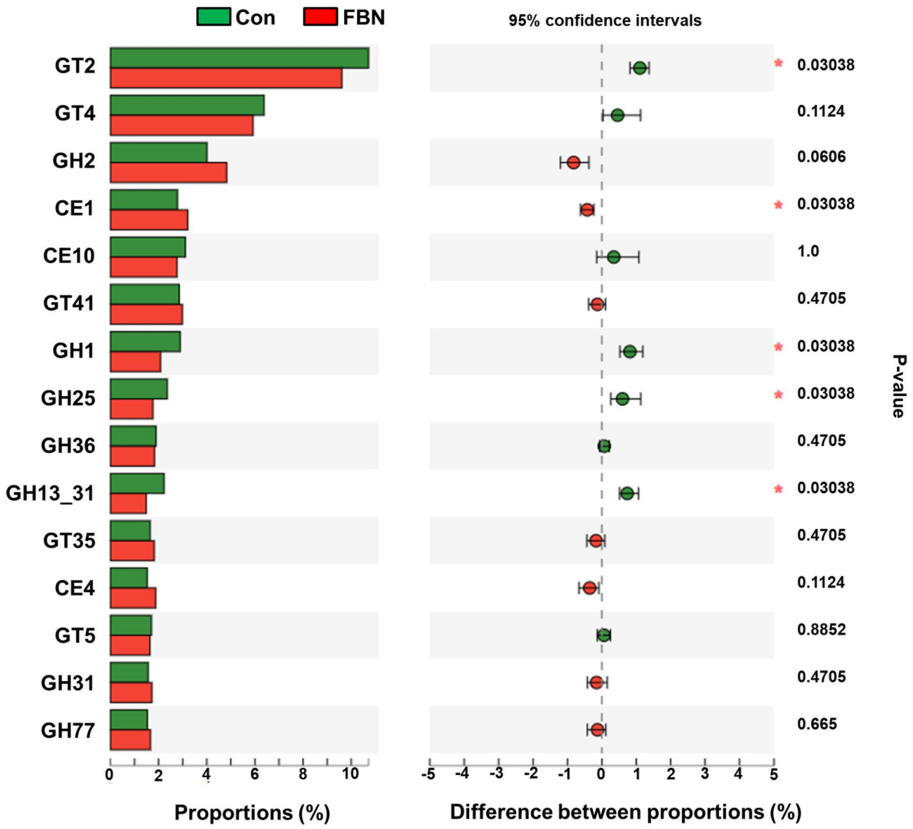


FIGURE 9
Putative carbohydrate-active enzyme (CAZyme) abundances of cecal microbes at the family level, annotated using the CAZy database. GT, glycosyltransferases; GH, glycoside hydrolases; CE, carbohydrate esterases.

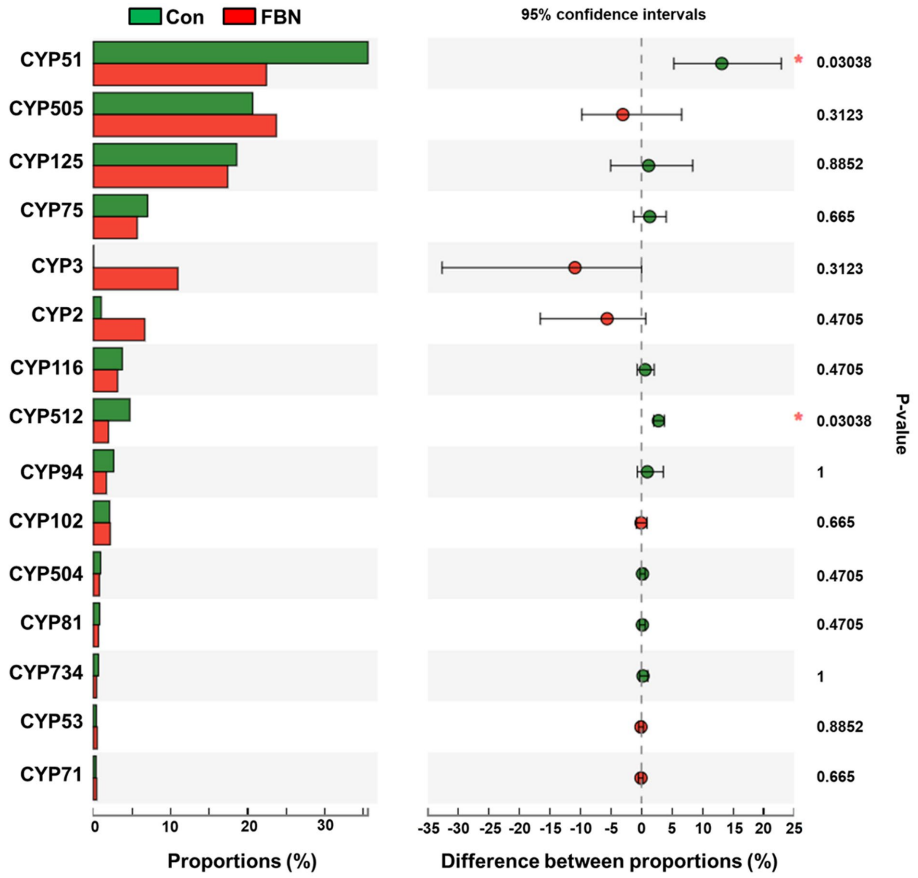


FIGURE 10
Putative CYP450 family of cecal microbes annotated using the Cytochrome P450 Engineering database.

Streptococcus species such as *S. alactolyticus* are prevalent in pigs from China but not in pigs from France or Denmark, which may be attributed to the widespread use of antibiotics in China (31). Several ARGs that confer resistance to aminoglycosides, macrolides, and fluoroquinolones have been found in the genome of *Streptococcus suis* isolated from diseased pigs (32). *S. suis* is an important hemolysin-producing pathogen of swine (33). The decrease in *Streptococcus* abundance may explain the reduced antibiotic resistance and hemolysin levels in the gut microbiome of FBN-fed pigs. Wylensek et al. identified several *Clostridium* species in the cecal microbiota of pigs, such as *C. innocuum*, *C. beijerinckii*, *C. perfringens*, *C. cochlearium*, *C. cadaveris*, *C. porci*, *C. celerecrescens*, and *C. scindens* (31). Among these, *C. porci* was a newly identified species, and a fucosyltransferase-encoding gene was found in its genome (31). *Eubacterium* and *Ruminococcus* are common intestinal genera and have been detected in sows in both prenatal and postnatal periods (34). Yang et al. (35) reported that the abundance of *Eubacterium* and *Ruminococcus* decreased in diarrheic piglets, suggesting that greater numbers of the two genera may indicate a healthier gut microbiota in pigs. A metagenomic analysis showed that *Roseburia inulinivorans* was enriched in the intestinal microbiota of FBN-fed pigs. *R. inulinivorans* is known to produce butyrate from dietary polysaccharides via the secretion of fructofuranosidase, α-amylase, and α-glucanotransferases (36). Function prediction based on the KEGG database indicated that ABC transporter function decreased with FBN supplementation, which is consistent with a previous report showing that traditional Chinese medicine significantly decreased the abundance of ABC transporters in mice (37). The enzymatic activities of gut microbes are very important for nutrient digestion and absorption. Functional insights into the microbiome based on the CAZy database showed that the activities of GT2, GH1, GH13, and GT2 were decreased with FBN treatment. Among these, GT2 was reported to be the most abundant enzyme (family level) in the ileum, cecum, and colon of pigs (16). The decreased activities of these enzyme families may be attributed to the inhibitory effects of some phytochemicals in FBN on microbes capable of producing glycoside transferase and glycoside hydrolase enzymes, which are involved in glycolysis/gluconeogenesis. Cytochrome P450s play an important role in the activation and detoxification of drugs, environmental toxicants, and dietary ingredients. The gut microbiome is involved in the biotransformation of xenobiotics in the host either directly via the production of enzymes that metabolize the xenobiotics or indirectly by influencing host receptors and signaling pathways via metabolite production (38). The CYP51 family is widely distributed in fungi (39). Unexpectedly, FBN supplementation in the diet reduced the abundance of CYP51 in the gut microbiome of the finishing pigs. This may be due to the low abundance of fungi in the gut microbiome of the animals. Additionally, AhR may be associated

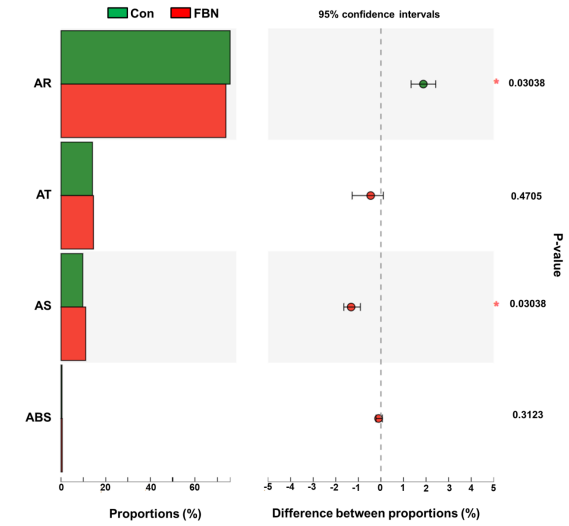


FIGURE 11
Putative antibiotic resistance of cecal microbes at the class level annotated using the CARD database (Comprehensive Antibiotic Resistance Database). AR, Antibiotic resistance; AT, Antibiotic Target; AS, Antibiotic Sensitive; ABS, Antibiotic Biosynthesis.

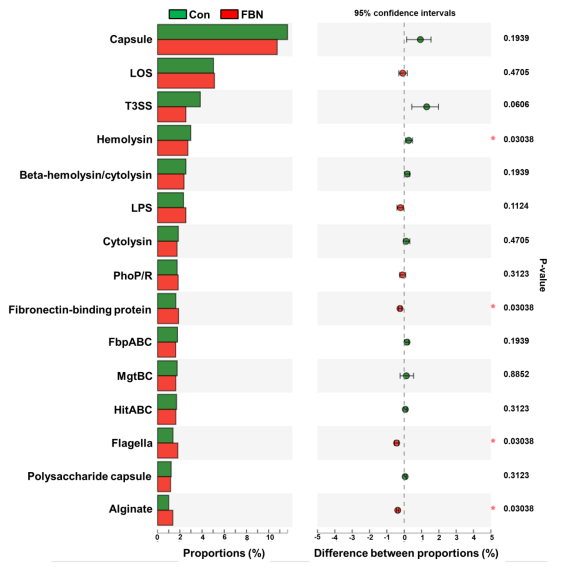


FIGURE 12
Putative virulence factors of cecal microbes annotated by the Virulence Factor Database (VFDB).

with the reduced abundance of CYP family proteins given that it acts upstream of CYP genes (27).

5. Conclusion

In conclusion, this study was the first to explore the effects of high dietary inclusion levels (20%, *w*/*w*) of *Aspergillus niger*-fermented *Boehmeria nivea* on the composition, structure, and putative functions of the cecal microbiota of finishing pigs. High levels of FBN supplementation in the diet did not affect serum antioxidant capacity, intestinal morphology, or the expression of genes encoding antioxidant-, immune-, and tight junction-related proteins in the liver, except for an observed decrease in *AHR* and *IL6* gene expression levels in the ileum. FBN treatment altered the composition of the gut microbiota at the genus level, as evidenced by the reduced abundance of *Streptococcus* and the enrichment of *Clostridium*, *Eubacterium*, *Ruminococcus*, and *Roseburia inulinivorans*. In addition, the inclusion of FBN in the diet reduced the predicted functions of glycoside hydrolases (GH1, GH13, GH25), which may be associated with pathways involved in glycolysis/gluconeogenesis. Gene functions related to antibiotic resistance and hemolysin-based virulence in the cecal microbiota were attenuated with FBN treatment. These results indicate that high dietary inclusion levels of FBN may not impact gut health in finishing pigs. However, the effects of FBN on growth performance, meat quality, and feed costs require further investigation.

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 all experimental procedures involving animals were approved (2015-8A) by the Animal Care and Use Committee of the Institute of Subtropical Agriculture, Chinese Academy of Science. The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

XL: Data curation, Formal analysis, Writing – original draft, Methodology, Software. ZZ: Data curation, Formal analysis, Writing – original draft. FR: Formal analysis, Investigation, Writing – original draft. YJ: Conceptualization, Writing – review & editing, Resources. S-KK: Conceptualization, Writing – review & editing. K-MN: Writing – review & editing, Data curation, Formal analysis, Funding acquisition, Writing – original draft. XW: Writing – review & editing, Funding acquisition, Conceptualization, Project administration.

Funding

This work was financially supported by the pilot demonstration project for the Overall Rationing System of the Jiangxi Academy of Sciences (Grant Nos. 2022YSBG22002 and 2022YJC2002), the cooperation project of the Chinese Academy of Sciences and local governments (Grant No. 2022-03JXZT-02), the Key Collaborative Research Program of the Alliance of International Science Organizations (Grant No. ANSO-CR-KP-2021-10), and the Central Government Guides Local Science and Technology Development Fund of China (Grant No. 20231zdf03076).

Conflict of interest

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

Publisher's note

All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2023.1253778/full#supplementary-material

References

1. Rehman M, Gang D, Liu QQ, Chen YL, Wang B, Peng DX, et al. Ramie, a multipurpose crop: potential applications, constraints and improvement strategies. *Ind Crop Prod*. (2019) 137:300–7. doi: 10.1016/j.indcrop.2019.05.029

2. Tang SX, He Y, Zhang PH, Jiao JZ, Han XF, Yan QX, et al. Nutrient digestion, rumen fermentation and performance as ramie (*Boehmeria nivea*) is increased in the diets of goats. *Anim Feed Sci Technol*. (2019) 247:15–22. doi: 10.1016/j.anifeedsci.2018.10.013

3. Du E, Guo W, Chen F, Fan Q, Zhao N, Zhang W, et al. Effects of ramie at various levels on ruminal fermentation and rumen microbiota of goats. *Food Sci Nutr*. (2020) 8:1628–35. doi: 10.1002/fsn3.1449

4. Gao S, Liao Y, Li Z, Hou Z, Zhong R, Wu D. Including ramie (*Boehmeria nivea* L. gaud) in the diet of dairy cows: effects on production performance, milk composition, rumen fermentation, and nutrient digestion. *Ital J Anim Sci*. (2020) 19:240–4. doi: 10.1080/1828051X.2020.1726831

5. Li YH, Liu YY, Li FN, Lin Q, Dai QZ, Sun JB, et al. Effects of dietary ramie powder at various levels on carcass traits and meat quality in finishing pigs. *Meat Sci*. (2018) 143:52–9. doi: 10.1016/j.meatsci.2018.04.019

6. Li YH, Liu YY, Li FN, Sun A, Lin Q, Huang XG, et al. Effects of dietary ramie powder at various levels on growth performance, antioxidative capacity and fatty acid profile of finishing pigs. *J Anim Physiol Anim Nutr*. (2019) 103:564–73. doi: 10.1111/jpn.13031

7. Coblentz WK, Akins MS. Silage review: recent advances and future technologies for baled silages. *J Dairy Sci*. (2018) 101:4075–92. doi: 10.3168/jds.2017-13708

8. Bansal N, Tewari R, Soni R, Soni SK. Production of cellulases from *aspergillus niger* NS-2 in solid state fermentation on agricultural and kitchen waste residues. *Waste Manag*. (2012) 32:1341–6. doi: 10.1016/j.wasman.2012.03.006

9. Li C, Zhou JW, Du GC, Chen J, Takahashi S, Liu S. Developing *Aspergillus niger* as a cell factory for food enzyme production. *Biotechnol Adv*. (2020) 44:107630. doi: 10.1016/j.biotechadv.2020.107630

10. Ma X, Gao M, Wang N, Liu S, Wang Q, Sun X. Lactic acid production from co-fermentation of food waste and spent mushroom substance with *Aspergillus niger* cellulase. *Bioresour Technol*. (2021) 337:125365. doi: 10.1016/j.biortech.2021.125365

11. Xue PY, Liao W, Chen Y, Xie JH, Chang XX, Peng GY, et al. Release characteristic and mechanism of bound polyphenols from insoluble dietary fiber of navel orange peel via mixed solid-state fermentation with *Trichoderma reesei* and *Aspergillus niger*. *LWT*. (2022) 161:113387. doi: 10.1016/j.lwt.2022.113387

12. Lynch SV, Pedersen O. The human intestinal microbiome in health and disease. *N Engl J Med*. (2016) 375:2369–79. doi: 10.1056/NEJMra1600266

13. Gresse R, Chaucheyras-Durand F, Fleury MA, Van de Wiele T, Forano E, Blanquet-Diot S. Gut microbiota dysbiosis in postweaning piglets: understanding the keys to health. *Trends Microbiol*. (2017) 25:851–73. doi: 10.1016/j.tim.2017.05.004

14. Niu KM, Lee BJ, Kothari D, Lee WD, Hur SW, Lim SG, et al. Dietary effect of low fish meal aquafeed on gut microbiota in olive flounder (*Paralichthys olivaceus*) at different growth stages. *Microbiology*. (2020) 9:e992. doi: 10.1002/mbo3.992

15. Jovel J, Patterson J, Wang W, Hotte N, O'Keefe S, Mitchel T, et al. Characterization of the gut microbiome using 16S or shotgun metagenomics. *Front Microbiol*. (2016) 7:459. doi: 10.3389/fmicb.2016.00459

16. Quan JP, Cai GY, Yang M, Zang ZH, Ding RR, Wang XW, et al. Exploring the fecal microbial composition and metagenomic functional capacities associated with feed efficiency in commercial DLY pigs. *Front Microbiol*. (2019) 10:52. doi: 10.3389/fmicb.2019.00052

17. Wu X, Xie CY, Long CM, Li J, Zhou XH, Fan ZY, et al. Effects of a daily three-meal pattern with different dietary protein contents on pig growth performance, carcass and muscle quality traits. *J Sci Food Agric*. (2018) 98:415–21. doi: 10.1002/jsfa.8467

18. Wu X, Gao LM, Liu YL, Xie CY, Cai LC, Xu K, et al. Maternal dietary uridine supplementation reduces diarrhea incidence in piglets by regulating the intestinal mucosal barrier and cytokine profiles. *J Sci Food Agric*. (2020) 100:3709–18. doi: 10.1002/jsfa.10410

19. Chen SF, Zhou YQ, Chen YR, Gu J. Fastp: an ultra-fast all-in-one FASTQ preprocessor. *Bioinformatics*. (2018) 34:i884–90. doi: 10.1093/bioinformatics/bty560

20. Li H, Durbin R. Fast and accurate short read alignment with burrows-wheeler transform. *Bioinformatics*. (2009) 25:1754–60. doi: 10.1093/bioinformatics/btp324

21. Noguchi H, Park J, Takagi T. MetaGene: prokaryotic gene finding from environmental genome shotgun sequences. *Nucleic Acids Res*. (2006) 34:5623–30. doi: 10.1093/nar/gkl723

22. Fu LM, Niu BF, Zhu ZW, Wu ST, Li WZ. CD-HIT: accelerated for clustering the next-generation sequencing data. *Bioinformatics*. (2012) 28:3150–2. doi: 10.1093/bioinformatics/bts565

23. Li RQ, Li YR, Kristiansen K, Wang J. SOAP: short oligonucleotide alignment program. *Bioinformatics*. (2008) 24:713–4. doi: 10.1093/bioinformatics/btn025

24. Buchfink B, Xie C, Huson DH. Fast and sensitive protein alignment using DIAMOND. *Nat Methods*. (2015) 12:59–60. doi: 10.1038/nmeth.3176

25. Hooper LV. You AhR what you eat: linking diet and immunity. *Cells*. (2011) 147:489–91. doi: 10.1016/j.cell.2011.10.004

26. Zhou L. AHR function in lymphocytes: emerging concepts. *Trends Immunol*. (2016) 37:17–31. doi: 10.1016/j.it.2015.11.007

27. Singh R, Chandrashekharappa S, Bodduluri SR, Baby BV, Hegde B, Kotla NG, et al. Enhancement of the gut barrier integrity by a microbial metabolite through the Nrf2 pathway. *Nat Commun*. (2019) 10:89. doi: 10.1038/s41467-018-07859-7

28. Kohle C, Bock KW. Activation of coupled ah receptor and Nrf2 gene batteries by dietary phytochemicals in relation to chemoprevention. *Biochem Pharmacol*. (2006) 72:795–805. doi: 10.1016/j.bcp.2006.04.017

29. Hirano T. IL-6 in inflammation, autoimmunity and cancer. *Int Immunol*. (2021) 33:127–48. doi: 10.1093/intimm/dxaa078

30. Wang AR, Ran C, Ringo E, Zhou ZG. Progress in fish gastrointestinal microbiota research. *Rev Aquac*. (2018) 10:626–40. doi: 10.1111/raq.12191

31. Wylensek D, Hitch TCA, Riedel T, Afrizal A, Kumar N, Wortmann E, et al. A collection of bacterial isolates from the pig intestine reveals functional and taxonomic diversity. *Nat Commun*. (2020) 11:6389. doi: 10.1038/s41467-020-19929-w

32. Yongkiettrakul S, Wongsurawat T, Jenjaroenpun P, Acheampong DA, Srimanote P, Maneerat K, et al. Genome sequences of antibiotic-resistant *Streptococcus suis* strains isolated from human patients and diseased and asymptomatic pigs in Thailand. *Infect Genet Evol*. (2021) 87:104674. doi: 10.1016/j.meegid.2020.104674

33. Jacobs AAC, Vandenberg AJG, Baars JC, Nielsen B, Johannsen LW. Production of suilysin, the thiol-activated hemolysin of Streptococcus-Suis, by field isolates from diseased pigs. *Vet Rec*. (1995) 137:295–6. doi: 10.1136/vr.137.12.295

34. Sun L, Zhang Y, Chen W, Lan T, Wang Y, Wu YB, et al. The dynamic changes of gut microbiota during the perinatal period in sows. *Animals*. (2020) 10:2254. doi: 10.3390/ani10122254

35. Yang QL, Huang XY, Zhao SG, Sun WY, Yan ZQ, Wang PF, et al. Structure and function of the fecal microbiota in diarrheic neonatal piglets. *Front Microbiol*. (2017) 8:502. doi: 10.3389/fmicb.2017.00502

36. Scott KP, Martin JC, Chassard C, Clerget M, Potrykus J, Campbell G, et al. Substrate-driven gene expression in *Roseburia inulinivorans*: importance of inducible enzymes in the utilization of inulin and starch. *Proc Natl Acad Sci U S A*. (2011) 108:4672–9. doi: 10.1073/pnas.1000091107

37. Zhang XP, Yang YN, Zhang F, Yu JQ, Sun WY, Wang RQ, et al. Traditional Chinese medicines differentially modulate the gut microbiota based on their nature (Yao-Xing). *Phytomedicine*. (2021) 85:153496. doi: 10.1016/j.phymed.2021.153496

38. Dempsey JL, Cui JY. Microbiome is a functional modifier of P450 drug metabolism. *Curr Pharmacol Rep*. (2019) 5:481–90. doi: 10.1007/s40495-019-00200-w

39. Chen WP, Lee MK, Jefcoate C, Kim SC, Chen FS, Yu JH. Fungal cytochrome P450 monooxygenases: their distribution, structure, functions, family expansion, and evolutionary origin. *Genome Biol Evol*. (2014) 6:1620–34. doi: 10.1093/gbe/evu132

frontiers | Frontiers in Veterinary Science

TYPE Review
PUBLISHED 13 October 2023
DOI 10.3389/fvets.2023.1272274

OPEN ACCESS

EDITED BY
Tao Wang,
Jilin Agricultural University, China

REVIEWED BY
Shuaijuan Han,
Hebei Agricultural University, China
Luigi Liotta,
University of Messina, Italy

*CORRESPONDENCE
Flavia Ferlisi
✉ flavia.ferlisi@studenti.unipg.it

†These authors have contributed equally to this work and share first authorship

‡These authors have contributed equally to this work and share last authorship

RECEIVED 03 August 2023
ACCEPTED 29 September 2023
PUBLISHED 13 October 2023

CITATION
Ferlisi F, Tang J, Cappelli K and Trabalza-Marinucci M (2023) Dietary supplementation with olive oil co-products rich in polyphenols: a novel nutraceutical approach in monogastric animal nutrition. *Front. Vet. Sci.* 10:1272274. doi: 10.3389/fvets.2023.1272274

Dietary supplementation with olive oil co-products rich in polyphenols: a novel nutraceutical approach in monogastric animal nutrition

Flavia Ferlisi[1*†], Jiayong Tang[2†], Katia Cappelli[1‡] and Massimo Trabalza-Marinucci[1‡]

[1]Department of Veterinary Medicine, University of Perugia, Perugia, Italy, [2]Animal Nutrition Institute, Sichuan Agricultural University, Chengdu, China

In recent years, the increased demand for agri-food products to feed livestock species has stimulated research to identify novel solutions for the valorization of natural waste, according to the modern concept of a circular economy. Numerous studies have shown the use of plant-derived and agro-industrial co-products that are sources of bioactive molecules for preparing animal feeds. Supplementation with co-products derived from the extraction of olive oil (i.e., olive pomace, olive mill wastewater, olive cake and olive leaf) in diet has been widely considered in recent decades, because these wastes are produced in high quantity and their re-use represents an innovative economic and environmental strategy. Olive oil co-products are characterized by various bioactive molecules such as polyphenols, carbohydrates, proteins, and lipids. Among them, polyphenols are the nutraceuticals most studied, showing to promote health effects in both humans and animals. Olive oil co-products and their phenolic extracts have shown many beneficial and promising effects when added to the diets of monogastric animals, by improving performance parameters and maintaining the oxidative status of meat and derived products. This review provides an update on the use of olive co-products in monogastric animal (swine, poultry and rabbit) diets and their effects on the productive performance, meat quality characteristics and gut health status.

KEYWORDS

antimicrobials, antioxidants, feed supplementation, monogastric, olive oil co-products, polyphenols

1. Introduction

The global consumption of olive oil has grown over the past three crop years due to its high nutritional value and health benefits, and should reach about 3.055 million tons in the 2022/2023 (1). It is known from the International Olive Council (IOC) that there are more than 800 million olive trees in the word, covering an area of 10 million hectares (2), and that the Mediterranean region accounts for 97% of global olive oil production (1). In Mediterranean countries, there are significant differences in the extraction methods of olive oil in different areas. The three-phase centrifugal extraction method is the most common; other techniques are the two-phase extraction and traditional discontinuous pressing (3). The three-phase decanter has become

popular for its ability to decrease labor requirements and simultaneously enhance processing capacity and production of oil. However, it requires a large amount of energy and water during malaxation and generates large quantities of wastewater during the production process. The two-phase decanter separates the liquid part (the oil) from a moist solid part without requiring water, resulting in oil with high antioxidant and aromatic compound levels. However, the two-phase process typically necessitates higher energy consumption and entails a certain degree of loss of oil, because it remains in the pomace (4).

Each production process generates high amounts of co-products, mainly solid and liquid residues including olive cake, olive mill wastewater (OMWW) and olive pomace, which pollute the environment and create disposal problems (5). After two-phase centrifugation, the percentage of wet olive pomace, extra virgin olive oil (EVOO) and OMWW are 78.3, 18.7 and 3%, respectively. After three-phase centrifugation, the percentages of wet olive pomace, EVOO and OMWW are, respectively, 37.6, 17.4 and 45% (4).

In the last decades, innovative strategies including the reutilization of these wastes have become necessary, in order to reduce a negative impact on the ecosystem (6). Interestingly, these co-products can be regarded as excellent economic sources for their content in various beneficial compounds including proteins, lipids, fibers (lignin, cellulose, hemicelluloses, and pectins), polyphenols (phenolic alcohols, phenolic acids, secoiridoids and flavonoids) and other phytochemicals such as tocopherols, triterpenoids and carotenoids (7–9). The content of bioactive molecules differs according to the type of product, and for this reason it is important to consider their individual features and composition, in order to find the most useful applications in different fields (7).

Extra virgin olive oil include a number of polyphenols, among which the phenyl alcohols tyrosol and hydroxytyrosol and the secoiridoid oleuropein, which are abundant and have a considerable antioxidant and antimicrobial activity (10). After extracting olive oil, some olive oil remains in the liquid and solid phases, and these co-products retain about 98% of total phenolic compounds (11). Thus, co-products of olive oil, including OMWW and olive pomace, are considered an excellent source of polyphenols [3.0–50.0 g/kg of dry matter (DM)] (11).

Among the various bioactive molecules contained in olive oil co-products, polyphenols are the most recently studied and recovered molecules, which in several human and animal studies showed multifunctional effects that need to be further investigated (8).

Polyphenols occur naturally in plants either as free aglycones or as esters with polysaccharides or monosaccharides (12). The polyphenols encompass over 8,000 molecules that are characterized by phenolic rings with one or more hydroxyl groups, which are crucial in determining their antioxidant functions (13). Hydroxytyrosol and tyrosol are the major components found in OMWW that could be exploited as high-value compounds (13, 14). Oleuropein and hydroxytyrosol represent as much as 10% of the composition in fresh olive leaves (15), while tyrosol and hydroxytyrosol can also be found in leaf extract and olive cake (16). Furthermore, oleuropein is the main polyphenol in olive leaf extract (17), and dried olive pulp also contains antioxidant phenols such as oleuropein and hydroxytyrosol (10). Membrane technologies such as microfiltration, ultrafiltration and nanofiltration have recently been developed to extract phenolic compounds from olive oil co-products. This innovative method could be a valorization to make the co-products of olive oil a nutritional source of high economic value. Moreover, the novel recovery processes concerning olive oil co-products, in general, helps also to simplify their disposal and management (18).

Phenolic compounds show important biological effects, including anti-inflammatory, antioxidant, antibacterial, antiproliferative, antifungal and hypoglycaemic activities (19). In particular, based on their antioxidant and antimicrobial power, they have become increasingly important for livestock. Olive oil co-products could provide an advantage for the agri-food and zootechnical industries, and their inclusion in livestock feed may provide benefits without compromising their production traits.

The use of these products for animal feeds could be considered a contribution to the circular economy of Mediterranean countries such as Italy, Spain and Greece, which are the main olive oil producers. Moreover, agro-industrial co-products are usually less expensive compared with the feeds traditionally used (20). Regarding animal nutrition, especially for monogastric animals, there has been extensive research into the use of plant extracts high in polyphenols (21). Plant extracts can be regarded as promising alternatives to synthetic antioxidants, given the characteristics of many natural compounds they contain, especially polyphenols (21). The potent antimicrobial properties of phenolic compounds can also potentially reduce the risk of bacterial infections (22). In this regard, it should be useful be remembered that the European Union (EU) has prohibited the use of antibiotics as growth promoters in livestock since 2006 (EC Regulation No. 1831/2003), owing to the potential detrimental impacts on food safety and animal health (23, 24). The use of polyphenols as potential natural feed supplements with a role in the immunity, antimicrobial, antioxidant power and overall production performance of swine and poultry has been confirmed by *in vivo* and *in vitro* studies (21, 25). Furthermore, dietary supplementation with polyphenols could enhance food oxidative stability originating from livestock animals (26). Phenolic compounds can also be applied as natural compounds protecting animals from the bad consequences of feed component oxidation, particularly when there is an high unsaturated fatty acid content (25). This review summarizes the studies available that have examined in monogastric animals the effects of olive oil co-products and extracts as sources of beneficial bioactive molecules, including polyphenols.

2. Chemical composition of olive oil co-products

2.1. Olive pomace and olive cake

Olive pomace is the primary solid product that remains after extracting olive oil from the fruit; it is composed of oil, skin, pulp and pits and has a relatively high water content. It is a good source of beneficial molecules such as carbohydrates, proteins, lipids and polyphenols (7, 27). Olive pomace is rich in fibrous compounds such as cellulose, lignin, pectins and hemicelluloses (xyloglucans, xylans, mannans and glucoroxylans) and also contains small amounts of organic nitrogen as well as minerals (especially potassium) (7, 27).

Interestingly, this co-product is composed by oleuropeosides, polyphenols and flavonoids. The extract of olive pomace contains at least 10% of triterpenes and 2% of polyphenols (28).

Olive cake is a semi-liquid paste produced by pressing and compressing the olive pomace to extract any remaining oil. The olive cake consists of stone (18–32%), pulp, olive skin, kernel, a portion of oil, and approximately 40–60% of water. The olive cake can be regarded as a source of various phytochemicals such as peptides, flavonoids (quercetin), tocopherols and polyphenols (7). Interestingly, vitamin E is a group of exogenous antioxidants including tocopherols that is lipophilic and can work in a synergically with phenolic acids, potentiating its action (29).

Olive cakes devoid of stones exhibit a significant presence of fiber and lignin (160–557 g/kg DM), while the crude protein (CP) content is relatively low and varies from 44 to 115 g/kg DM (30, 31). Some olive cake can be used in the diets of finishing pigs after partial defatting. On dry matter basis, olive cake contains 52–92 g/kg of CP, 7.9 g/kg of Ca, 9 g/kg of total phosphorus, 122 g/kg of ether extract, 8.6 g/kg of total polyphenols (gallic acid equivalents) and 170 g/kg of lignin (32, 33). The semisolid destoned olive cake, called pâté, has an olive oil concentration varying from approximately 8 to 12%, depending on the moisture content. Pâté also has high levels of sugars, structural carbohydrates and a moderate CP content. Fatty acids in pâté mainly comprise oleic acid and polyunsaturated fatty acids (34). The main components of pâté are the following: total polyphenols 120 mg/kg, hydroxytyrosol 54 mg/kg, tyrosol 60 mg/kg, pinoresinol 3 mg/kg, and verbascoside 3.5 mg/kg (34).

2.2. Olive pulp

Olive pulp refers to the fleshy part of the olive fruit that is left over after the oil is extracted. It consists of fragments of olive stones, skin and a small portion of olive oil; it is what remains of the olive cake after drying. Olive pulp is rich in essential fatty acids (73% oleic acid, 13% palmitic acid and 7% linoleic acid) and has a high residual oil (35). Because of this high residual content, dried olive pulp could be a low-cost source of energy. Olive pulp contains fibers, whose contents may change depending on the processing (36). It is also composed by protein, fat, calcium, copper and cobalt, even if it does not have a high nutritional value because of its decreased digestible protein and mineral content and a high lignin concentration (36). Dried olive pulp is considered a relevant reservoir of natural antioxidants, with a high concentration of hydroxytyrosol (228.6 mg/kg DM) and oleuropein (1,007 mg/kg DM), and has antibacterial and antifungal effects (10). The proximate chemical composition of dried olive pulp includes 88–95% of DM, 7–12% of CP, 28–35% of crude fiber, 7.9–19.0 g/kg DM of total phenolic compounds, and 229 mg/kg DM of 3,4-dihydroxyphenylethanol (DHPEA) (10, 37, 38).

2.3. Olive mill wastewater

Olive mill wastewater is the liquid waste generated during the production of olive oil. It consists of the vegetation water of olives diluted during the extraction of oil and has a high polyphenolic content (up to 53% of olive fruit phenolic compounds) for the elevated water solubility of these molecules (39). This liquid product presents a high content of organic compounds such as proteins, lipids, carbohydrates, tocopherols, carotenoids and polyphenols (7).

The amount of OMWW produced every year in the Mediterranean region is approximately 30 million m^3 (40). Polyphenolic powder can be extracted from OMWW by separating the solids; acidifying the solution; extracting with a solvent; purifying through filtration; and precipitating, drying and storing the isolated polyphenols (40). Different extraction processes have a greater impact on the content of phenolic compounds. The content of total polyphenols is approximately 100 mg/g, with hydroxytyrosol and tyrosol being 0.5 and 0.55 mg/g, respectively (41). In terms of g/kg DM, the content of total polyphenolic is approximately 96.6 g/kg, with hydroxytyrosol and tyrosol representing, respectively, 20.8 and 3.9 g/kg of the composition (13).

2.4. Olive leaf extract

Another interesting co-product of olive fruit is the olive leaf extract, which is a bitter-tasting, dark brown liquid derived from olive leaves; it represents approximately 10% of the material deriving from the olive oil press (42, 43). Olive leaves are remnants from the agricultural process of harvesting olives, where the olive trees are beaten to collect the fruits. Pruning yields about 25 kg of olive leaves per tree. The chemical composition of olive leaves consists of a high quantity of extractives (36.52%), lignin (39.6%), cellulose (5.7%) and hemicelluloses (3.8%), as well as crude proteins (ranging from 8.10 to 39.6%) (7).

Fresh olive leaves contain approximately 10% polyphenols (15), specifically oleuropein and hydroxytyrosol. After drying for 2 days at 37°C, olive leaves have up to 25 g/kg total polyphenols and 22 g/kg oleuropein (43). Deriving from the leaves of the olive tree, olive leaf extract is a dark brown liquid with a bitter taste and is also rich in polyphenols, which have a strong antimicrobial effect (44). Among them, oleuropein is the most abundant, with a concentration ranging from 60 to 90 mg/g in dried olive leaves (45).

3. Antimicrobial properties and effects on gut health

In plants and plant extracts, polyphenols are the main secondary metabolites widely known to have antimicrobial action (46). The antimicrobial effects of co-products from olive oil production (especially olive mill waste water and olive leaf extracts) have been related to the presence of phenolic compounds (47), among which oleuropein and hydroxytyrosol are the two most powerful bioactive molecules contained (48, 49). Many polyphenols are able to inhibit the microbial growth and also interfere with oxidative reactions (46). Oleuropein can stimulate the increase of nitric oxide inside the macrophages, protecting them from the endotoxins produced by the gram-negative bacterial species (50). Additionally, extracts can be considered more effective than insolated compounds, because each constituent can be affected by the others contained. In this way, the use of extracts, including the ones from olive oil production, is considered essential for enhancing the synergistic actions of their bioactive compounds (49), with possible applications in animal feed as natural antimicrobial compounds (51). Moreover, a greater antimicrobial activity of olive co-products such as olive leaf extract could be attributed to many phenolic metabolites, including the

products of oleuropein hydrolysis (elenolic acid and oleuropein aglycon) rather than the oleuropein glucoside form (49).

The antimicrobial effect of natural extracts can be also related to their the ability to change the intestinal bacterial population (52) and/or modulating the immune response in the gut. In the body, the primary defense system against adverse microorganisms and harmful internal and external substances is the intestinal tract. However, oxidative stress can compromise this function, resulting in intestinal cell damage, apoptosis and reduction in tight junction proteins' expression, that contribute to the function of mucosal barrier (53). Gut health is crucial for animal growth and is closely related to intestinal immune function. In farm animals, oxidative stress is often associated with gut dysbiosis and pathogenic infections, which can lead to reductions in the antioxidant capacity and increased lipid peroxidation, potentially resulting in severe conditions like sepsis (54). In broiler chickens and weaned piglets, oxidative distress may also contribute to microbial infections, causing inflammation and a reduction of feed efficiency related to a gut function impairment (13).

Interestingly, polyphenols from olive oil have been found to maintain the integrity of gut barrier by increasing the expression of genes involved in tight junction stability and modulating the oxidative state, immune and inflammatory response in the intestinal epithelium (55). Polyphenols also act during weaning stress in piglets and calves by improving not only nutrient absorption and digestion, but also intestinal barrier and intestinal microbiota function (56). Furthermore, olive oil co-products rich in phenolic compounds can induce a modification of gut microbiota populations not only by decreasing intestinal pathogens (i.e., *Helicobacter pylori* and *Escherichia coli*) (42), but also exerting a prebiotic action in the gut through an induction of the growth of helpful bacteria such as *Clostridium* (44, 57) and *Lactobacillus* (44, 58). In every animal species, particularly during the first days of life, is important to have a good microbial composition in the gut, in order to maintain homeostasis and avoid intestinal tract disorders that may occur, especially in the early period (59). In piglets, gut microbiota colonization at birth also derives from the microbiota of the maternal intestinal tract (59). For this reason, the increase of *Lactobacillus* spp. and *Bifidobacterium* spp. in the sow's intestinal tract after dietary administration with olive oil co-products (such as olive pulp) is an important strategy to transfer these effects to the offspring (60). In laying hens, supplementation with olive oil co-products (e.g., with fermented defatted olive pomace) in early life induced an increase of the abundance of Firmicutes, Proteobacteria and Actinobacteria, also stimulating bacterial diversity. These actions can strengthen the innate immune response, help to compete with microbial pathogens and could potentially reduce the antibiotic use (61).

3.1. Olive cake

Dietary supplementation of piglets with an extract of olive cake partially restores intestinal villi height and reduces the depth of crypts by alleviating the lipopolysaccharide (LPS)-induced inflammatory response (53). Furthermore, in the same study the olive cake extract group had a reduced impairment of small intestine morphology, showing that this supplementation can contribute to the morphological and structural integrity of intestine by alleviating LPS-induced damage. Additionally, olive cake extract has been shown to increase the amount of the pro-biotic bacteria *Clostridium* and *Lactobacillus* by lowering the pH, displaying a beneficial effect on pig intestine (53) (Table 1).

3.2. Olive mill wastewater

Bonos et al. (57) recently demonstrated that silage supplemented with OMWW significantly reduced the amounts of harmful bacteria such as *Clostridium* spp., *E. coli*, and *Campylobacter jejuni*, suggesting a good correlation between gastrointestinal tract modifications and bird health. Olive mill vegetation water is also able to improve meat microbial quality (62). In poultry, dietary treatment with a phenolic extract from OMWW decreased *Campylobacter* spp. prevalence, by controlling the spread of the bacterium when the production lifespan ended, which could reduce the risk of *Campylobacter* infection in processed poultry meat (5) (Table 1).

3.3. Olive leaves

In one study, olive leaf extract dietary supplementation (100 and 200 mg/kg) was more efficient in decreasing the number of total coliform bacteria and *E. coli* compared to a control diet (44). Additionally, feeding broiler chickens with an olive leaf extract reduced the level of total coliform bacteria. This extract exerted also a beneficial effect on the microflora content of broiler chicken ileum (by increasing the concentration of *Lactobacillus*) and could be used as natural antioxidant alternatives to probiotics (44). Olive leaf extracts have also been shown to successfully inhibit *in vitro* the adhesion of *C. jejuni* and hamper its colonization (63). Olive leaf extract polyphenols exhibit antimicrobial activity against various pathogenic microorganisms, particularly *E. coli*, and the combined phenolics exert antioxidant and antimicrobial effects comparable to or better than individual phenolics (64). Additionally, in broiler chickens, dietary inclusion of different levels of an olive leaf powder improved the intestinal morphology by enhancing the length and surface of villi and the depth of crypts (65).

4. Effects on animal performance

A current issue in livestock industry is the promotion of animal performances without increasing production costs, minimizing environmental impacts and maximizing welfare (66). In the animal body, different stress conditions including farm practices, changes of environment, transportation, lairage and fasting preceding slaughter can disrupt the balance between the antioxidant defense and the production of free radicals (67). Reactive oxygen species (ROS) and nitrogen species (RNS), the most common groups of free radicals in biological systems, are usually produced at low or moderate levels in livestock animals being exposed to the stress conditions of the extensive or rangeland production (68). The excess of ROS in the body can be detrimental. Moreover, animals with faster growth rates, and thus high metabolic rates, are more prone to oxidative stress (68), causing negative effects on livestock. Accumulating evidence suggests that oxidative stress can reduce the health status, leading to an impaired immune system and lower productivity and welfare (67).

TABLE 1 Effects of olive oil co-products on microbial populations.

Co-product	Content of polyphenols (g/kg)	Dosage (g/kg)	Species	Effect	References
Olive leaf extract	97	0.5, 1.0	Ross 308 male broilers	Total aerobic bacteria →, Total coliform bacteria ↓, *Lactobacilli* bacteria ↑	(44)
OMWPE, DOC	0.0125, 0.0625	160, 330	Ross 308 female broilers	*Campylobacter* spp. ↓	(5)
Olive cake	8.6	120	Finishing pigs	Total aerobic bacteria →, *Enterobacteria* →, *Bifidobacteria* →, *Lactobacilli* →	(32)
Olive oil cake extract	-	1.0	Weaned piglets (DLL)	*Clostriudium _sensu_ scricto_* ↑, *Lactobacillus* ↑	(53)

DLL, Duroc × Landrace × Large White; DOC, dried olive cake; GA, gallic acid; OMWPE, Olive mill wastewater polyphenolic extract. ↑, increased; ↓, decreased; →, no difference.

Stress regulates the functions of the cells by inducing physiological or pathological modifications, and consequently affecting metabolism and the fate of nutrients in the body. Furthermore, oxidative stress could induce DNA, lipid and protein damage, changes that are correlated to various harmful consequences which alter animal production and performances (69). This may contribute to deterioration of the qualitative characteristics of meat products (68). Additionally, in piglets, weaning is a very stressful condition characterized by high production of free radicals, leading to all the oxidative-stress induced negative effects (70). Post-weaning stress is often linked to a decreased growth performance, as well as to an higher susceptibility to microbial infections (71, 72).

Animal performance and quality of the derived products are markedly affected in monogastrics, especially chickens (16, 28, 33, 44, 73) and pigs (43, 74, 75). On the farm, the use of antioxidants is required during animal life conditions such as reproduction and growth, in order to maintain the oxidative balance in the biological systems of both cells and tissues (68). Recent research has focused on the addition of natural antioxidants in livestock feed, in order to improve the productivity and health of animals, protecting them from the harmful effects of oxidative stress (76). More specifically, the use of phenolic compounds can reduce the oxidative stress-induced damages in animals due to the antibiotic-like action of these molecules (77). In this way, these molecules can also improve the antioxidant potential of products of animal origin, e.g., meat.

Many *in vitro* and *in vivo* animal studies have demonstrated that polyphenols present in olive oil, olive fruit and extracts (such as OMWW) exert strong biological activities, mostly-but not only exclusively-linked to their antioxidant power (78). For example, pretreatment with hydroxytyrosol protects renal cells from membrane oxidative damage induced by free radicals by improving also the morphology and biochemical functions in the proximal tubular epithelium (79). The protective action of hydroxytyrosol from EVOO has been also demonstrated against the damage induced by ochratoxins in a Madin–Darby canine kidney cell line (MDCK) and rabbit kidney cell line (RK 13) (80).

Regarding the *in vivo* effects, the use of natural plants and their extract rich in polyphenols in diet has been recently considered as a novel strategy in farm animals, in order to improve their growth and reduce mortality (81). Polyphenols can be considered as good promoters of farm animal growth by stimulating saliva, enzyme digestion, secretion of mucus and bile, and by protecting gut morphology thanks to their anti-inflammatory and anti-oxidant action (81). Moreover, the improvement of chicken growth rate is probably related to a reduction of the passage rate induced by polyphenols during digestion, which increases the digestibility of feed (38). Among the co-products of olive oil extraction, olive leaf extract supplementation in animal feed regulates the digestion and enhances digestive juices stimulating appetite and the consumption of food by the animal. This extract may additionally prevent diseases for its antibacterial and antifungal activity, and improves animal performance (73).

4.1. Olive pomace and olive cake

Herrero-Encinas et al. (28) demonstrated that a bioactive olive pomace extract added to chicken diets (750 mg/kg) improved animal growth given its anti-inflammatory activities and did not induce a negative effect on body weight (BW) and feed conversion ratio (FCR) (Table 2). Moreover, olive cake supplementation at 10% to broiler chicken diets significantly improved the FCR (33), while its inclusion up to 15% in laying hen diets increased feed intake (FI) and improved feed efficiency (82). Branciari et al. (5) confirmed these positive effects in poultry: they found that dietary supplementation with a dehydrated olive cake could increase live weights compared to the controls. Dietary olive cake (10%) with citric acid improved final BW and daily and total body weight gain (BWG) in broiler chickens (88). In rabbit diets, supplementation with olive cake at 30% and bentonite led to improvements of final BW, daily weight gain and FCR parameters (89). Olive cake pulp (up to 25%) added in growing rabbits did not induce harmful effects on their growth performance parameters (90). Furthermore, dietary supplementation with 82.5 and 165 g/kg of pâté olive cake led to greater BW in chickens compared with the control diet (34). A diet based on wet crude olive cake in Iberian pigs resulted in a better growth performance compared with dry olive pulp (91), and the addition of olive cake in finishing pig diets also increased FI and BWG (37). Similarly, Joven et al. (75) reported in pigs an higher

TABLE 2 Effects of olive co-products supplementation on animal growth performances.

Co-product	Content of polyphenols (g/kg)	Dosage (g/kg)	Species	Effect	References
Olive cake	-	100, 150	Ross 308 broilers	BWG →, FI →, FCR ↓	(82)
Olive pomace extract	20	0.75	Ross 308 broilers	ADG↑, FCR ↓, ADFI→, AID →	(28)
Olive pulp	2.41	30, 60	Ross broilers	BW →, FC →, FCR →, hock burn →, feather cleanliness ↑, foot pad dermatitis ↓	(83)
Olive leaf extract	97	0.5,1.0	Ross 308 broilers	BWG ↑, FCR ↓, FI →	(44)
Olive leaf extract	4.4	5, 10, 15	Arbor acre broilers	ADG ↑, ADFI ↑, FCR ↓	(16)
Olive cake	-	50, 100	Ross broilers	BW →, FI →, FCR →, survival rate ↑	(33)
Pâté olive cake	0.17	82.5, 165	Ross 308 broilers	LW ↑, ADG ↑, FCR ↓	(34)
Olive leaf and grape	7	2	Ross 308 broilers	ADG →, ADFI →, FCR →, EBI →	(84)
Olive pulp	-	25–50, 50–50, 50–80	Cobb 500 broilers	ADG →, ADFI →, FCR ↑	(10)
Olive pulp	7.9	50, 100	Hubbard broilers	BW →, ADG →, ADFI →, FCR →, mortality →	(85)
OMWW extract	100	0.2, 0.5	Hubbard-Sasso broilers	BW →, water consumption →	(41)
Olive cake	-	50, 100, 150	Finishing pig	BW ↑, FI ↑, ADG →, FCR →, ADE ↑	(75)
Olive leaf	25	50, 100	Growing-finishing pigs	FW↓, ADG↓, ADFI↓, F/G↑, ATTD↓	(43)
Olive cake	8.6	120	Finishing pigs	BW →, ADG →, ADFI →, FCR →, LD ↓	(32)
Olive cake	-	100	Finishing Bísaro pigs	BW →, pH →, color →, CW →	(86)
Olive cake	-	50, 100	Pietrain pigs	BW ↑, FCR ↑	(87)

ADFI, average daily feed intake; ADG, average daily gain; ADE, apparent digestible energy; AID, apparent ileal digestibility; ATTD, apparent total tract digestibility; BW, body weight; BWG, body weight gain; EBI, European broiler index; FCR, feed conversion ratio; FI, feed intake; FW, feed weight; GA, gallic acid; LD, loin depth; LW, live weight; OMWW, olive mill wastewater. ↑, increased; ↓, decreased; →, no difference.

consumption of feeds and higher growth rates after a dietary supplementation with 5% or 10% of olive cake (replacing an equivalent proportion of barley in the diet) (Table 2). In contrast, during the pig finishing period, dietary supplementation with 100 mg/kg of partially defatted olive cake had no effect on growth performances, showed only a tendency to increase FCR ($p = 0.059$) and significantly reduced loin depth (32).

4.2. Olive pulp

Dietary supplementation with dried olive pulp can be considered a good strategy for feeding slow-growing broilers, as it has no adverse effects on animal productive performance, carcass weight, yield of breast, and fatty acid composition of breast meat (10). An increase in FI has been observed when supplementing broiler chicken diets with olive pulp (37). In addition, researchers reported an increase in BW and BWG of broilers subjected to heat stress after dietary treatment with olive pulp (92).

4.3. Olive mill wastewater

A natural polyphenolic product from OMWW included in post-weaning pig feed induced higher BW and average daily gain (ADG) (70). Furthermore, this phenolic administration improved clinical performance by decreasing the frequency of post-weaning diarrhea (70). Branciari et al. (5) demonstrated that in poultry, supplementation with an OMWW polyphenolic extract could increase final BW and carcass weight compared to the control. Sabino et al. (93) did not find differences in feed conversion efficiency after dietary supplementation with OMWW in chicken diets compared to controls, but the observed morphological changes in the jejunum of the OMWW-supplemented

group suggest that this co-product could have a beneficial effect on the intestinal ecosystem.

4.4. Olive leaves

Researchers have demonstrated that olive leaves can be added to feeds without adversely affecting growth performance (94). The healthful effects of the olive leaf extract have been attributed to the known antioxidant, anti-microbial and anti-inflammatory activity (12) (Table 2). Paiva-Martins et al. (43) investigated the effect of the dietary addition of olive leaf extract on feed digestion, growth performance and meat qualitative characteristics of pigs (Table 2). Olive leaf supplementation improved growth performance, with a better feed-to-gain ratio by comparing with a conventional diet. Sarıca and Ürkmez (44) reported in broiler chickens an increase in BWG and a decrease in the FCR during the 6 weeks after supplementation with a composition of either 100 or 200 mg/kg of olive leaf extract (Table 2). In broiler chickens reared in humid and warm temperature, supplementation of drinking water with olive leaf extract at 15 mL/L (containing 66 mg/L oleuropein) improved BW, BWG, FI and the FCR (16). Furthermore, supplementation of growing rabbits with until 1.5 mL/kg of an olive leaf extract improved several performance parameters (final BW and the FCR) (95). The supplementation of an aqueous extract of olive leaf also increased ADG in growing rabbits (96).

5. Effects on meat quality

Antioxidants can contribute to animal welfare and productivity by delaying or preventing lipid oxidation through the reduction of free radical activity in meat (43). An excess of reactive oxygen species (such as ROS and RNS) reduce the quality of meat, causing many defects in flavor and taste that compromise the biological and reduce meat shelf life (67). Polyunsaturated fatty acids are highly susceptible to oxidation, and oxidation products can destroy the nutritional, chemical and sensory characteristics of meat, especially tenderness, juiciness, flavor, drip loss and shelf life (15, 43, 97). Nutritional supplementation is a novel strategy that can improve meat stability by changing the profile of fatty acids or the content of tocopherols in the muscle. Notably, the addition of anti-oxidants in animal feed is considered a useful method to increase meat stability (98). Recently, the use of natural antioxidants including polyphenols has been recommended, in order to restrict lipoperoxidation and maintain qualitative characteristics (flavor, color, tenderness) and shelf life of animal-derived products, ensuring their healthfulness for the consumers (99). Co-products deriving from olive oil could be employed as potential animal nutrients to produce high-quality meat based on their strong radical scavenger activity from its very high levels of polyphenols, including oleuropein, hydroxytyrosol and verbascoside (100). Hydroxytyrosol is able to scavenge the peroxyl radicals near the surface of the membranes, and oleuropein can interfere with their chain propagation (101). Increased dietary absorption of polyphenols can also exert a protective effect on the low-molecular-weight antioxidant tocopherol by acting as a barrier against the oxidation of lipids (68).

Oxidative stress can be evaluated with the thiobarbituric acid-reactive substance (TBARS) content, which measures lipoperoxidation due to free radical generation. Branciari et al. (34) reported the TBARS content of meat from animals fed with olive polyphenols. They found that this supplementation improved the oxidative status of the meat. Recent *in vivo* studies have reported that co-products from olive oil rich in polyphenols improve the antioxidant state and welfare of monogastric species including chickens, pigs and rabbits, as well as the quality of their meat (34). Tufarelli et al. (38) also demonstrated an improvement of antioxidant status with lower TBARS in the liver of chickens fed with a supplementation of EVOO. Additionally, a reduction in saturated fatty acids and a monosaturated fatty acids increase improves the meat chemical composition, and these effects can be induced by polyphenols from olive oil co-products (32, 75) (Table 3). The analysis of fat content in animal-based products usually consists in the evaluation of nutritional parameters including the ratio between polyunsaturated/saturated fatty acids and of n-6/n-3 fatty acids (104), which are important to ensure healthy human nutrition.

5.1. Olive pomace and olive cake

De Oliveira et al. (105) demonstrated that in chickens olive pomace can induce a modification of the lipid composition in meat by increasing the monounsaturated fatty acids content and decreasing the amount of the saturated ones. The same co-product type (from 5 to 16%) added in rabbit diets significantly increased meat monounsaturated fatty acids, with a correlated reduction in the polyunsaturated ones (100). Moreover, a reduced meat peroxidation has been observed in the olive pomace–supplemented group, in comparison to the control (100).

Dietary supplementation of a chicken diet with a high concentration of pâté led to a reduction of TBARS values in meat (34). Increasing levels of olive cake (up to 15%) led to healthy fatty acid profiles in pig fat by promoting a linear decrease in the proportion of total saturated fatty acids and an increase in the percentage of the total monounsaturated ones (75). The addition of partially defatted olive cake in pig diets did not shown a significant effect on carcass quality, microbial counts and subcutaneous fatty acids profile, but induced a lower pH and polyunsaturated fatty acid content and higher monounsaturated fatty acid concentration in the meat (32). Additionally, the inclusion of olive cake silage in the diet (up to 40%) of Apulo-Calabrese pigs promoted a higher proportion of monounsaturated fatty acid, especially oleic acid, and a lower concentration of polyunsaturated acids (102).

5.2. Olive pulp

The results reported by Papadomichelakis et al. (10) confirmed that feeding broiler chickens with dried olive pulp can increase the content of monounsaturated fatty acids in meat. The addition of this olive co-product in broiler diets also induce a significant improvement in meat color, as indicated by higher meat lightness (L*) and redness (a*) values (85). Moreover, in finishing pigs, meat yellowness decreased and meat oxidation stability tended to be improved after dried olive pulp supplementation (74).

TABLE 3 Effects of olive co-products supplementation on meat quality.

Co-product	Content of polyphenols (g/kg)	Dosage (g/kg)	Species	Effect	References
OMWW extract, DOC	0.125, 0.0625	160, 330	Ross broilers	LW ↑, CW ↑, dressing percentage →	(5)
Olive leaf and grape	7	2	Ross 308 broilers	CY →, color →, drip loss →	(84)
Olive pulp	-	25–50, 50–50, 50–80	Cobb 500 broilers	SFA ↓, MUFA ↑, MDA →, cooking loss →, shear force →, pH_{24} ↓, a* →, b* → L*↑	(10)
OMWW extract	100	0.2, 0.5	Hubbard-Sasso broilers	CAT ↑, GSH ↑, TAC ↑, protein oxidation ↓, lipid peroxidation ↓	(41)
Olive pulp	7.9	50, 100	Hubbard broilers	Carcass traits →, pH_{24} ↑, FA →, L* ↑, a* ↑, b* ↓, TBARS ↓, SFA ↓, MUFA ↑, PUFA/SFA →, Lipid hydroperoxides ↓	(85)
Pâté olive cake	0.17	82.5, 165	Ross 308 broilers	pH →, drip loss →, cooking loss →, shear force →, TBARS ↓, DPPH ↑	(34)
Olive pulp	-	50	Finishing pigs	CW →, pH →, cooking loss →, b* ↓, shear force →	(74)
Olive leaf	25	50, 100	growing-finishing pigs	α-tocopherol ↑, pH →, color →, cooking loss →, drip loss ↓	(43)
Olive cake	-	50, 100, 150	Finishing pigs	CW ↑, fat depth ↓, SFA ↓, MUFA ↑, PUFA →	(75)
Olive cake	-	200, 400	pigs	MUFA ↑, OA ↑, PUFA ↓, LA ↓	(102)
Olive leaf	67	25, 50	Finishing pigs	Improvement of tocopherol content	(103)
Olive pulp	-	50	Finishing pigs	CW →, pH →, cooking loss →, b* ↓, shear force →	(74)

a*, redness; ADE, apparent digestible energy; b*, yellowness; CAT, catalase; CW, carcass weight; CY, carcass yield; DOC, dried olive cake; DPPH, 2,2-diphenyl-1-picrylhydrazyl; FA, fatty acid; GSH, glutathione; L*, lightness; LA, linoleic acid; LW, live weight; MDA, malondialdehyde; MUFA, monounsaturated fatty acids; OA, oleic acid; OMWW, olive mill wastewater; PUFA, polyunsaturated fatty acids; SFA, saturated fatty acids; TAC, total antioxidant capacity; TBARS, thiobarbituric acid reactive substances. ↑, increased; ↓, decreased; →, no difference.

5.3. Olive mill wastewater

Olive mill wastewater supplementation in chicken feed can induce a decrease in the oxidation of lipids and proteins in meat without influencing its color stability. Moreover, piglets fed with an OMWW polyphenolic co-product showed lesser damages in proteins and lipids related to oxidative stress, indicated by a decrease in TBARS and protein carbonyl contents (106). The addition of a polyphenolic powder from OMWW to chicken diets reduced oxidative stress–induced damage (41). Gerasopoulos et al. (14) indicated that in piglets, feed containing polyphenolic additives from processed OMWW improved the lipid ratio and the quality of the meat. OMWW supplementation into rabbit diets also decreased *Pseudomonas* spp. growth in the meat (107).

5.4. Olive leaves

Olive leaves contain a large amount of polyphenols, which are strong natural antioxidants potentially able to decrease the excess of free radicals and harmful DNA modifications (41). Olive leaf extract could be used to produce high-quality meat due its very high level of polyphenols, including oleuropein (108). Supplementation of pig diets with 5% or 10% olive leaf extract significantly increased α-tocopherol content in *Longissimus dorsi* muscle and backfat compared with a control diet (43) (Table 3). Even if added at low dosages, olive leaf extract is a beneficial source of biologically-active molecules and could increase tocopherols in meat (43). In broiler chickens, dietary supplementation with olive leaf and a grape co-product (2 g/kg) changed breast color, by increasing its yellowness (b*) values and color

intensity (84), compared with protected sodium butyrate. The same dietary supplementation seemed to decrease drip loss, a change related to enhanced breast meat quality (84) (Table 3). The combination of oleuropein, magnesium, betaine and vitamin E in pig diet could improve the oxidative state and maintain the stability of lipids in meat (67).

6. Effects on gene transcription

Dietary nutrients may modify gene and protein expression and metabolism directly or indirectly (109, 110). Diet is one of the external factors that can influence directly the expression of genes through the biologically-active nutrients contained, which interact with transcriptional factors to positively or negatively affect signal transduction pathways (111–113). Moreover, bioactive molecules in food and feed can have an impact on epigenetics, e.g., methylation of DNA and modifications occurring in histones (111). Transcription factors are found in organs which are metabolically active, like liver, adipose tissue and intestines. Their function consists in acting as molecular sensors through a modification of gene transcriptions as a response to changes in nutrient composition (109). Polyphenols can affect various transcription factors and gene expression (114).

Due to their antioxidant properties, polyphenols can inhibit the negative consequences of excessive ROS production. Two essential regulatory mechanisms inside the cells are the gene expression regulation and the adaptive homeostasis, both of which are redox-induced (115). When the oxidation rate is high, a stress response started inside the cell to control free radicals excess and support redox homeostasis (115). Specifically, stress situations stimulate the translocation of transcription cellular factors [like nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB)] inside the nucleus; they then bind to specific DNA sites to exert a protective function, but can often exert the opposite effect (115). Oxidative stress induces different cellular processes linked to inflammation, proliferation and apoptosis (116). Polyphenols act by reducing ROS and, consequently, inhibit NF-κB, the most important regulator of the transcription of inflammatory markers (interleukins, tumor-necrosis factor etc). Cappelli et al. (116) showed for the first time the downregulation of the tumor necrosis factor-alpha (*TNFA*), advanced glycosylation end-product specific receptor (*AGER*) and BCL2-associated X apoptosis regulator (*BAX*) genes in rabbit liver after dietary supplementation with OMWW polyphenols. These effects suggest a possible inhibition from polyphenols of the effect of oxidative stress on NF-κB. Dietary OMWW also decreased *BAX* expression in rabbit's ovary system, which highlighted the beneficial action of polyphenols on reproduction linked to inhibition of apoptosis (117).

Additionally, Sabino et al. (93) demonstrated that the incorporation of OMWW into broiler chicken diets modulated, in their jejunum, the expression of innate immune response genes against viral infections [recombinant inhibitory subunit of NF kappa B Epsilon (*IKBE*), Toll-like receptor 3 (*TLR3*), eukaryotic translation initiation factor 2 alpha kinase 2 (*EIF2AK2*), oligoadenylate synthetase like (*OASL*), myxovirus resistance gene (*MX*) and radical S-adenosyl methionine domain containing 2 (*RSAD2*)]. The same study supported that an OMWW-supplemented diet could regulate sterol biosynthesis and lipid metabolism by downregulating farnesyl diphosphate synthase (*FDPS*), matrix metalloproteinase 1 (*MMP1*) and fatty acid binding protein 3 (*FABP3*) expression in chicken small intestine (93). This nutritional strategy reduced fatty acid transportation as well as body fat accumulation in chickens (93). A few genes involved in lipid metabolism [acetyl CoA carboxylase (*ACC*) and fatty acid synthase (*FAS*)] had an upregulated expression in the serum of laying hens fed with a diet supplemented with olive cake (118). Furthermore, olive oil increased the expression of many genes encoding for heat shock proteins in broiler chickens, improving their tolerance to heat stress (119).

7. Conclusion

The supplementation of co-products from olive oil extraction–olive pomace, olive cake, OMWW, olive pulp and olive leaf–in monogastric animal nutrition is advisable, as these co-products are found to be harmless, sustainable and are sources of several valuable bioactive compounds. In particular, olive co-products retain the majority of EVOO polyphenols, which are the secondary metabolites most studied and recovered, given their multifunctional effects (antioxidant, antimicrobial, and anti-inflammatory) widely demonstrated in both humans and animals. Additionally, the use of these co-products in animal diets represents an innovative and efficient strategy which contributes to the circular economy, ensuring economic and environmental improvements. The suitability of co-products depends on their specific chemical features. Co-products such as OMWW can be added more easily in monogastric diets compared to others (such as olive pomace, olive pulp and olive leaf), which contain a high level of structural carbohydrates and decrease digestibility and palatability in poultry. Furthermore, the beneficial functions of the phenolic compounds contained in olive co-products and the derived extracts are related to many factors including type, dosage, absorption and metabolism. This review suggests that dietary supplementation with olive oil co-products may improve animal health, productive performances and meat quality characteristics, reduce the adverse effect of lipid peroxidation and improve the antioxidant status.

Author contributions

FF: Writing – original draft. JT: Writing – original draft. KC: Writing – review & editing. MT-M: Writing – review & editing.

Funding

This study was partially funded by a doctoral grant (Doctorate Program in Health and Experimental Veterinary Sciences, University of Perugia, XXXVII cycle) for FF.

Acknowledgments

The authors thank Samanta Mecocci for her helpful comments and assistance.

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.

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.

Publisher's note

References

1. Home. *International Olive Council*. (2023). Available at: https://www.internationaloliveoil.org/ (Accessed July 30, 2023).

2. Abu-Rumman G. Effect of olive mill solid waste on soil physical properties. *Int J Soil Sci*. (2016) 11:94–101. doi: 10.3923/ijss.2016.94.101

3. Fernández-Lobato L, López-Sánchez Y, Blejman G, Jurado F, Moyano-Fuentes J, Vera D. Life cycle assessment of the Spanish virgin olive oil production: a case study for Andalusian region. *J Clean Prod*. (2021) 290:125677. doi: 10.1016/j.jclepro.2020.125677

4. Restuccia D, Prencipe SA, Ruggeri M, Spizzirri UG. Sustainability assessment of different extra virgin olive oil extraction methods through a life cycle thinking approach: challenges and opportunities in the Elaio-technical sector. *Sustainability*. (2022) 14:15674. doi: 10.3390/su142315674

5. Branciari R, Ranucci D, Ortenzi R, Roila R, Trabalza-Marinucci M, Servili M, et al. Dietary administration of Olive Mill Waste Water extract reduces Campylobacter spp. *Prevalence Broiler Chickens Sustain.* (2016) 8:837. doi: 10.3390/su8090837

6. Alimenti C, Lianza M, Antognoni F, Giusti L, Bistoni O, Liotta L, et al. Characterization and biological activities of in vitro digested olive pomace polyphenols evaluated on ex vivo human immune blood cells. *Molecules*. (2023) 28:2122. doi: 10.3390/molecules28052122

7. Gullón P, Gullón B, Astray G, Carpena M, Fraga-Corral M, Prieto MA, et al. Valorization of by-products from olive oil industry and added-value applications for innovative functional foods. *Food Res Int*. (2020) 137:109683. doi: 10.1016/j.foodres.2020.109683

8. Rivero-Pino F, Millan-Linares MC, Villanueva-Lazo A, Fernandez-Prior Á, Montserrat-de-la-Paz S. In vivo evidences of the health-promoting properties of bioactive compounds obtained from olive by-products and their use as food ingredient. *Crit Rev Food Sci Nutr*. (2023) 2023:1–13. doi: 10.1080/10408398.2023.2203229

9. Otero P, Garcia-Oliveira P, Carpena M, Barral-Martinez M, Chamorro F, Echave J, et al. Applications of by-products from the olive oil processing: revalorization strategies based on target molecules and green extraction technologies. *Trends Food Sci Technol*. (2021) 116:1084–104. doi: 10.1016/j.tifs.2021.09.007

10. Papadomichelakis G, Pappas AC, Tsiplakou E, Symeon GK, Sotirakoglou K, Mpekelis V, et al. Effects of dietary dried olive pulp inclusion on growth performance and meat quality of broiler chickens. *Livest Sci*. (2019) 221:115–22. doi: 10.1016/j.livsci.2019.01.023

11. Silvan JM, Martinez-Rodriguez AJ. Food by-products as natural source of bioactive compounds against Campylobacter In: P Ferranti, EM Berry and JR Anderson, editors. *Encyclopedia of food security and sustainability*. Oxford: Elsevier (2019). 336–50.

12. Abdel-Moneim A-ME, Shehata AM, Alzahrani SO, Shafi ME, Mesalam NM, Taha AE, et al. The role of polyphenols in poultry nutrition. *J Anim Physiol Anim Nutr*. (2020) 104:1851–66. doi: 10.1111/jpn.13455

13. Varricchio E, Coccia E, Orso G, Lombardi V, Imperatore R, Vito P, et al. Influence of polyphenols from olive mill wastewater on the gastrointestinal tract, alveolar macrophages and blood leukocytes of pigs. *Ital J Anim Sci*. (2019) 18:574–86. doi: 10.1080/1828051X.2018.1548911

14. Gerasopoulos K, Stagos D, Krouezas A, Karaveli C, Barda C, Gkika H, et al. Assessment of fatty acid allocation in plasma and tissues in piglets, using feed supplemented with byproducts from processed olive mill wastewater. *In Vivo*. (2016) 30:291–301.

15. Savournin C, Baghdikian B, Elias R, Dargouth-Kesraoui F, Boukef K, Balansard G. Rapid high-performance liquid chromatography analysis for the quantitative determination of oleuropein in *Olea europaea* leaves. *J Agric Food Chem*. (2001) 49:618–21. doi: 10.1021/jf000596+

16. Oke OE, Emeshili UK, Iyasere OS, Abioja MO, Daramola JO, Ladokun AO, et al. Physiological responses and performance of broiler chickens offered olive leaf extract under a hot humid tropical climate. *J Appl Poult Res*. (2017) 26:376–82. doi: 10.3382/japr/pfx005

17. Leouifoudi I, Harnafi H, Zyad A. Olive mill waste extracts: polyphenols content, antioxidant, and antimicrobial activities. *Adv Pharmacol Sci*. (2015) 2015:714138:1–11. doi: 10.1155/2015/714138

18. Al-Qodah Z, Al-Zoubi H, Hudaib B, Omar W, Soleimani M, Abu-Romman S, et al. Sustainable vs. conventional approach for olive oil wastewater management: a review of the state of the art. *Water*. (2022) 14:1695. doi: 10.3390/w14111695

19. Leo M, Muccillo L, Dugo L, Bernini R, Santi L, Sabatino L. Polyphenols extracts from oil production waste products (OPWPs) reduce cell viability and exert anti-inflammatory activity via PPARγ induction in colorectal Cancer cells. *Antioxidants (Basel)*. (2022) 11:624. doi: 10.3390/antiox11040624

20. Vastolo A, Calabrò S, Liotta L, Musco N, Di Rosa AR, Cutrignelli MI, et al. In vitro fermentation and chemical characteristics of Mediterranean by-products for swine nutrition. *Animals*. (2019) 9:556. doi: 10.3390/ani9080556

21. Mahfuz S, Shang Q, Piao X. Phenolic compounds as natural feed additives in poultry and swine diets: a review. *J Anim Sci Biotechnol*. (2021) 12:48. doi: 10.1186/s40104-021-00565-3

22. Servili M, Esposto S, Fabiani R, Urbani S, Taticchi A, Mariucci F, et al. Phenolic compounds in olive oil: antioxidant, health and organoleptic activities according to their chemical structure. *Inflammopharmacology*. (2009) 17:76–84. doi: 10.1007/s10787-008-8014-y

23. Fernando U, Biswas D, Allan B, Willson P, Potter AA. Influence of *Campylobacter jejuni* fliA, rpoN and flgK genes on colonization of the chicken gut. *Int J Food Microbiol*. (2007) 118:194–200. doi: 10.1016/j.ijfoodmicro.2007.07.038

24. European Union. *The Community Summary Report on Trends and Sources of Zoonoses and Zoonotic Agents in the European Union in 2007, EFSA*. (2009). Available at: https://www.efsa.europa.eu/en/efsajournal/pub/rn-223 (Accessed April 13, 2023).

25. Lipiński K, Mazur M, Antoszkiewicz Z, Purwin C. Polyphenols in Monogastric nutrition–a review. *Ann Anim Sci*. (2017) 17:41–58. doi: 10.1515/aoas-2016-0042

26. Starčević K, Krstulović L, Brozić D, Maurić M, Stojević Z, Mikulec Ž, et al. Production performance, meat composition and oxidative susceptibility in broiler chicken fed with different phenolic compounds. *J Sci Food Agric*. (2015) 95:1172–8. doi: 10.1002/jsfa.6805

27. Medouni-Haroune L, Zaidi F, Medouni-Adrar S, Kecha M. Olive pomace: from an olive mill waste to a resource, an overview of the newtreatments. *JCR*. (2018) 56:1–6. doi: 10.22159/jcr.2018v5i5.28840

28. Herrero-Encinas J, Blanch M, Pastor JJ, Mereu A, Ipharraguerre IR, Menoyo D. Effects of a bioactive olive pomace extract from Olea europaea on growth performance, gut function, and intestinal microbiota in broiler chickens. *Poult Sci*. (2020) 99:2–10. doi: 10.3382/ps/pez467

29. Shahidi F, Pinaffi-Langley ACC, Fuentes J, Speisky H, de Camargo AC. Vitamin E as an essential micronutrient for human health: common, novel, and unexplored dietary sources. *Free Radic Biol Med*. (2021) 176:312–21. doi: 10.1016/j.freeradbiomed.2021.09.025

30. Molina-Alcaide E, Yáñez-Ruiz DR. Potential use of olive by-products in ruminant feeding: a review. *Anim Feed Sci Technol*. (2008) 147:247–64. doi: 10.1016/j.anifeedsci.2007.09.021

31. De Blas JC, Rodriguez CA, Bacha F, Fernandez R, Abad-Guamán R. Nutritive value of co-products derived from olivecake in rabbit feeding. *World Rabbit Sci*. (2015) 23:255–62. doi: 10.4995/wrs.2015.4036

32. Ferrer P, Calvet S, García-Rebollar P, De Blas C, Jiménez-Belenguer AI, Hernández P, et al. Partially defatted olive cake in finishing pig diets: implications on performance, faecal microbiota, carcass quality, slurry composition and gas emission. *Animal*. (2020) 14:426–34. doi: 10.1017/S1751731119002040

33. Al-Harthi MA. The efficacy of using olive cake as a by-product in broiler feeding with or without yeast. *Ital J Anim Sci*. (2016) 15:512–20. doi: 10.1080/1828051X.2016.1194173

34. Branciari R, Galarini R, Giusepponi D, Trabalza-Marinucci M, Forte C, Roila R, et al. Oxidative status and presence of bioactive compounds in meat from chickens fed polyphenols extracted from olive oil industry waste. *Sustainability*. (2017) 9:1566. doi: 10.3390/su9091566

35. Ibrahim NS, Sabic EM, Abu-Taleb AM. Effect of inclusion irradiated olive pulp in laying quail diets on biological performance. *J Radiat Res Appl Sci*. (2018) 11:340–6. doi: 10.1016/j.jrras.2018.06.004

36. Sayehban P, Seidavi A, Dadashbeiki M, Ghorbani A, Araújo W, Albino L. Effects of different dietary levels of two types of olive pulp and exogenous enzyme supplementation on the gastrointestinal tract size, immunology and hematology of broilers. *Rev Bras Cienc Avic*. (2015) 17:73–85. doi: 10.1590/1516-635073-086

37. Sayehban P, Seidavi A, Dadashbeiki M, Ghorbani A, De Araújo WAG, Durazzo A, et al. Olive pulp and exogenous enzymes feed supplementation effect on the carcass and offal in broilers: a preliminary study. *Agriculture*. (2020) 10:359. doi: 10.3390/agriculture10080359

38. Tufarelli V, Laudadio V, Casalino E. An extra-virgin olive oil rich in polyphenolic compounds has antioxidant effects in meat-type broiler chickens. *Environ Sci Pollut Res Int*. (2016) 23:6197–204. doi: 10.1007/s11356-015-5852-1

39. La Scalia G, Micale R, Cannizzaro L, Marra FP. A sustainable phenolic compound extraction system from olive oil mill wastewater. *J Clean Prod*. (2017) 142:3782–8. doi: 10.1016/j.jclepro.2016.10.086

40. Zahra El Hassani F, El Karkouri A, Errachidi F, Merzouki M, Benlemlih M. The impact of olive mill wastewater spreading on soil and plant in arid and semi-arid areas. *Environ Nanotechnol Monit Manage*. (2023) 20:100798. doi: 10.1016/j.enmm.2023.100798

41. Papadopoulou A, Petrotos K, Stagos D, Gerasopoulos K, Maimaris A, Makris H, et al. Enhancement of antioxidant mechanisms and reduction of oxidative stress in chickens after the administration of drinking water enriched with polyphenolic powder from olive mill waste waters. *Oxidative Med Cell Longev*. (2017) 2017:8273160–10. doi: 10.1155/2017/8273160

42. Sudjana AN, D'Orazio C, Ryan V, Rasool N, Ng J, Islam N, et al. Antimicrobial activity of commercial *Olea europaea* (olive) leaf extract. *Int J Antimicrob Agents*. (2009) 33:461–3. doi: 10.1016/j.ijantimicag.2008.10.026

43. Paiva-Martins F, Barbosa S, Pinheiro V, Mourão JL, Outor-Monteiro D. The effect of olive leaves supplementation on the feed digestibility, growth performances of pigs and quality of pork meat. *Meat Sci*. (2009) 82:438–43. doi: 10.1016/j.meatsci.2009.02.014

44. Sarıca Ş, Ürkmez D. *The use of grape seed-, olive leaf-and pomegranate peel-extracts as alternative natural antimicrobial feed additives in broiler diets*. *80*. Stuttgart: Verlag Eugen Ulmer, pp. 1612–9199. (2016).

45. Benavente-García O, Castillo J, Lorente J, Ortuño A, del Rio JA. Antioxidant activity of phenolics extracted from *Olea europaea* L. leaves. *Food Chem*. (2000) 68:457–62. doi: 10.1016/S0308-8146(99)00221-6

46. Sánchez-Gutiérrez M, Bascón-Villegas I, Rodríguez A, Pérez-Rodríguez F, Fernández-Prior Á, Rosal A, et al. Olive leaves through the evaluation of their extracts: antioxidant and antimicrobial activity. *Foods*. (2021) 10:966. doi: 10.3390/foods10050966

47. Brenes M, García A, De Los SB, Medina E, Romero C, De Castro A, et al. Olive glutaraldehyde-like compounds against plant pathogenic bacteria and fungi. *Food Chem*. (2011) 125:1262–6. doi: 10.1016/j.foodchem.2010.10.055

48. Medina E, Brenes M, Romero C, García A, De CA. Main antimicrobial compounds in Table olives. *J Agric Food Chem*. (2007) 55:9817–23. doi: 10.1021/jf0719757

49. Pereira A, Ferreira I, Marcelino F, Valentão P, Andrade P, Seabra R, et al. Phenolic compounds and antimicrobial activity of olive (*Olea europaea* L. cv. Cobrançosa) leaves. *Molecules*. (2007) 12:1153–62. doi: 10.3390/12051153

50. Özkaya FD, Özkaya MT. Oleuropein using as an additive for feed and products used for humans. *J Food Process Technol*. (2011) 2:113. doi: 10.4172/2157-7110.1000113

51. Yakhlef W, Arhab R, Romero C, Brenes M, De Castro A, Medina E. Phenolic composition and antimicrobial activity of Algerian olive products and by-products. *LWT*. (2018) 93:323–8. doi: 10.1016/j.lwt.2018.03.044

52. Selma MV, Espín JC, Tomás-Barberán FA. Interaction between Phenolics and gut microbiota: role in human health. *J Agric Food Chem*. (2009) 57:6485–501. doi: 10.1021/jf902107d

53. Zhang Y, Deng Z-X, He M-L, Pastor JJ, Tedo G, Liu J-X, et al. Olive oil cake extract stabilizes the physiological condition of lipopolysaccharide-challenged piglets by reducing oxidative stress and inflammatory responses and modulating the ileal microbiome. *Food Funct*. (2021) 12:10171–83. doi: 10.1039/D0FO03012K

54. Scott MB, Styring AK, McCullagh JSO. Polyphenols: bioavailability, microbiome interactions and cellular effects on health in humans and animals. *Pathogens*. (2022) 11:770. doi: 10.3390/pathogens11070770

55. Shehata AA, Yalçın S, Latorre JD, Basiouni S, Attia YA, Abd El-Wahab A, et al. Probiotics, prebiotics, and phytogenic substances for optimizing gut health in poultry. *Microorganisms*. (2022) 10:395. doi: 10.3390/microorganisms10020395

56. Jiao X, Wang Y, Lin Y, Lang Y, Li E, Zhang X, et al. Blueberry polyphenols extract as a potential prebiotic with anti-obesity effects on C57BL/6 J mice by modulating the gut microbiota. *J Nutr Biochem*. (2019) 64:88–100. doi: 10.1016/j.jnutbio.2018.07.008

57. Bonos E, Skoufos I, Petrotos K, Giavasis I, Mitsagga C, Fotou K, et al. Innovative use of olive, winery and cheese waste by-products as functional ingredients in broiler nutrition. *Vet Sci*. (2022) 9:290. doi: 10.3390/vetsci9060290

58. Almuhayawi M, Alruhaili M, Gattan H, Alharbi M, Nagshabandi M, Almehayawi M, et al. Evaluation of antimicrobial effect of olive leaves powder and its role in improving the broiler productivity, carcass traits, blood metabolites, and caecal microbiota. *Poult Sci*. (2023) 102:103054. doi: 10.1016/j.psj.2023.103054

59. Kubasova T, Davidova-Gerzova L, Merlot E, Medvecky M, Polansky O, Gardan-Salmon D, et al. Housing systems influence gut microbiota composition of sows but not of their piglets. *PLoS One*. (2017) 12:e0170051. doi: 10.1371/journal.pone.0170051

60. Sánchez CJ, Barrero-Domínguez B, Martínez-Miró S, Madrid J, Baños A, Aguinaga MA, et al. Use of olive pulp for gestating Iberian sow feeding: influence on performance, health status indicators, and fecal microbiota. *Animals*. (2022) 12:3178. doi: 10.3390/ani12223178

61. Rebollada-Merino A, Bárcena C, Ugarte-Ruiz M, Porras N, Mayoral-Alegre FJ, Tomé-Sánchez I, et al. Effects on intestinal mucosal morphology, productive parameters and microbiota composition after supplementation with fermented defatted Alperujo (FDA) in laying hens. *Antibiotics*. (2019) 8:215. doi: 10.3390/antibiotics8040215

62. Vastolo A, Calabrò S, Cutrignelli MI. A review on the use of agro-industrial CO-products in animals' diets. *Ital J Anim Sci*. (2022) 21:577–94. doi: 10.1080/1828051X.2022.2039562

63. Šikić Pogačar M, Klančnik A, Bucar F, Langerholc T, Smole MS. Anti-adhesion activity of thyme (*Thymus vulgaris* L.) extract, thyme post-distillation waste, and olive (*Olea europea* L.) leaf extract against *Campylobacter jejuni* on polystyrene and intestine epithelial cells. *J Sci Food Agric*. (2016) 96:2723–30. doi: 10.1002/jsfa.7391

64. Lee O-H, Lee B-Y. Antioxidant and antimicrobial activities of individual and combined phenolics in *Olea europaea* leaf extract. *Bioresour Technol*. (2010) 101:3751–4. doi: 10.1016/j.biortech.2009.12.052

65. Sabetan Shiraze AA, Hassanabadi A, Agah MJ, Nasiri MH. Eeffect of dietary inclusion of olive leaf powder on performance, small intestine morphology and nutrient digestibility in broiler chickens. *Anim Prod*. (2017) 19:371–87. doi: 10.22059/jap.2017.62053

66. Hume DA, Whitelaw CBA, Archibald AL. The future of animal production: improving productivity and sustainability. *J Agric Sci*. (2011) 149:9–16. doi: 10.1017/S0021859610001188

67. Rey AI, De Cara A, Segura JF, Martí P, Hechavarría T, Calvo L. Dietary oleuropein extract supplementation and its combination with α-tocopheryl acetate and selenium modifies the free fatty acid profile of pork and improves its stability. *J Sci Food Agric*. (2021) 101:2337–44. doi: 10.1002/jsfa.10855

68. Ponnampalam EN, Kiani A, Santhiravel S, Holman BWB, Lauridsen C, Dunshea FR. The importance of dietary antioxidants on oxidative stress, meat and Milk production, and their preservative aspects in farm animals: antioxidant action, animal health, and product quality–invited review. *Animals*. (2022) 12:3279. doi: 10.3390/ani12233279

69. Xing T, Gao F, Tume RK, Zhou G, Xu X. Stress effects on meat quality: a mechanistic perspective. *Compr Rev Food Sci Food Saf*. (2019) 18:380–401. doi: 10.1111/1541-4337.12417

70. Papakonstantinou GI, Meletis E, Petrotos K, Kostoulas P, Tsekouras N, Kantere MC, et al. Effects of a natural polyphenolic product from olive mill wastewater on oxidative stress and post-weaning diarrhea in piglets. *Agriculture*. (2023) 13:1356. doi: 10.3390/agriculture13071356

71. Kluess J, Schoenhusen U, Souffrant WB, Jones PH, Miller BG. Impact of diet composition on ileal digestibility and small intestinal morphology in early-weaned pigs fitted with a T-cannula. *Animal*. (2010) 4:586–94. doi: 10.1017/S1751731109991455

72. Heo JM, Opapeju FO, Pluske JR, Kim JC, Hampson DJ, Nyachoti CM. Gastrointestinal health and function in weaned pigs: a review of feeding strategies to control post-weaning diarrhoea without using in-feed antimicrobial compounds. *J Anim Physiol Anim Nutr*. (2013) 97:207–37. doi: 10.1111/j.1439-0396.2012.01284.x

73. Agah M, Mirakzehi MT, Saleh H. Effects of olive leaf extract (*Olea europea* l.) on growth performance, blood metabolites and antioxidant activities in broiler chickens under heat stress. *J Anim Plant Sci*. (2019) 29:657–66.

74. Tsala A, Mpekelis V, Karvelis G, Tsikakis P, Goliomytis M, Simitzis P. Effects of dried olive pulp dietary supplementation on quality characteristics and antioxidant capacity of pig meat. *Foods*. (2020) 9:81. doi: 10.3390/foods9010081

75. Joven M, Pintos E, Latorre MA, Suárez-Belloch J, Guada JA, Fondevila M. Effect of replacing barley by increasing levels of olive cake in the diet of finishing pigs: growth performances, digestibility, carcass, meat and fat quality. *Anim Feed Sci Technol*. (2014) 197:185–93. doi: 10.1016/j.anifeedsci.2014.08.007

76. Skaperda Z-V, Veskoukis A, Kouretas D. Farm animal welfare, productivity and meat quality: interrelation with redox status regulation and antioxidant supplementation as a nutritional intervention (review). *World Acad Sci J*. (2019) 1:177–83. doi: 10.3892/wasj.2019.19

77. Scicutella F, Mannelli F, Daghio M, Viti C, Buccioni A. Polyphenols and organic acids as alternatives to antimicrobials in poultry rearing: a review. *Antibiotics*. (2021) 10:1010. doi: 10.3390/antibiotics10081010

78. Cardinali A, Pati S, Minervini F, D'Antuono I, Linsalata V, Lattanzio V. Verbascoside, isoverbascoside, and their derivatives recovered from olive mill wastewater as possible food antioxidants. *J Agric Food Chem*. (2012) 60:1822–9. doi: 10.1021/jf204001p

79. Loru D, Incani A, Deiana M, Corona G, Atzeri A, Melis MP, et al. Protective effect of hydroxytyrosol and tyrosol against oxidative stress in kidney cells. *Toxicol Ind Health*. (2009) 25:301–10. doi: 10.1177/0748233709103028

80. Crupi R, Palma E, Siracusa R, Fusco R, Gugliandolo E, Cordaro M, et al. Protective effect of Hydroxytyrosol against oxidative stress induced by the Ochratoxin in kidney cells: in vitro and in vivo study. *Front Vet Sci*. (2020) 7:136. doi: 10.3389/fvets.2020.00136

81. Valenzuela-Grijalva NV, Pinelli-Saavedra A, Muhlia-Almazan A, Domínguez-Díaz D, González-Ríos H. Dietary inclusion effects of phytochemicals as growth promoters in animal production. *J Anim Sci Technol*. (2017) 59:8. doi: 10.1186/s40781-017-0133-9

82. Al-Harthi MA, Attia YA, El-Shafey AS, Elgandy MF. Impact of phytase on improving the utilisation of pelleted broiler diets containing olive by-products. *Ital J Anim Sci*. (2020) 19:310–8. doi: 10.1080/1828051X.2020.1740896

83. Dedousi A, Kotzamanidis C, Kritsa M-Z, Tsoureki A, Andreadelli A, Patsios SI, et al. Growth performance, gut health, welfare and qualitative behavior characteristics of broilers fed diets supplemented with dried common (*Olea europaea*) olive pulp. *Sustainability*. (2023) 15:501. doi: 10.3390/su15010501

84. De-Cara A, Saldaña B, Vázquez P, Rey AI. Dietary protected sodium butyrate and/or olive leaf and grape-based by-product supplementation modifies productive performance, antioxidant status and meat quality in broilers. *Antioxidants*. (2023) 12:201. doi: 10.3390/antiox12010201

85. Tufarelli V, Passantino L, Zupa R, Crupi P, Laudadio V. Suitability of dried olive pulp in slow-growing broilers: performance, meat quality, oxidation products, and intestinal mucosa features. *Poult Sci*. (2022) 101:102230. doi: 10.1016/j.psj.2022.102230

86. Leite A, Vasconcelos L, Ferreira I, Domínguez R, Pateiro M, Rodrigues S, et al. Did the addition of olive cakes obtained by different methods of oil extraction in the finishing diet of Bísaro pigs affect the volatile compounds and sensory characteristics of dry-cured loin and Cachaço? *Foods*. (2023) 12:2499. doi: 10.3390/foods12132499

87. Liotta L, Chiofalo V, Lo Presti V, Chiofalo B. In vivo performances, carcass traits, and meat quality of pigs fed olive cake processing waste. *Animals*. (2019) 9:1155. doi: 10.3390/ani9121155

88. Azazi I, Gadelrab S, Elkomy H, Ahmed A. Growth performance and feed utilization of growing rabbits fed diets containing olive cake meal supplemented with or without citric acid. *Egypt J Rabbit Sci*. (2018) 28:241–62. doi: 10.21608/ejrs.2018.44114

89. Salama WA, Basyony M, Suliman M, Matari R, Hassanein H. Effect of feeding olive cake supplemented with or without bentonite on performance of growing rabbits. *Egypt J Rabbit Sci*. (2016) 26:211–30. doi: 10.21608/ejrs.2016.42076

90. Bakr EOA, Shetaewi MM, El-Desoky AEIMI. Effect of olive cake pulp as a partial ora a complete substitute of wheat bran in growing rabbit's diet on growth performance, carcass traits and blood constituents under North Sinai conditions. *J Anim Polut Prod Mansoura Univ*. (2019) 10:83–8. doi: 10.21608/jappmu.2019.40530

91. García-Casco JM, Muñoz M, Martínez-Torres JM, García-López A, Fernández-Barroso MA, González-Sánchez E. Alternative feeding in Iberian pigs during growth period: incorporation of olive cake in a dry or wet (silage) form. *Agric Conspec Sci*. (2017) 82:147–50.

92. Dedousi A, Kritsa M-Z, Sossidou EN. Thermal comfort, growth performance and welfare of olive pulp fed broilers during hot season. *Sustainability*. (2023) 15:10932. doi: 10.3390/su151410932

93. Sabino M, Cappelli K, Capomaccio S, Pascucci L, Biasato I, Verini-Supplizi A, et al. Dietary supplementation with olive mill wastewaters induces modifications on chicken jejunum epithelial cell transcriptome and modulates jejunum morphology. *BMC Genomics*. (2018) 19:576. doi: 10.1186/s12864-018-4962-9

94. Yeñicerī M, Fīlīk AG, Fīlīk G. The effect of some selected fruit wastes for poultry feed on growth performance of broilers. *Palandöken J Anim Sci Technol Econ*. (2022) 1:33–41.

95. Younan GE, Mohamed MS, Morsy WA. Effect of dietary supplementation of olive leaf extract on productive performance, blood parameters and carcass traits of growing rabbits. *Egypt J Nutr Feeds*. (2019) 22:173–82. doi: 10.21608/ejnf.2019.103477

96. Héla Y, Aymen H, Jihen J, Khalil A, Mohamed K, Jamel R, et al. Use of olive leaf aqueous extract (*Olea europaea*) in growing rabbits' diets. *J N Sci*. (2019) 68:4209–13.

97. Hedji C, Houndonougbo F, Tougan UP, Marcel H, Fiogbe E. Technological, sensorial and nutritional meat quality traits from pig fed with conventional and unconventional diets. *Food Nutr Sci*. (2015) 6:1514–21. doi: 10.4236/fns.2015.616156

98. Maraschiello C, Sárraga C, García Regueiro JA. Glutathione peroxidase activity, TBARS, and alpha-tocopherol in meat from chickens fed different diets. *J Agric Food Chem*. (1999) 47:867–72. doi: 10.1021/jf980824o

99. North MK, Dalle Zotte A, Hoffman LC. The use of dietary flavonoids in meat production: a review. *Anim Feed Sci Technol*. (2019) 257:114291. doi: 10.1016/j.anifeedsci.2019.114291

100. Dal Bosco A, Mourvaki E, Cardinali R, Servili M, Sebastiani B, Ruggeri S, et al. Effect of dietary supplementation with olive pomaces on the performance and meat quality of growing rabbits. *Meat Sci*. (2012) 92:783–8. doi: 10.1016/j.meatsci.2012.07.001

101. Saija A, Trombetta D, Tomaino A, Lo Cascio R, Princi P, Uccella N, et al. `in vitro' evaluation of the antioxidant activity and biomembrane interaction of the plant phenols oleuropein and hydroxytyrosol. *Int J Pharm*. (1998) 166:123–33. doi: 10.1016/S0378-5173(98)00018-0

102. Caparra P, Chies L, Scerra M, Foti F, Bognanno M, Cilione C, et al. Effect of dietary ensiled olive cake supplementation on performance and meat quality of Apulo-Calabrese pigs. *Animals*. (2023) 13:2022. doi: 10.3390/ani13122022

103. Paiva-Martins F, Ribeirinha T, Silva A, Gonçalves R, Pinheiro V, Mourão JL, et al. Effects of the dietary incorporation of olive leaves on growth performance, digestibility, blood parameters and meat quality of growing pigs. *J Sci Food Agric*. (2014) 94:3023–9. doi: 10.1002/jsfa.6650

104. World Health Organization. *Diet, nutrition and the prevention of chronic diseases: report of a joint WHO/FAO expert consultation, Geneva, 28 January–1 February 2002*. Geneva: World Health Organization, p. 149. (2003). Available at: https://apps.who.int/iris/handle/10665/42665 (Accessed June 23, 2023).

105. de Oliveira CO, Roll AAP, Medeiros Gonçalves FM, Lopes DCN, Xavier EG. Olive pomace for the feeding of commercial poultry: effects on performance, meat and eggs quality, haematological parameters, microbiota and immunity. *Worlds Poult Sci J*. (2021) 77:363–76. doi: 10.1080/00439339.2021.1894409

106. Gerasopoulos K, Stagos D, Petrotos K, Kokkas S, Kantas D, Goulas P, et al. Feed supplemented with polyphenolic byproduct from olive mill wastewater processing improves the redox status in blood and tissues of piglets. *Food Chem Toxicol*. (2015) 86:319–27. doi: 10.1016/j.fct.2015.11.007

107. Branciari R, Galarini R, Trabalza-Marinucci M, Miraglia D, Roila R, Acuti G, et al. Effects of olive mill vegetation water phenol metabolites transferred to muscle through animal diet on rabbit meat microbial quality. *Sustainability*. (2021) 13:4522. doi: 10.3390/su13084522

108. Xie P, Deng Y, Huang L, Zhang C. Effect of olive leaf (*Olea europaea* L.) extract addition to broiler diets on the growth performance, breast meat quality, antioxidant capacity and caecal bacterial populations. *Ital J Anim Sci*. (2022) 21:1246–58. doi: 10.1080/1828051X.2022.2105265

109. Hashemi SR, Davoodi H, Arabiyan E. Nutrigenomics: a new approach to feed formulation. *J Qazvin Univ Med Sci*. (2020) 24:80–93. doi: 10.32598/JQUMS.23.5.8

110. Fekete SG, Brown DL. Veterinary aspects and perspectives of nutrigenomics: a critical review. *Acta Vet Hung*. (2007) 55:229–39. doi: 10.1556/AVet.55.2007.2.9

111. Sabino M, Capomaccio S, Cappelli K, Verini-Supplizi A, Bomba L, Ajmone-Marsan P, et al. Oregano dietary supplementation modifies the liver transcriptome profile in broilers: RNASeq analysis. *Res Vet Sci*. (2018) 117:85–91. doi: 10.1016/j.rvsc.2017.11.009

112. Dauncey M, Katsumata M, White P. Nutrition, hormone receptor expression and gene interactions: Implications for development and disease. *Proc Nutr Soc*. (2004) 60:103–24. doi: 10.1079/9780851998114.0103

113. Kaput J, Rodriguez RL. Nutritional genomics: the next frontier in the postgenomic era. *Physiol Genomics*. (2004) 16:166–77. doi: 10.1152/physiolgenomics.00107.2003

114. Surai PF. Integrated antioxidant Defence network in animals. *EC Nutr*. (2023) 18:18–20.

115. Surai PF, Kochish II, Fisinin VI, Kidd MT. Antioxidant Defence systems and oxidative stress in poultry biology: an update. *Antioxidants (Basel)*. (2019) 8:235. doi: 10.3390/antiox8070235

116. Cappelli K, Ferlisi F, Mecocci S, Maranesi M, Trabalza-Marinucci M, Zerani M, et al. Dietary supplementation of olive mill waste water polyphenols in rabbits: evaluation of the potential effects on hepatic apoptosis, inflammation and metabolism through RT-qPCR approach. *Animals*. (2021) 11:2932. doi: 10.3390/ani11102932

117. Maranesi M, Dall'Aglio C, Acuti G, Cappelli K, Trabalza Marinucci M, Galarini R, et al. Effects of dietary polyphenols from olive mill waste waters on inflammatory and apoptotic effectors in rabbit ovary. *Animals*. (2021) 11:1727. doi: 10.3390/ani11061727

118. Safwat GM, Kandiel MA, Abozaid OAR, Arafa MM, Mohamed SO. Biochemical effect of olive cake as feed additive on antioxidants and molecular expression of FAS, ANS, ACC in laying hens. *Adv Anim Vet Sci*. (2022) 10:738. doi: 10.17582/journal.aavs/2022/10.4.731.738

119. Tari AR, Sadeghi AA, Mousavi SN. Dietary vegetable oils inclusion on the performance, hormonal levels and hsp 70 gene expression in broilers under heat stress. *Acta Sci Anim Sci*. (2020) 42:–e45517. doi: 10.4025/actascianimsci.v42i1.45517

frontiers | Frontiers in Veterinary Science

TYPE Review
PUBLISHED 25 October 2023
DOI 10.3389/fvets.2023.1279506

OPEN ACCESS

EDITED BY
Tao Wang,
Jilin Agricultural University, China

REVIEWED BY
Chunyin Geng,
Yanbian University, China
Xiaofeng Xu,
College of Animal Science and Technology, China

*CORRESPONDENCE
Achraf Adib Lesaux
✉ a.adiblesaux@phileo.lesaffre.com

RECEIVED 18 August 2023
ACCEPTED 09 October 2023
PUBLISHED 25 October 2023

CITATION
Maturana M, Castillejos L, Martin-Orue SM, Minel A, Chetty O, Felix AP and Adib Lesaux A (2023) Potential benefits of yeast *Saccharomyces* and their derivatives in dogs and cats: a review.
Front. Vet. Sci. 10:1279506.
doi: 10.3389/fvets.2023.1279506

Potential benefits of yeast *Saccharomyces* and their derivatives in dogs and cats: a review

Marta Maturana[1], Lorena Castillejos[1], Susana M. Martin-Orue[1], Anaelle Minel[2], Olivia Chetty[2], Ananda P. Felix[3] and Achraf Adib Lesaux[2]*

[1]Animal Nutrition and Welfare Service (SNiBA), Department of Animal and Food Science, Universitat Autònoma de Barcelona (UAB), Bellaterra, Spain, [2]Department of Research & Development, Phileo by Lesaffre, Marcq-en-Baroeul, France, [3]Department of Animal Science, Federal University of Paraná, Curitiba, Brazil

Yeast *Saccharomyces* and its derivatives have been largely used in livestock and poultry nutrition for their potential positive impact on growth, performance, and general health. Originally included in animal diets as a source of protein, yeasts can also offer a wide range of by-products with interesting bioactive compounds that would confer uses beyond nutrition. Although its supplementation in livestock, poultry and even in humans is well documented, the available body of literature on the use of yeast and its derivatives in companion animals' food, mainly dogs and cats' diets, is still developing. Despite this, gut microbiota modulation, immune system enhancement or decreasing of potentially pathogenic microorganisms have been reported in pets when using these products, highlighting their possible role as probiotics, prebiotics, and postbiotics. This review attempts to provide the reader with a comprehensive on the effects of *Saccharomyces* and its derivatives in pets and the possible mechanisms that confer their functional properties.

KEYWORDS

companion animals, prebiotics, probiotics, postbiotics, yeast

1. Introduction

As the pet food industry keeps steadily growing annually, so does the demand for functional ingredients in dogs and cats' diets. Owners look for ingredients that can provide additional health benefits beyond basic nutrition, echoing the functional foods trend already established in humans. Examples of these ingredients are omega-3 fatty acids, probiotics/prebiotics/postbiotics, or plants and vegetables' extracts. Yeasts may also represent a good candidate. There are about 500 different yeasts species and some of them have been used both in humans and animals, especially livestock, for many years, *Saccharomyces cerevisiae* (*S. cerevisiae* or SC) being the predominant one. In this review, we will specifically focus on the use of *S. cerevisiae*, referring to it also as yeast.

Although the use of yeast in livestock and poultry feed has been widely reviewed, there is still a lack of information about the effects on dogs and cats, admittedly the number of studies is increasing. Moreover, despite the many beneficial health effects reported, the supplementation of yeast-based products has not always resulted in consistent effects, and the precise mode of action of these compounds has been difficult to determine. Thus, the aim of this review is to

provide an overview on the use of *Saccharomyces* and/or their derivatives, as a functional ingredient in pet food, discussing its general possible modes of action with a specific focus on *in vivo* studies in companion animals.

2. *Saccharomyces* composition and products

Yeasts cells intracellular composition includes amino acids, peptides, carbohydrates, salts, vitamins, monosodium glutamate, nucleic acids, enzymes, and cofactors. On the other hand, yeast cell wall (YCW) is mostly formed by Beta-glucans (β-glucans), glycoproteins, and mannan oligosaccharides (MOS), and to a lesser extent by chitin. There are different forms of yeasts being studied, ranging from viable yeast cells to purified components of the cell wall, as shown in Table 1. From primary yeast cultures that undergo propagation and fermentation steps, yeasts by-products are obtained via downstream processing (1, 2). Live yeast cells can be inoculated in specific culture media to ferment it and subsequently be dried, forming a yeast culture containing a wide variety of metabolic products. Yeast cells can also undergo a process of hydrolysis or autolysis to obtain yeast lysates that contain both intracellular and cell wall fractions. However, intracellular components and carbohydrates of the cell wall can also be found separately as yeast extract and cell wall products, respectively.

Most of the yeast-based products available in the European market are registered under the European Food Safety Authority (EFSA) category of zootechnical additives, and subcategories of gut microbiota stabilizers or digestibility enhancers, depending on the product and target species. Whereas dried yeast is used for its nutritional value as a specialty amino acid, vitamin and mineral supplement, the rest of the products could fall under the definitions of probiotics, prebiotics, and postbiotics established by the International Scientific Association for Probiotics and Prebiotics (ISAPP). Active dry yeast is formed by live yeast cells and acts as a probiotic, defined by ISAPP as "live microorganisms that when administered in adequate amounts, confer a health benefit on the host" (3). The probiotic role of *Saccharomyces cerevisiae* var. *boulardii* (*S. boulardii*), a strain of the same genus as SC, nearly identical at a molecular level but physiologically different (4), is well established. However the probiotic role of *S. cerevisiae* needs further investigation, although some recent studies in dogs reported positive and promising results on modulation of fermentative metabolites and microbiota composition and function (5). On the other hand, yeast lysates, yeast extracts, and YCW components, mostly MOS and β-glucans, could be considered prebiotics and their use is more extended, with more studies reporting benefits on both human and animal health (1, 6). Prebiotics are defined by the ISAPP as "a substrate that is selectively utilized by host microorganisms conferring a health benefit" (7). Lastly, yeast culture, also known as *Saccharomyces cerevisiae* fermentation product (SCFP), would pertain to the still recent term "postbiotics" defined by the ISAPP as "preparation of inanimate microorganisms and/or their components that confers a health benefit on the host" (8).

3. *Saccharomyces* as a functional ingredient: modes of action

3.1. Microbiota improvement

A balanced gut microbiome is of vital importance for the health of the host and yeasts have been proven to cause shifts in the bacteria population of the gastrointestinal tract in several species (9, 10). *In vitro* studies reported that the association of some strains of *Saccharomyces* and *Lactobacillus rhamnosus* exert a protective effect on human microbiota following antibiotic administration (11) or against pathogenic bacteria (12). The use of yeast β-glucan also promoted the growth of *Bifidobacterium longum* and other health-promoting gut microbiota in a study simulating human saliva, gastric and intestinal digestion (13). Similarly, mannan and MOS derived from yeast were able to grow specific lactic acid bacteria strains *in vitro* and beneficial *Bacteroides* in humans (14, 15). Thus, yeast and yeast-based products may potentially promote host's gut health.

TABLE 1 Most common *Saccharomyces cerevisiae* products added in animal feed, with its production process and the major composition (1).

Product	Process	Composition
Dried yeast (also dehydrated primary yeast)	Drying of whole yeast cells (high temperature).	Biomass of dead yeast cells.
Active dry yeast (also dehydrated active yeast or live yeast)	Drying of whole yeast cells.	Live yeast cells.
Yeast culture (also dehydrated yeast culture or SCFP in the case of *Saccharomyces cerevisiae*)	Inoculation of live yeast cells in a specific culture medium followed by controlled fermentation and drying.	Yeast biomass (residual viable cells, dead cells, and yeast cell wall fragments), fermentation metabolites, and residual growth medium.
Lysate yeast (also hydrolyzed yeast /hydrolysates or autolyzed yeast/autolysates)	Hydrolysis or autolysis of whole yeast cells.	B vitamins, peptides, amino acids, glutamic acid, nucleotides, minerals, mannan oligosaccharides, and β-glucans.
Yeast cell wall components	Centrifugation after the hydrolysis/autolysis of the yeast.	Mannan oligosaccharides and β-glucans (together or individualized).
Yeast extract	Centrifugation after the hydrolysis/autolysis of the yeast.	B vitamins, peptides, amino acids, glutamic acid, nucleotides, and minerals.

SCFP, *Saccharomyces cerevisiae* fermentation product.

3.2. Immune system enhancement

The supplementation of yeast products can modulate the immune system through changes in the innate and adaptative response. It is thought that the main responsible of these properties are the β-glucan and MOS present in yeast. The specific changes are different depending mainly on the type of product and the general health status of the animals. As reviewed by many authors, yeast products can affect concentrations of platelets and WBC populations, phagocytic capacity of cells, secretion of pro- and anti-inflammatory cytokines, acute phase proteins concentrations, and antibodies production by binding with complement receptor type three (CR3), dectin-1 or TLRs on different cells (16–18). Yeasts may also have an indirect effect on the immune system through microbiota; as previously mentioned, β-glucans and MOS could increase *Bifidobacterium* population which, in return, has been shown to decrease the expression of toll-like receptors (TLRs), NFkB, and cytokines, *in vitro* and *in vivo* with mice (19–21). Moreover, emerging studies also point out the potential of yeast of altering the metabolism, including availability of glucose and fatty acids, as another factor contributing to their capacity of modulate the immune system (16). In conclusion, the immune response is a complex process that goes beyond the response of a single immune parameter; thus, to truly understand the mechanisms behind the possible beneficial effects of the use of yeast as an immunomodulator, integration of the different factors is needed.

3.3. Increased digestibility

Glucans present in yeasts could improve nutrient digestibility through the modification of passage rate in the small intestine. This effect would be mediated by the formation of a semisolid gel promoted by yeast's alkali-soluble glucans that could slow the passage and increase the time that the chyme is in contact with enzymes, resulting in a higher digestibility of nutrients (22). As a result of this, bacterial fermentation in the large intestine would be reduced as less fermentable substrate would be available. However, one should be cautious with this hypothesis as *in vivo* studies with yeast β-glucans have reported increases in apparent total tract digestibility (ATTD) in pre-ruminant calves (23) or no changes at all in dogs (24). Other possible mechanism of yeast to modulate nutrient digestibility is the production of enzymes, such as amylases, lipases, and proteases. Some studies with fish and hens have reported increases in digestive enzymes with yeast supplementation, resulting in improved nutrients digestibility (25, 26).

Despite this, other studies have reported decreases in crude protein (CP), nitrogen-free extracts and dry matter (DM) apparent digestibility in dogs supplemented with MOS (27). These reductions in digestibility were accompanied by increased total fecal water and decreased unbound water which could mean different physical properties of intestinal chyme. Thus, the higher water binding could have had an impact on the solubility of nutrients and affected digestibility.

3.4. Protection against toxins and pathogens

Saccharomyces cerevisiae and other genetically close yeasts can exert a protective effect against toxins and pathogens through different mechanisms that involve the binding of bacteria and toxins, enhancement of immunoglobulins production by the host, and releasing of proteases that destroy certain toxins, among others (28, 29).

One of the most frequent protection mechanisms of yeasts that can be found in the literature is the capacity of binding pathogenic bacteria, avoiding its union with the host's receptors, being MOS the main responsibles for this binding activity. Tiago et al. (30) showed both *in vivo* with mice and *in vitro* that selected strains of *S. cerevisiae* were able to capture some enteropathogenic bacteria on the surface, regardless of the yeast viability. However, this capturing ability was limited to specific Gram-negative enteropathogens such as *Salmonella* and *Escherichia coli* (*E. coli*). Mannan oligosaccharides form part of *Saccharomyces* cell walls, and their concentration is especially high in yeast derivatives such as YCW. The mechanism would be that mannose can occupy binding sites on fimbrial adhesins on the surface of bacteria. This way, mannose residues in live yeast or derivatives could prevent certain bacteria that possess fimbria type-1 from adhering to the intestinal mucosa, thus avoiding colonization and multiplication to disease-causing levels (31–33).

Saccharomyces cerevisiae var. *boulardii* has been widely used in treating *Clostridium difficile* diarrhea and colitis. The mechanisms of this effect include increasing the activity of intestinal disaccharidases (34) and stimulation of the intestinal secretion of s-IgA and of the secretory component of immunoglobulins (35, 36). Other authors characterized a serine protease released by the yeast that hydrolyzes toxins A and B, which are *C. difficile*'s mediators of diarrhea and colitis, thus inhibiting the binding of the toxins to its intestinal receptor (37–39). More recent studies have found that *S. boulardii* exerts an effect on the structural component of the microbe's biofilm matrix *in vitro*, possibly affecting intestinal colonization (40). Reports about the use of *S. cerevisiae* against this pathogen are scarce but some demonstrate a protective effect of certain strains of the yeast in rats and mice (41, 42). Inhibition of other toxins such as cholera toxin or aflatoxin B1 has also been observed in *in vitro* and *in vivo* trials using rats, with *S. cerevisiae* and *S. boulardii*, hypothesizing that in this case the mode of action included binding of the toxin through a receptor located in its surface (43, 44).

Saccharomyces cerevisiae was also able to reduce heat-labile enterotoxins production from *Enterotoxigenic Escherichia coli* (ETEC), associated with the removal of the toxin by the yeast and the reduction in the expression of its corresponding gene *eltB* (45). In the same series of assays, the yeast also interacted directly with ETEC through agglutination in a mannose-related manner.

Lastly, *S. cerevisiae*-based probiotic has shown benefits in the treatment of bacterial vaginosis produced by *Gardnerella vaginalis* in mice (46). In this case, the suggested mechanisms of action include direct interference with adherence to vaginal tissues, inhibition of sialidase activity and reduction of vaginal epithelial exfoliation.

3.5. Antioxidant effect

The activity of *S. cerevisiae* and derivatives has been assessed to explore their potential as natural antioxidants, protecting cells against oxidative stress. Although it is probably a result of the combined action of different factors, cell wall β-glucans and mannans are thought to be the main actors in the antioxidant capacity of yeasts. As widely review in the literature, these

compounds can boost the antioxidant status of the body by improving the activity of antioxidant enzymes such as glutathione peroxidase (GPx), catalase or superoxide dismutase (SOD) (17). Experiments with porcine jejunum epithelial cell lines damaged by deoxynivalenol, a strong inducer of oxidative stress, reported promising results. The association of mannan and β-glucan from yeast cell walls was able to down-regulate the production of malondialdehyde (MDA) and reactive oxygen species (ROS), while up-regulating the production of glutathione (47). *In vivo* studies with weaned piglets, supplemented with *S. cerevisiae* cell wall, also resulted in decreased serum MDA and increased activity of the enzymes GPx, catalase and SOD (48). The addition of yeast β-glucans in broilers exposed to mycotoxins improved serum levels of antioxidant enzymes and lower lipid peroxidation as well (49). Moreover, the naturally good antioxidant capacities of selenium seem to be improved by the enrichment with yeast, due to a synergic effect, as shown in *in vitro* and *in vivo* assays with mice (50).

3.6. Other effects

3.6.1. Skin health

Saccharomyces cerevisiae extract, known occasionally as "skin respiratory factor," has largely been used by humans for the treatment of burns, wounds and hemorrhoids (51). Studies with human and animal models indicate that it accelerates healing stages: inflammation, angiogenesis, granulation tissue and epithelial migration (52–55). The active fraction of the extract has been identified as a mixture of peptides from 6 to 17 kD formed by: copper zinc superoxide-dismutase ubiquitin, glucose-lipid regulated protein and acyl-CoA binding protein (51, 52). Gruenstein et al. (56) used a human monocyte-derived cell line, THP-1, to study the mechanisms involved in the extract and wound healing. These cells are widely used to study monocyte functions and mechanisms, being monocytes one of the first immune cells to reach the wound site. Apparently, the yeast derivative activates phospholipase-C which results in an increase of cytoplasmic Ca^{2+}. This in turn initiates the transcription of early genes for tissue repair, such as c-fos. β-glucans are other components of the yeast cell wall that can have an effect on wound healing, facilitating the transportation of macrophages to the wound site, stimulating the tissue granulation and re-epithelization, and improving collagen deposition (57).

Topical application of *S. cerevisiae* has been shown to improve the healing of burn wounds and to enhance the action of a collagen hydrogel-scaffold, increasing epithelialization and biomechanical performance of wound area and reducing the scar size, in rats (58). Studies with seabreams and trouts showed that feed supplementation with *S. cerevisiae* improved skin health and skin recovery from wounds (59, 60).

The evidence of *S. cerevisiae* wound healing effect and its antioxidant properties have targeted the yeast as a potential biotechnological raw material in dermo-cosmetic formulations. Dermatological formulations that include *S. cerevisiae* extract or derivatives showed positive effects in skin moisture and roughness in animal and human models (15, 57, 61); protection against UV irradiation both *in vitro* and in humans (62, 63) and itch relief in chronic pruritus in humans (64, 65).

Saccharomyces cerevisiae has also been used as a skin cell model in experimental biology. Lastly, this yeast has been linked to different skin diseases such as psoriasis or atopic dermatitis playing a pro- or anti-inflammatory effect, but the exact mechanism has yet to be determined (66).

3.6.2. Mood modulator

Probiotic ingestion has been linked to reduced anxiety-like behaviors via the hypothalamic–pituitary–adrenal axis and the gut-brain axis (67). Supplementing a mixture of *Bifidobacterium lactis*, *Lactobacillus acidophilus*, and *Lactobacillus fermentum* to rats exposed to noise stress decreased behaviors related to anxiety and also serum corticosterone (68). *Saccharomyces* sp. has also been proven to attenuate anxiety-like behaviors in rats exposed to LPS, possibly through modulation of the gut microbiota and hypothalamic–pituitary–adrenal axis (69). In this previous study, yeast supplementation led to significantly lower serum levels of cortisol and corticosterone. Supplementation prevented the reduction in the serum levels of serotonin and brain-derived neurotrophic factor (downregulated during stress) in rats challenged with LPS, compared with those not supplemented and challenged; however, the difference was not significant. Healthy medical students presented a decreased salivary serotonin concentration when facing a stressful event after 30-day supplementation with *Saccharomyces* sp., although an association with anxiety, depression or eating attitudes was not found (70). Hemiplegic spastic cerebral palsy rats that exhibited depression behavior improved their mental state when a *Saccharomyces* sp. treatment was applied. This yeast increased gut microbiota diversity and decreased the level of inflammation and the hypothalamic–pituitary–adrenal axis activity (71). In a study with healthy human volunteers, subjects consuming the *S. cerevisiae* yeast hydrolysate showed better marks in questionnaires that assessed stress and anxiety, suggesting that this derivative could be effective in modulating behavior (72).

4. *Saccharomyces* in dogs and cats

There are limited publications examining the impact of yeast or yeast by-products in the dog and cat, although the number is increasing. This contrasts with livestock, where there are a wide number of publications on the use of yeast or yeast by-products. Nevertheless, there is an ever-increasing demand for functional ingredients that improve companion animal health and yeast may prove to provide such benefits.

Pets could benefit from the inclusion of yeast products in their diet, as some promising results have been described in the scientific literature including the modulation of the intestinal microbiota (with potential increases in *Bifidobacterium* or *Lactobacillus*), enhancing of the immune function, reduction of potentially pathogenic microorganisms such as *E. coli*, improvement of blood parameters related with obesity and possible improvement in the antioxidant status, among others. In the following sections we review some of the most recent evidence on the potential benefits of using yeast or derivatives as functional ingredients in petfood. The main outputs of studies with live yeast *S. cerevisiae* and its by-products in dogs and cats are shown in Tables 2, 3, respectively.

TABLE 2 Main outputs of different studies on the use of live yeast *Saccharomyces cerevisiae* and by-products in dogs.

Product	Dose	Effects of the yeast product	Ref.
LY SC (strain CNCM I-5660; Actisaf®; Phileo by Lesaffre)	0 (CON) or 0.12 (SUP) g daily of LY SC (1×10^{10} CFU/g) for 49 days with abrupt change of diet at day 22. **Abrupt dietary change as challenge*	Fecal microbiota: - ↓ Dysbiosis index, ↑ abundance of *Bifidobacterium* (days 35 and 49) and *Turicibacter*, and ↓ abundance of *Lactobacillus* and *E. coli.* - ↓ Firmicutes and ↑ Actinobacteria after the challenge. Hindgut fermentation products (fecal): - ↓ Ammonia (day 23), ↓ total biogenic amines (days 21 and 49), and aromatic compounds. ↑ butyrate concentration. - Others: - CON showed upregulation in genes related to virulence factors, antibiotic resistance, and osmotic stress.	(5)
LY SC (strain MUCL 39885; Biosprint®, Prosol S.p.A.)	0 (CON) or 7×10^{10} CFU SC/kg feed daily for 35 days (SUP)	Digestion: - ↓ Fecal DM; the magnitude of the effect is questionable. - =BW and feed intake.	(73)
LY SC (strain CNCM I-4407; Actisaf Sc 47; Phileo by Lesaffre)	0 (CON) or 2.9×10^{8} CFU SC/kg BW (SUP) daily for 42 days	Fecal microbiota: - ↓ *E. coli* and enterococci counts. - Digestion: - ↑ Weight gain- - ↑ ATTD of NDF, but not of the rest of nutrients.	(74)
Different fermentation media with SC	0 (CON), 10 g/kg food brewer's yeast (SUP1), 10 g/kg food brewer's yeast + corn yeast (SUP2) and 10 g/kg food brewer's yeast + corn yeast + cell wall fractions (SUP3) for 20-day periods.	Fecal microbiota: - ↑ *Bacteroides, Faecalibacterium, Phascolarctobacterium, Coprococcus.* Hindgut fermentation products (fecal): - ↑ Total SCFA, acetate and butyrate. - ↓ Indole. Digestion: - ↑ Preference.	(75)
SCFP (TruMune, Diamond V Mills, Inc.)	0 (CON) or 250 mg SCFP daily (equivalent to approximately. 8.6 mg/kg BW) (SUP) for 10 weeks *Exercise challenge*	Fecal microbiota: - After 21 days of supplementation, before the first exercise challenge, ↑ *Clostridium* in CON whereas not change in SUP. Others: - For the rest of the parameters, exercise challenge was the primary cause of the shifts observed, but not the yeast.	(76)
SCFP (TruMune, Diamond V Mills, Inc.)	0 (CON) or 30 mg SCFP/kg BW (SUP) daily for 11-weeks periods. **Dogs challenged with transport stress*	Digestion: - Change in fecal DM: ↓ (tendency) in CON but stable in SUP after transport. Antioxidant properties: - Change in serum malondyaldehide ↑ and in serum 8-isoprostane ↑ (tendency), after transport, whereas in CON ↓. - No changes in the expression. Of genes associated with blood cyclooxygenase-2 and myeloperoxidase mRNA, after transport, whereas in CON ↑.	(77)
SCFP (Diamond V Mills, Inc.)	0 (CON) or 30 mg SCFP/kg BW (SUP) daily for 10-weeks periods.	Inmmunomodulation: - ↓ Changes in unstimulated lymphocytes and stimulated IFN-γ secreting T cells. - ↓ Platelets; variable in CON. - Variable basophils; ↑ in CON. - Antioxidant properties: - Change in serum superoxide dismutase concentration was ↑ and in catalase mRNA expression was ↓. Skin: - ↑ (tendency) sebum concentration. - ↑ Transepidermal water loss values in back region of CON and in ear region of SUP.	(78)
SCFP (XPC™, Diamond V Mills, Inc.)	0% (CON), 0.3% (SUP1), or 0.6% (SUP2) SCFP of diet for 25-day periods.	Fecal microbiota: - ↑ Actinobacteria and Firmicutes, and ↓ Fusobacteria. - ↓ *Allobaculum* and *Fusobacterium*, and ↑ *Clostridium*. - ↑ *Collinsella* and ↓ *Prevotella* in SUP2. Hindgut fermentation products (fecal): - ↑ Propionate. Immunomodulation: - ↑ phagocytosis index- Digestion: - CF ATTD, ↓ CP and NFE ATTD, and ↓ ME in supplemented groups.	(79)
SCFP (Diamond V Mills, Inc.)	0 (CON), 250 mg (SUP1) or 500 mg (SUP2) daily for 14 weeks. ** Dogs underwent exercise and transport stress*	Immunomodulation: - ↑ TNF-α, ↓ IgE and IgG. - ↑ Haptogoblin, serum amyloid A, and C-reactive protein Antioxidant properties: - ↓ Thiobarbituric acid reactive substances, creatine kinase, and ↑ total antioxidant capacity. Others: - ↑ Activity, running speed, and stool quality.	(80)

(Continued)

TABLE 2 (Continued)

Product	Dose	Effects of the yeast product	Ref.
SCFP (Diamond V Mills, Inc.)	0 (CON), 125 (SUP1), 250 (SUP2), or 500 (SUP3) mg SCFP daily for 28-day periods (12.1, 24.3, and 48.5 mg/kg BW) Sub-study: palatability test, 0.2% SCFP of diet.	Fecal microbiota: - ↑ Actinobacteria, ↑ (tendency) Firmicutes, ↓ Fusobacteria. - ↑ *Bifidobacterium*, ↑ (tendency) *Prevotella*, and ↓ *Fusobacterium*. Hindgut fermentation products (fecal): - ↓ Phenol and phenol+indoles, linearly with dosage. - Immunomodulation: - ↓ WBC with increasing SCFP dose; SUP2 had ↓ WBC than CON. - ↑ MHC-II + B cell and monocyte expressing surface MHC-II populations. - ↓ TNF-α secretion from cells stimulated with TLR2, TLR3, TLR4 and TLR7/8 agonists. - ↑ IgE with dosage. - CON had the highest TNF-α concentrations. Digestion: - Slightly softer when comparing all SCFP treatments with CON. - TTD was not influenced. - ↑ Preference.	(81)
Hydrolyzed SC (Progut®, Suomen Rehu, patented EP 1387620 hydrolysis process)	0% (CON) and 0.3% (SUP) hydrolyzed SC of diet for 14 days (day 0 to day 14). Whole study 42 days.	Fecal microbiota: - ↑ *Bifidobacterium* (day 14 and day 42), lactic acid bacteria (day 42) and *Clostridium* (day 42). Hindgut fermentation products (fecal): - ↑ pH (day 28). Obesity: - ↓ Serum lipid profile (triglyceride, cholesterol) (day 42). Kidney and liver: - ↑ ALT (day 14), AST (day 28 and 42), and urea serum levels.	(82)
Hydrolyzed SC (Brewer's Yeast, BY)	Control diet (CON) vs. control diet substituted by 15% BY (SUP-BY), 7.5 and 15% autolyzed sugar cane (ASCY) or integral sugar cane (ISCY). 10 days.	Digestion: - ↓ Gross energy ATTD in ASCY and ISCY compared to CON. - ↓ Fecal DM with the addition of yeast. - ↑ Preference.	(83)
Yeast extract (Nupro®, Alltech Brasil Ltda.)	0 (CON), 40, 80, or 120 g yeast extract/kg diet daily for 10-day periods Sub-study: palatability test, 0 vs. 40 g yeast extract/kg diet.	Hindgut fermentation products (fecal): - ↑ Total SCFA, propionate and butyrate with dosage. - ↓ Isovalerate and pH with dosage. Digestion: - ↓ ATTD of CP and AHEE with dosage. - ↑ Preference.	(84)
YCW (Biolex MB 40, Leiber GmbH, Bramsche, Germany)	*In vitro* study, simulating the canine gastrointestinal tract. Doses: 0 (CON), 0.5 (SUP1), 1.0 (SUP2) and 2.0 (SUP3) g/d corresponding to 0.05, 0.1 and 0.2% in pet food, for 3 weeks.	Fecal microbiota: - ↑ Bacteroidetes and ↓ Fusobacteria. - ↓ Enterobacteriaceae and Fusobacteriaceae. Hindgut fermentation products (fecal): - ↑ Gas production. - ↑ Acetate and butyrate. - ↑ Propionate for all SUP groups compared to CON in PC, but only for SUP3 in DC. - BCFA were low overall but significantly higher for SUP1 and SUP2 in both PC and DC, and only for SUP2 in DC. - ↑ Ammonia for SUP2 in PC and DC.	(85)
YCW (Safmannan®, Phileo by Lesaffre)	0 (CON) or 365 mg yeast daily (0.2% of diet) (SUP) for 28-day periods with an abrupt diet transition at day 15 **Abrupt dietary change as a challenge.*	Fecal microbiota: - ↑ (tendency) *C. perfringens*. Immunomodulation: - ↑ (tendency) fecal IgA, after abrupt diet transition.	(86)
Conventional YCW and specific high solubility of MOS YCW (YCWs) (Biorigin, Brazil)	0 (CON), 0.3% YCW (SUP1) and 0.3% YCWs (SUP2) of the diet for 32-day periods	Hindgut fermentation products (fecal): - ↑ Butyrate and ↓ lactate in SUP2. - ↑ Putrescine and ↑ (tendency) spermidine in SUP2. Immunomodulation: - ↓ Serum IL-6 in SUP2. - ↓ (tendency) serum IL-6 and TNF-α in SUP1. - ↑ Phagocytic index in peripheral monocytes. Digestion: - ↓ AHEE ATTD in SUP2.	(87)
YCW (DMB; Bio-Mos, Alltech Nicholasville, KY)	0 (CON) and 1.4% YCW (SUP) of the diet for 21-day periods **Raw diets: beef and chicken.*	Fecal microbiota: - ↑ *Bifidobacterium* and ↓ *Clostridium celerecrescens*.	(88)
YCW (DMB; Bio-Mos, Alltech Nicholasville, KY)	0 (CON) and 1.4% YCW (SUP) of the diet for 21-day periods **Raw diets: beef and chicken.*	Hindgut fermentation products (fecal): - When used in the chicken diet: ↓ pH, ↓ indole and ↑ spermine. - When used in the beef diet: ↑ fecal acetate, total SCFA, and spermine. Digestion: - When used in the beef diet: ↑ (tendency) fecal output and softer feces.↓ DM, OM, CP and energy ATTD.	(89)

(Continued)

TABLE 2 (Continued)

Product	Dose	Effects of the yeast product	Ref.
YCW (Safmannan®, Phileo by Lesaffre)	0 (CON), 0.07 (SUP1), 0.35 (SUP2) or 0.91 (SUP3) g of YCW per animal twice daily for 14-day periods. Doses correspond to 0, 0.05, 0.25, 0.45, or 0.65% of food offered.	Fecal microbiota: - ↓ *E. coli*. *Clostridium perfringens* responded cubically to supplementation. Immunomodulation: - ↓ (tendency) quadratically of WBC and eosinophils, and ↓ linearly of monocyte with dosage. - Serum IgA responded cubically to supplementation whereas ileal IgA tended to respond quadratically, with the greatest value in SUP2. Digestion: - ↑ (tendency) apparent Ileal digestibility of DM, OM, CP, and GE. - ATTD of DM, OM, CP, AHEE, and GE responded cubically to supplementation, being lowest in SUP2.	(90)
Yeast-derived MOS, high in mannoproteins (Actigen, Alltech Inc.)	0 (CON), 400 mg (SUP1), or 800 mg MOS/kg (SUP2) of diet daily for 28 days, **Use of adult and elderly dogs*	Immunomodulation: - ↑ (tendency) neutrophil phagocytic activity in SUP1 compared with SUP2. Antioxidant properties: - ↑ (tendency) production of H_2O_2 when neutrophils were stimulated with LPS. Skin: - Diet influenced the delayed cutaneous hypersensitivity test upon saline inoculation.	(91)
Yeast-derived MOS (Provimi Animal Nutrition)	0 (CON) or 15 g MOS/kg of diet (SUP) for 150 days.	Immunomodulation: - ↑ Cell-mediated immune response, CD4+ lymphocyte population and CD4+:CD8+ ratio. Obesity: - ↓ Serum total- and LDL- cholesterol.	(92)
Yeast-derived MOS (Bio-MOS, Alltech Inc.)	0 (CON) or 2 g/kg BW (SUP) daily for 20 days from 24 h after infection. **Challenge with enteropathogenic Escherichia coli*	Management of gastrointestinal disorders: - Faster recovery from infection.	(93)
Yeast-derived MOS	0 (CON), 1% dietary chicory (SUP1), 1% dietary MOS (SUP2) or 1% chicory +1% MOS (SUP3) for 28 days.	Fecal microbiota: - ↑ *Bifidobacterium* in SUP1 and SUP2. - ↓ *E.coli* in SUP2 Immunomodulation: - ↓ (tendency) lymphocytes in SUP2 and SUP3. - ↑ (tendency) neutrophils in SUP1 and SUP3. Digestion: - ↑ (tendency) feed intake in SUP2 and SUP3. - ↑ Fecal score in SUP3, although still in desirable range.	(94)
Yeast-derived MOS (Bio-MOS, Alltech Inc.)	0 FOS/MOS (CON), 1 g FOS (SUP1), 1 g MOS (SUP2), 1 g FOS + 1 g MOS (SUP3) twice daily for 14-day periods	Fecal microbiota: - ↓ Total aerobes in SUP2. - ↓ (tendency) total anaerobes in SUP3. Hindgut fermentation products (fecal): - ↓ Phenols + indoles, and ↓ (tendency) indoles in SUP1 and SUP3. - ↑ (tendency) pH in SUP2. Immunomodulation: - ↑ Ileal IgA in SUP3. - ↑ lymphocytes in SUP2.	(95)
Yeast-derived MOS (Bio-MOS, Alltech Inc.)	0 (CON) vs. 1 g/kg BW (SUP) daily for 10 days (5% of diet)	Hindgut fermentation products (fecal): - ↓ Unbound water, fecal pH, and ammonia. Digestion: - ↓ ATTD ofCP, DM and nitrogen-free extracts. ↑ ATTD of CF.	(27)
Yeast-derived MOS (DP607, Alltech Inc.)	0 (CON) vs. 5 g/kg of diet DM (SUP) daily for 21-day periods.	Hindgut fermentation products (fecal): - ↑ (tendency) ileal butyrate in CON. Digestion: - ↓ (tendency) amount of feces excreted on a DM in CON.	(96)
Concentrated BY (not specified, possibly β-glucans) (F.L. Emmert Company, USA).	0 (CON) vs. 7 mg/kg BW of β-glucan (SUP) daily for 10 weeks.	Hindgut fermentation products (fecal): - ↑ Arabinose. Immunomodulation: - ↓ (tendency) gut permeability. - ↓ Serum haptoglobin levels.	(97)
Yeast-derived β-glucan	0 (CON) vs. 0.1% (SUP) of diet, for 90 days, in obese dogs.	Obesity: - ↓ Plasma glucose triglyceride, and cholesterol concentrations. - ↑ GLP-1. Immunomodulation: - ↓ TNF-α.	(98)
Yeast-derived β-glucan (Wellmune for Pet™, Kerry Incorporated, Beloit, WI)	0 (CON), 0.012 (SUP1) and 0.023% (SUP2) of diet for 28 days.	- No effect of the β-glucan in the analyzed parameters.	(24)

(Continued)

TABLE 2 (Continued)

Product	Dose	Effects of the yeast product	Ref.
Yeast-derived β-glucan	0 (CON), 150 ppm β-glucan top-dressed (SUP1), 150 ppm retorted β-glucan included in diet formulation (SUP2), during 42 d. *Vaccine challenge*	Digestion: - ↑ ATTD of DM, CP and GEin SUP2. ATTD of GE was greater for SUP2 than for both CON and SUP1.	(99)
Yeast-derived β-glucan (BG01 and BG02, Biorigin)	0 (CON), 15 mg/kg/day of BG01 (SUP1) or 25 mg/kg/day of BG02 (SUP2)	Immunomodulation: - ↑ Phagocytic activity of blood monocytes and neutrophils. - ↑ IL-2 and anti-ovalbumin antibodies production (antibody response). - ↓ Blood glucose in dogs with hyperglycemia.	(100)
Yeast-derived β-glucan (Biolex-Beta HP. Inter Yeast Poland).	0 (CON) vs. 7 mg/kg BW (SUP) daily for 6 weeks **Dogs with IBD*	Immunomodulation: - ↓ IL6 and ↑ IL-10. Management of gastrointestinal disorders: - Most efficient treatment efficacy by producing the quickest therapeutic effect. - No recurrence of IBD at least for 6 months after the experiment.	(101)
Yeast-derived β-glucan (MacroGuard, Biorigin)	0 (CON) vs. 800 ppm of β-glucan (SUP) daily for 8 weeks. **Dogs with atopic dermatitis signs*	Immunomodulation: - Numerical improvement in the scores of itching, redness, scaling, thickening, and stripping of skin, but notstatistical significance compared with CON. - Global improvement (sum of all the parameters) of the atopy index was 63% comparing SUP with CON. - ↑ Coat quality.	(102)
Yeast-derived β-glucan (MacroGuard, Biorigin)	0 (CON) vs. 800 ppm of β-glucan (SUP) daily for 8 weeks. **Dogs with osteoarthritis signs*	Immunomodulation: - Numerical improvement in the scores of activity, stiffness, swelling of joing, lameness, and pain, but the differences between CON and SUP did not reach statistical significance.	(103)
Yeast-derived β-glucan (MacroGuard, Biorigin)	0 (CON) vs. 225 mg of β-glucan (SUP) daily for 4 weeks. *Experiment 1. No vaccine.* *Experiment 2. Vaccine challenge*	Immunomodulation: *Experiment 1:* - ↓ IgA and ↑ IgM serum concentrations. - ↑ - ↓ Saliva and tears IgA. Increase of saliva IgA after the end of the supplementation was also higher for SUP group. *Experiment 2:* - After vaccination, antigen-specific IgM increased similarly to total IgM. - Antigen-specific IgA remained nearly unchanged, whereas it was increased in CON.	(104)
Dried yeast	Different diets with different primary protein source: poultry by-product meal (CON), dried yeast product (SUP1, 30% of the diet), garbanzo beans (SUP2), green lentils (SUP3) and peanut flour (SUP4), for 14-day periods	Fecal microbiota: - ↑ β-diversity in SUP1. - ↑ Several genera belonging to Firmicutes. Hindgut fermentation products (fecal): - ↑ Acetate, butyrate and total SCFA. - ↑ Valerate. - ↓ Phenols and total phenol/indoles. Digestion: - ↓ DM, OM and AHEE ATTD, although still >80%. ↓ Metabolizable energy content. - ↑ Fecal output.	(105)
Dried yeast	0 (CON) or 0.19 g/kg BW (SUP) daily for 28–63 days	Obesity: - Removal of the yeast in hyperthyroid dogs resulted in lower intake and anorexia. **This study is about hyperthyroidism, B1 deficiency and yeast supplementation.*	(106)

AHEE, acid-hydrolized ether extract; ALT, alanine transaminase; AST, aspartate transferase; ATTD, apparent total tract digestibility; BCFA, branched-chain fatty acids; BW, body weight; CF, crude fiber; CFU, colony forming units; CP, crude protein; CON, control group; DC, distal colon; DM, dry matter; GE, gross energy; IBD, intestinal bowel disease; IFN, interferon; Ig, immunoglobulin; IL, interleukin; LY, live yeast; MHC, major histocompatibility complex; MOS, mannan oligosaccharides; NFE, nitrogen-free extract; NDF, neutral detergent fiber; ME, metabolizable energy; OM, organic matter; PC, proximal colon; Ref., reference; SC, *Saccharomyces cerevisiae*; SCFA, short-chain fatty acids; SCFP, *Saccharomyces cerevisiae* fermentation product; SUP, supplemented group; TLR, toll like receptor; TNF, tumor necrosis factor; YCW, yeast cell wall; WBC, white blood cells.

4.1. Modulation of gut microbiota and hindgut fermentation

The addition of yeast by-products in dogs' diet produces changes in gut's bacterial population. Supplementation with SCFP has resulted in increases in the relative abundance of the phyla Firmicutes and/or Actinobacteria, and decreases of Fusobacteria (79, 81). Accordingly, use of dried yeast as primary protein source in a dog diet resulted in higher relative abundances of several genera belonging to Firmicutes phylum (105). An *in vivo* study simulating the canine intestinal microbial ecosystem also reported a decrease in Fusobacteria and an increase in Bacteroidetes with the addition of YCW (85).

At the genus level, *Bifidobacterium* increased with live yeast, SCFP, yeast hydrolysate, or MOS addition (5, 81, 82, 88, 94). *Prevotella* relative abundance increased while *Fusobacterium* decreased with higher doses of SCFP supplementation (81). Santos et al. (79) also observed reduced *Fusobacterium* and *Allobaculum*, as well as increased *Clostridium* with the inclusion of SCFP; in this same study, a level of 0.6% SCFP of the diet resulted in a decrease of *Prevotella* and an increase of genus *Collinsella*. Increases in genera considered beneficial and related with the production of SCFA, such as *Bacteroides*, *Faecalibacterium*, *Coprococcus*, and *Phascolarctobacterium*, were reported with the use of yeast products (75). Fecal *Lactobacillus* tended to be higher and total aerobes tended to be lower in dogs

TABLE 3 Main outputs of different studies on the use of live yeast *Saccharomyces cerevisiae* and by-products in cats.

Product	Dose	Results	Ref.
LY SC (strain MUCL 39885; Biosprint®, Prosol S.p.A.)	0 (CON) or 7.5×10^{10} CFU SC/kg feed daily for 35 days (SUP)	Digestion: - ↑ Fecal DM (day 28).	(73)
SCFP (XPC™, Diamond V)	0% (CON), 0.3% (SUP1) or 0.6% (SUP2) SCFP of diet for 25-day periods	Fecal microbiota: - ↓ *Clostridium perfringens*. Hindgut fermentation products (fecal): - ↑ Lactic acid. - ↓ Isovaleric in SUP1. Digestion: - ↑ ATTD of CF and ash. - ↓ GE in SUP2.	(107)
Yeast extract (Nupro®, Alltech Brasil Ltda.)	Exp. 1: 0% (CON) vs. 30% replacement by yeast extract (SUP). 10-day periods. Exp. 2: 0% (CON) vs. 2% (SUP1), 4% (SUP2), 6% (SUP3), 8% (SUP4) and 10% (SUP5) replacement. 15-day periods. Exp. 3: 0% vs. 2% replacement with yeast extract. 4 days.	Exp. 1: yeast extract as an ingredient: - According to ATTD, yeast extract is comparable to other protein sources. Exp. 2: yeast extract as a functional additive: - No changes. Exp. 3: yeast extract as a palatability agent: - Preference for control diet was observed.	(108)
Yeast extract (Nupro®, Alltech Brasil Ltda.)	0% (CON), 0.6% phosphoric acid (SUP1), 1.5% yeast extract (SUP2), combination of A + Y (SUP3) for 15-day period.	Digestion: - ↑ ATTD of DM and ashes in SUP3. - ↓ DM intake in SUP1 and SUP2.	(109)
Yeast extract	0 (CON), 0.2 (SUP1), 0.4 (SUP2) or 0.6% (SUP3) yeast extract in wet food	- Changes on the intestinal mucosa in SUP3 were noticed, related with an inflammatory response.	(110)
YCW	Exp. 1: 0 (CON), 0.2 (SUP1), 0.4 (SUP2) or 0.6% (SUP3) YCW in wet food, for 30 days. Exp. 2 (25 days): 0 (CON), 0.2 (SUP1), 0.4 (SUP2) or 0.6% (SUP3) YCW in dry food, for 25 days. Exp. 3: 0 and 0.4% YCW in wet food for 10 days.	Exp. 1: no changes. Exp. 2: ↑ ATTD of DM, linearly with dosage. Exp. 3: ↓ preference.	(111)
Yeast-derived MOS (Bio-MOS, Alltech Inc.)	0% (CON), 0.2% (SUP1) 0.4% (SUP2) and 0.6% (SUP) inclusion of YCW for 34-day periods	Fecal microbiota: - ↓ *Clostridium perfringens* (linear) and *Escherichia coli* (quadratic). - ↑ *Bifidobacterium* and *Lactobacillus* with dosage. Hindgut fermentation products (fecal): - ↑ Butyrate, valerate, total biogenic amines, putrescine, cadaverine and histamine increased with dosage. Digestion: - ↑ Ash ATTD of ash. Increased with YCW content.	(112)

AHEE, acid-hydrolyzed ether extract; ATTD, apparent total tract digestibility; BW, body weight; CF, crude fiber; CFU, colony forming units; CON, control group; CP, crude protein; DM, dry matter; GE, gross energy; LY, live yeast; OM, organic matter; Ref., reference; SC, *Saccharomyces cerevisiae*; SCFA, short-chain fatty acids; SUP, supplemented group; YCW, yeast cell wall.

supplemented with MOS whereas supplementation with both FOS and MOS resulted in a tendency of lower total anaerobe concentration compared with control (95), although no impact on *Bifidobacterium* was noted in this case.

Increases in *Lactobacillus* and *Bifidobacterium* would likely yield increased lactic acid and short-chain fatty acids (SCFA), which could be the reason behind the lower fecal pH observed by some authors after the inclusion of yeast derivatives in dogs' diets along with decreased fecal ammonia (27, 84). Bastos et al. (5) also observed lower fecal ammonia levels after an abrupt dietary change in dogs supplemented with live yeast compared to control ones. However, these finding have not been consistent as other authors have reported higher fecal pH (82, 90, 95) or no change (24, 74, 81, 86).

Short chain fatty acids are rapidly absorbed by colonocytes, thus determining their levels *in vivo* can be challenging, perhaps contributing to the varying results reported. Unaltered fecal concentrations of SCFA have been reported when yeast or yeast by-products were used (24, 74, 81, 95, 97). However, decreases in isovalerate and/or increases in propionate, butyrate, acetate and total SCFA when using yeast products have been reported by different authors (5, 75, 81, 95). Van den Abbeele et al. (85) also observed increases in acetate, butyrate and propionate when adding YCW in their *in vitro* experiment simulating the canine gut. Moreover, concentration of fecal phenol and/or total phenol + indole were lower in dogs supplemented with yeast products (5, 75, 81, 95). Total fecal biogenic amines decreased significantly in dogs receiving live yeast (5) (5). These results may indicate the possible beneficial effects of yeast and yeast by-products on the canine intestinal functionality.

Yeast cell wall supplementation in cats also led to increased fecal *Bifidobacterium* and *Lactobacillus*, and reductions of *Clostridium perfringens* (*C. perfringens*), *E. coli*, *Hafnia alvei*, and *Shigella* spp. (112). The inclusion of SCFP in cats' diet reduced *C. perfringens*

abundance, however no change for *Bifidobacterium*, *Lactobacillus*, or *E. coli* was observed (107). Supplementation with a microencapsulation of probiotic strains which include *S. cerevisiae* for 20 days resulted in fecal *Lactobacillus* count increase, but the effect of yeast cannot be isolated in this study (113). Aquino et al. (110) did not find differences in lactic acid bacteria counts nor clostridium-reductor when substituting up to 0.6% of the wet diet with yeast extract in cats.

There were no differences in cats' fecal pH when SCFP or yeast extract was supplemented (107, 109). Matheus et al. (107) compared a control diet without SCFP with two different inclusion levels (0.3 and 0.6% of the diet), and failed to find differences in fecal pH, ammonia, or SCFA between treatments (except for isovaleric acid, which was lower in 0.3% SCFP inclusion group), However they did report higher fecal lactic acid concentration in cats supplemented with SCFP compared with control ones. On the other hand, lactic acid was not changed and neither propionate, isovalerate, acetate, isobutyrate, total SCFA, and total BCFA when YCW was included in the feed at 0.2, 0.4, or 0.6% of the diet (112). These last authors also found that the fecal concentrations of cadaverine, histamine, putrescine, and total biogenic amines increased linearly with the inclusion of YCW. Collectively, these studies present a lack of consistent responses to yeast or yeast derivatives, which may suggest strain specific impact, but that too will require additional assessments.

4.2. Immunomodulatory properties

Immunity is certainly an area of interest for functional ingredients and may be positively influenced by yeast intake, at least in dogs. Supplementation of dogs with YCW, alone or in combination with FOS, resulted in increased ileal and fecal IgA (86, 90, 95), indicating enhanced mucosal immunity. Some of these authors also found serum IgA increases of around 20% compared with control when including YCW at 0.45–0.5% of the diet. Lin et al. (81), on the other hand, did not find differences in serum IgA, IgE, IgG, and IgM when supplementing SCFP compared to control, but higher SCFP dosages led to higher IgE concentrations. However, these latter authors found that dogs supplemented with SCFP had increased population of antigen-presenting cells, including B-cells and monocytes expressing surface major histocompatibility complex class II. One hypothesis would be that fewer immune cells are needed when immune capacity is enhanced. Moreover, in the same study cells obtained from dogs supplemented with SCFP had a lower TNF-α secretion when stimulated with toll-like receptor (TLR) agonists compared to control dogs, suggesting an anti-inflammatory effect of SCFP. Accordingly, lower pro-inflammatory interleukin 6 (IL-6) and TNF-α, as well as higher blood monocyte phagocytic index, were shown by dogs receiving YCW in the study by Theodoro et al. (87). This increased phagocytic index was also observed with the inclusion of SCFP (79), althought others authors did not report any changes with yeast hydrolysate supplementation (82). The ingestion of yeast-derived β-glucan also produced lower levels of TNF-α in obese dogs (98), as well as lower serum concentrations of IL-6, increased anti-inflammatory IL-10, reduced haptoglobin (marker of inflammatory status), and tended to reduce gut permeability (97, 101).

Pawar et al. (92) found an enhanced cell-mediated immune response, CD4+ lymphocyte population and CD4+:CD8+ ratio in dogs supplemented with MOS. In contrast, Kroll et al. (91), did not observe changes in those cell communities when supplementing adult or senior dogs a second-generation MOS, rich in mannoproteins.

Studies with dogs involving stress challenges suggest some benefits related to the modulation of the immune system as well. Dogs supplemented with SCFP and challenged with exercise and transport stress had increased serum TNF-α and decreased IgE and IgG, although the vitality of these animals was higher (80). In another study involving transport stress challenge, SCFP addition caused stable or decreased cyclooxygenase-2 and myeloperoxidase mRNA expression, two key inflammatory mediators. These findings suggest a positive impact through suppression of innate immune cell activation against stress.

The supplementation of β-glucans derived from *S. cerevisiae* also influenced the humoral immune response to vaccination, affecting IgA and IgM responses (104), whereas Traughber et al. (99) did not observe any effect.

The modulatory effect of yeasts in the immune system is especially relevant in the treatment of some inflammatory diseases; in fact, β-glucan was superior to levamisole in the treatment of dogs affected by Inflammatory Bowel Disease (IBD) (101) and had promising results treating dogs with signs of canine atopy and osteoarthritis (102, 103).

Hematological changes due to yeast supplementation, similar to other parameters, vary greatly among the studies. Some studies have found an increase within the reference ranges in mean corpuscular volume and mean corpuscular hemoglobin concentration and no effect on white blood cells (WBC) counts in dogs fed live yeast (74). Others noted a decrease in WBC, monocyte and eosinophils counts in dogs given SCFP or YCW components (81, 90). Although these last authors did not find differences in lymphocyte counts, others found an increase (95) and a decrease (78, 94) when supplementing yeast derivatives to dogs.

Although the number of studies evaluating immune modulation in dogs is limited, even less is known regarding to cat. Matheus et al. (107) did not report any difference in cats supplemented with SCFP when comparing CD4+ or CD8+ lymphocytes, CD4+/CD8+ ratio, or phagocytosis index, among other parameters involved in immune response which also did not change. Hematological changes were neither observed when yeast extract was supplemented up to 10% in the diet for cats (108).

4.3. Inhibition of pathogens and management of gastrointestinal disorders

As previously discussed, supplementation of yeast and its derivatives can modulate gut microbiota, providing possible benefits. This subsection will go through the changes in potentially pathogenic microorganisms that also inhabit the gastrointestinal tract, and the role that yeast can have in the management of some gastrointestinal disorders.

Although *E. coli* can sometimes have a positive effect in the gut, such as stimulation of the immune system or vitamin K production, its proliferation is more often associated with a negative impact. In this regard, the supplementation of live yeast, autolyzed YCW and MOS from *S. cerevisiae* has resulted in lower counts of *E. coli* (5, 74, 90, 94), although some other authors have not found differences with addition of MOS, FOS, or the combination of both (95). Even in an *in vitro*

experiment simulating the canine gut, adding YCW decreased the presence of the Enterobacteriaceae family, which includes *E. coli* (85). Another potentially harmful bacteria that could be modulated by yeast is *C. perfringens*. Whereas some authors did not find differences with MOS supplementation (95), other studies have reported higher *C. perfringens* counts with yeast extract supplementation but neither was accompanied by diarrhea (86, 90). However, Oba et al. (76) observed that *C. perfringens* abundance increased in control dogs and was stable for dogs fed SCFP for 21 days. Similarly, decreases in other species of *Clostridium* such as *Clostridium celerecrescens* and *Clostridium saccharolyticum* were observed in dogs fed YCW (88, 89). Bastos et al. (5) reported that control dogs, compared with dogs receiving a live yeast supplementation, showed upregulation in genes related to virulence factors, antibiotic resistance, and osmotic stress.

The use of *S. boulardii* in dogs with gastrointestinal disorders has also been studied, with promising results. One publication focused on the use of this yeast as a probiotic in dogs with diarrhea induced by lincomycin (114). Twenty-four dogs were given lincomycin intramuscularly (150 mg/kg BW/d) and three groups were formed ($n = 8$): (I) no *S. boulardii*, (II) *S. boulardii* (1,000 mg/d orally for 10 days) after lincomycin induced diarrhea onset, and (III) *S. boulardii* (1,000 mg/d orally for 10 days) together with lincomycin. The results showed that groups I and II had diarrhea and lower fecal SCFA concentrations, but not group III, and the duration of this gastrointestinal alteration was shorter in group II compared to control. Another publication used *S. boulardii* in dogs with chronic enteropathy (CE) (115). Four healthy dogs and 20 dogs with CE were used and *S. boulardii* was administered at a dose of 1×10^9 CFU/kg BW orally in capsules every 12 h. In healthy dogs, the administration of *S. boulardii* for 10 days reached a steady state in 5 days and was eliminated completely 4 days after administration, without side effects. Dogs with CE received placebo or *S. boulardii* for 60 days: the yeast significantly improved clinical activity index, stool frequency, and consistency and body condition score over control dogs.

Lastly, yeast cell wall could also have a positive impact in recovering gut health. Dogs fed MOS and experimentally infected with different strains of enteropathogenic *E. coli* recovered faster than control ones, indicating an improvement in the defense system of the animals (93). On the other hand, yeast-derived β-glucan were superior against levamisole in the treatment of dogs affected by IBD, achieving a fast recovery with no recurrence, at least in 6 months (101).

4.4. Management of obesity

Components of yeasts such as β-glucan could play a role in obesity prevention and treatment, as obese dogs fed yeast-derived β-glucan had lower plasma basal glycemic values, serum cholesterol and triglyceride levels (98). Reduced serum total- and LDL-cholesterol concentration was also reported in dogs supplemented with MOS (92) and β-glucans (97, 98) from SC.

However, the potential effect of yeast on weight is not clear. Several studies reported no effect on feed intake or body weight (BW) in dogs (24, 73, 81, 86, 90, 97, 105). However, Stercova et al. (74) observed that dogs fed capsulated live yeast SC showed a higher increase of BW compared with the control ones, but the difference was very limited. Increases in intake ratio for diets containing yeast products when compared to diets without yeast have also been reported, although they were not accompanied by differences in dry matter intake or body weight increase (83, 84).

Lastly, a study involving dogs with hyperthyroidism showed that the cessation of yeast as a vitamin B1 source in hypothyroid dogs resulted in loss of appetite and anorexia (106).

4.5. Effects on digestion

Regarding the potential effect of yeast on digestive function and digestibility of nutrients, results in the literature are variable and sometimes contradictory, although the most common findings are decreases in CP ATTD and occasionally increases in fiber ATTD.

Live yeast supplementation had no significant effect on DM, ash, crude fiber (CF), CP and fat ATTD (5); only non-digestible fiber ATTD was higher in supplemented dogs (74). Santos et al. (79) also found increased ATTD of crude fiber when SCFP was added, but decreased ATTD of CP, nitrogen-free extract and metabolizable energy.

On the other hand, Middelbos et al. (90) tried different doses of YCW inclusion on the diet (from 0 to 0.65% of the diet, mostly MOS) and reported increases in DM, organic matter (OM), CP, and gross energy (GE) ileal digestibility compared to control animals but decreases in DM and CP ATTD. Similarly, YCW included as 1.4% of the diet resulted in reduced ATTD of DM, OM, CP, fat, and GE (89) and MOS as 5% of the diet reduced ATTD of DM, CP and nitrogen-free extracts (27). Addition of yeast extract or dried yeast also lowered ATTD of different macronutrients (84, 105). Protein excretion may result from increased bacterial growth and excretion due to increased fermentation, underestimating CP digestibility and, in turn, lowering OM and DM digestibility. However, other studies with various kinds of yeast products (MOS, β-glucans, SCFP and SC from Brewer's yeast) did not find significant differences neither in ileal nor in apparent total tract nutrient digestibilities when compared to control diet (24, 75, 81, 83, 92, 94–96).

Fecal score was higher (wetter feces) in some studies supplementing SCFP, YCW or MOS to dogs when compared to control groups, although the values remained within the ideal score range (81, 89). Martins et al. (83) observed that dietary yeast inclusion of 30% of the diet resulted in diarrhea and soft feces but an inclusion of up to 15% was suitable, without changes in fecal consistency. However, the inclusion of a live SC yeast as in 7×10^{10} CFU/kg feed resulted in significant higher fecal dry matter, although the magnitude of the changes was of questionable biological relevance (73). Nevertheless, many of the other studies mentioned in this section did not find any differences in fecal score with the dietary inclusion of yeast products generally up to 5% of the diet, with the exception of the 12% inclusion of yeast extract in Kaelle et al. (84).

As for cats, regarding digestibility, there were no differences in nutrient and energy utilization when yeast extract was included in the feed up to 10% in the diet (108) or when yeast extract was added without an acidifying agent (109). Matheus et al. (107) also found relatively similar results, except for CF and ash digestibility, which were higher with SCFP inclusion, although the authors recommend taking these results with cautions as they warn that there may be calculation errors due to low fiber concentration of the diets. Yeast cell wall inclusion also resulted in improved DM and ash digestibility in cats (112).

4.6. Kidney and liver health

Dogs kidney and liver function assessed through serum urea, creatinine, alkaline phosphatase (ALP), and alanine transaminase (ALT) seems to be unaltered in some studies where dogs were supplemented with live yeast or yeast by-products (74, 81). However, the activity of ALT and aspartate aminotransferase (ASP) was increased in dogs supplemented with yeast hydrolysate during and after 28 days of supplementation (82). This same study showed increased serum urea levels in dogs receiving the hydrolysate in the post-treatment period whereas total protein, albumin, glucose, and ALP did not change significantly.

4.7. Potential antioxidant properties

Very few studies have assessed the effect of *S. cerevisiae* on the oxidative status of dogs, although some positive results have been obtained. Supplementation with SCFP at a dose of 500 mg/day decreased thioboarbituric acid reactive substance and increased total antioxidant capacity in serum of dogs exposed to exercise and transport stress, which could indicate an enhanced protection against oxidative damage (80). Wilson et al. (78) also measured markers and expression of genes related to oxidative stress in dogs fed SCFP compared to control ones and observed that superoxide dismutase enzyme increased from baseline and mRNA expression of catalase was lower. However, more investigation is necessary as another study of the same authors showed that SCFP-fed dogs increased malondialdehyde and 8-isoprostane levels due to transport stress (77). Lastly, the supplementation of MOS or SCFP (125, 250, and 500 mg/day) did not exert any effect on erythrocytic antioxidant indices when compared to the non-supplemented group (81, 92).

4.8. Skin and hair

To the knowledge of the authors, only two studies have investigated the effects of *S. cerevisiae* supplementation on skin and hair health of dogs. A tendency for higher change from baseline in sebum concentrations was observed in dogs fed SCFP that did not result in greasy hair coat (78). In this study, higher Transepidermal Water Loss (TEWL) in the ear region was also measured in supplemented dogs, possibly indicating enhanced skin integrity. However, TEWL in the back region was higher in control dogs but the authors of the study warn that this may be due to alterations during sampling. In fact, Beloshapka et al. (88) found no difference in the skin condition score in the tail region or any other skin and coat scores when dogs were supplemented with YCW.

4.9. Impact on palatability

Palatability tests comparing standard diets with diets including yeast products have resulted in a preference for the latter, probably due to the presence of glutamic acid and nucleotides, which can intensify flavors of the food (75, 81, 84). These results indicate that yeast or yeast by-products are well accepted by dogs, so their inclusion in commercial diets would not cause refusal and thereby negatively affect the food intake and body condition.

Regarding *S. boulardii*, there is one publication about the production of meat pet snacks containing this yeast that studied the production process, viability and acceptability of the snacks, showing that the addition of yeast had a good acceptance (116).

Contrary to dogs, in which the use of live yeast or derivatives seemed to increase palatability, results in cats are not uniform and tend to show worse palatability with yeast supplementation. No difference in feed intake compared to control group was found when evaluating the use of live yeast, SCFP or YCW (73, 107, 112).

On the other hand, Lima et al. (108) evaluated the use of yeast extract as a palatability agent observing that cats preferred the control diet over 2% inclusion yeast diet. Similar to Lima, Ogoshi et al. (109) found that the inclusion of yeast extract at 1.5% of the diet reduced feed consumption. Aquino et al. (111) reported lower palatability with wet diets that contained a 0.4% of YCW. Lower preference for yeast extract seems to be countered with the addition of sodium pyrophosphate; this blend was more preferred than control (117). Excess of umami taste and monosodium glutamate could reduce palatability and cats could have a lower threshold of acceptance. Moreover, processing may affect the flavor profile so thermal processes can decrease the flavor-enhancing effect and increase the acceptance of the ingredient. It is also hypothesized that the rejection of yeast extract is due to its high content in leucine as cats reject amino acids "bitter" for humans such as this one.

5. Concluding remarks and future perspectives

Pet humanization has led to an increase in the demand for functional ingredients in the pet food industry. Owners are more concerned about pet health and wellness and are willing to invest in diets with superior properties even if it means that they are more expensive, as long as their dogs and cats are healthier. The wide range of yeast-based products with potential to be used as prebiotic, probiotic, or postbiotic is of great interest at present, as they are becoming more related to the successful treatment of diseases. Based on the fact that the main scientific knowledge is gathered from human and livestock studies, further investigations are still required in pet species to better understand the mode of action, doses, safety, and guidelines for use of these products.

While the gut will probably remain the heartland of research, new clinical applications will continue to expand in other research areas including immune system, skin, nervous system, oral cavity, kidney, respiratory system, and weight management field. Thanks to significant and accelerating advances in biotechnologies and bioinformatics methodologies, new research will better characterize mechanistic insights into the mode of action of yeast and their derivatives, leading to a more targeted-oriented development of *S. cerevisiae*-based products.

Author contributions

MM: Methodology, Writing – review & editing. LC: Methodology, Writing – review & editing, Supervision. SM-O: Methodology, Supervision, Writing – review & editing. AM: Writing – review & editing. OC: Writing – review & editing. AF: Writing – review & editing. AA: Writing – review & editing, Funding acquisition, Methodology, Supervision.

Funding

The author(s) declare that no financial support was received for the research, authorship, and/or publication of this article.

Conflict of interest

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

Publisher's note

All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

References

1. Shurson GC. Yeast and yeast derivatives in feed additives and ingredients: sources, characteristics, animal responses, and quantification methods. *Anim Feed Sci Technol*. (2018) 235:60–76. doi: 10.1016/j.anifeedsci.2017.11.010

2. Patterson R, Rogiewicz A, Kiarie EG, Slominski BA. Yeast derivatives as a source of bioactive components in animal nutrition: a brief review. *Front Vet Sci*. (2022) 9:1067383. doi: 10.3389/fvets.2022.1067383

3. Hill C, Guarner F, Reid G, Gibson GR, Merenstein DJ, Pot B, et al. The international scientific association for probiotics and prebiotics consensus statement on the scope and appropriate use of the term probiotic. *Nat Rev Gastroenterol Hepatol*. (2014) 11:506–14. doi: 10.1038/nrgastro.2014.66

4. Fietto JLR, Araújo RS, Valadão FN, Fietto LG, Brandão RL, Neves MJ, et al. Molecular and physiological comparisons between *Saccharomyces cerevisiae* and *Saccharomyces boulardii*. *Can J Microbiol*. (2004) 50:615–21. doi: 10.1139/w04-050

5. Bastos TS, Souza CMM, Legendre H, Richard N, Pilla R, Suchodolski JS, et al. Effect of yeast *Saccharomyces cerevisiae* as a probiotic on diet digestibility, fermentative metabolites, and composition and functional potential of the fecal microbiota of dogs submitted to an abrupt dietary change. *Microorganisms*. (2023) 11:506. doi: 10.3390/microorganisms11020506

6. Moyad MA, Robinson LE, Kittelsrud JM, Reeves SG, Weaver SE, Guzman AI, et al. Immunogenic yeast-based fermentation product reduces allergic rhinitis-induced nasal congestion: a randomized, double-blind, placebo-controlled trial. *Adv Ther*. (2009) 26:795–804. doi: 10.1007/s12325-009-0057-y

7. Gibson GR, Hutkins R, Sanders ME, Prescott SL, Reimer RA, Salminen SJ, et al. Expert consensus document: the international scientific association for probiotics and prebiotics (ISAPP) consensus statement on the definition and scope of prebiotics. *Nat Rev Gastroenterol Hepatol*. (2017) 14:491–502. doi: 10.1038/nrgastro.2017.75

8. Salminen S, Collado MC, Endo A, Hill C, Lebeer S, Quigley EMM, et al. The international scientific association of probiotics and prebiotics (ISAPP) consensus statement on the definition and scope of postbiotics. *Nat Rev Gastroenterol Hepatol*. (2021) 18:649–67. doi: 10.1038/s41575-021-00440-6

9. Elghandour MMY, Tan ZL, Abu Hafsa SH, Adegbeye MJ, Greiner R, Ugbogu EA, et al. *Saccharomyces cerevisiae* as a probiotic feed additive to non and pseudo-ruminant feeding: a review. *J Appl Microbiol*. (2020) 128:658–74. doi: 10.1111/jam.14416

10. Perricone V, Sandrini S, Irshad N, Savoini G, Comi M, Agazzi A. Yeast-derived products: the role of hydrolyzed yeast and yeast culture in poultry nutrition. A review. *Animals*. (2022) 12:1426. doi: 10.3390/ani12111426

11. Duysburgh C, Van den Abbeele P, Morera M, Marzorati M. *Lacticaseibacillus rhamnosus* GG and *Saccharomyces cerevisiae* boulardii supplementation exert protective effects on human gut microbiome following antibiotic administration in vitro. *Benefic Microbes*. (2021) 12:365–79. doi: 10.3920/BM2020.0180

12. Moens F, Duysburgh C, van den Abbeele P, Morera M, Marzorati M. *Lactobacillus rhamnosus* GG and *Saccharomyces cerevisiae* boulardii exert synergistic antipathogenic activity in vitro against enterotoxigenic *Escherichia coli*. *Benefic Microbes*. (2019) 10:923–35. doi: 10.3920/BM2019.0064

13. Wang H, Chen G, Li X, Zheng F, Zeng X. Yeast β-glucan, a potential prebiotic, showed a similar probiotic activity to inulin. *Food Funct*. (2020) 11:10386–96. doi: 10.1039/D0FO02224A

14. Hernández-Macias S, Comas-Basté O, Jofré A, Bover-Cid S, Latorre-Moratalla ML, Vidal-Carou MC. Growth-promoting effect of cava lees on lactic acid Bacteria strains: a potential revalorization strategy of a winery by-product. *Foods*. (2021) 10:1636. doi: 10.3390/foods10071636

15. Tanihiro R, Sakano K, Oba S, Nakamura C, Ohki K, Hirota T, et al. Effects of yeast mannan which promotes beneficial bacteroides on the intestinal environment and skin condition: a randomized, double-blind, placebo-controlled study. *Nutrients*. (2020) 12:1–16. doi: 10.3390/nu12123673

16. Sanchez NCB, Broadway PR, Carroll JA. Influence of yeast products on modulating metabolism and immunity in cattle and swine. *Animals*. (2021) 11:1–14. doi: 10.3390/ani11020371

17. Liu Y, Wu Q, Wu X, Algharib SA, Gong F, Hu J, et al. Structure, preparation, modification, and bioactivities of β-glucan and mannan from yeast cell wall: a review. *Int J Biol Macromol*. (2021) 173:445–56. doi: 10.1016/j.ijbiomac.2021.01.125

18. Thomas S, Rezoagli E, Abidin IZ, Major I, Murray P, Murphy EJ. Beta-glucans from yeast. Immunomodulators from novel waste resources. *Appl Sci*. (2022) 12:5208. doi: 10.3390/app12105208

19. Heuvelin E, Lebreton C, Grangette C, Pot B, Cerf-Bensussan N, Heyman M. Mechanisms involved in alleviation of intestinal inflammation by *Bifidobacterium Breve* soluble factors. *PLoS One*. (2009) 4:5184. doi: 10.1371/journal.pone.0005184

20. Okada Y, Tsuzuki Y, Hokari R, Komoto S, Kurihara C, Kawaguchi A, et al. Anti-inflammatory effects of the genus Bifidobacterium on macrophages by modification of phospho-IκB and SOCS gene expression. *Int J Exp Pathol*. (2009) 90:131–40. doi: 10.1111/j.1365-2613.2008.00632.x

21. Riedel CU, Foata F, Philippe D, Adolfsson O, Eikmanns BJ, Blum S. Anti-inflammatory effects of bifidobacteria by inhibition of LPS-induced NF-kappaB activation. *World J Gastroenterol*. (2006) 12:3729–35. doi: 10.3748/wjg.v12.i23.3729

22. Nguyen TH, Fleet GH, Rogers PL. Composition of the cell walls of several yeast species. *Appl Microbiol Biotechnol*. (1998) 50:206–12. doi: 10.1007/s002530051278

23. Ma T, Tu Y, Zhang NF, Guo JP, Deng KD, Zhou Y, et al. Effects of dietary yeast β-glucan on nutrient digestibility and serum profiles in pre-ruminant Holstein calves. *J Integr Agric*. (2015) 14:749–57. doi: 10.1016/S2095-3119(14)60843-1

24. Kilburn-Kappeler LR, Aldrich CG. Evaluation of a yeast β-glucan blend in a pet food application to determine its impact on stool quality, apparent nutrient digestibility, and intestinal health when fed to dogs. *Front Anim Sci*. (2023) 4:1125061. doi: 10.3389/fanim.2023.1125061

25. El-Bab AFF, Saghir SAM, El-Naser IAA, El-Kheir SMMA, Abdel-Kader MF, Alruhaimi RS, et al. The effect of dietary *Saccharomyces cerevisiae* on growth performance, oxidative status, and immune response of sea bream (*Sparus aurata*). *Life*. (2022) 12:1013. doi: 10.3390/life12071013

26. Matur E, Ergul E, Akyazi I, Eraslan E, Cirakli ZT. The effects of *Saccharomyces cerevisiae* extract on the weight of some organs, liver, and pancreatic digestive enzyme activity in breeder hens fed diets contaminated with aflatoxins. *Poult Sci*. (2010) 89:2213–20. doi: 10.3382/ps.2010-00821

27. Zentek J, Marquart B, Pietrzak T. Intestinal effects of mannanoligosaccharides, transgalactooligosaccharides, lactose and lactulose in dogs. *J Nutr*. (2002) 132:1682S–4S. doi: 10.1093/jn/132.6.1682S

28. Kaźmierczak-Siedlecka K, Ruszkowski J, Fic M, Folwarski M, Makarewicz W. *Saccharomyces boulardii* CNCM I-745: a non-bacterial microorganism used as probiotic agent in supporting treatment of selected diseases. *Curr Microbiol*. (2020) 77:1987–96. doi: 10.1007/s00284-020-02053-9

29. Czerucka D, Rampal P. Diversity of *Saccharomyces boulardii* CNCM I-745 mechanisms of action against intestinal infections. *World J Gastroenterol*. (2019) 25:2188–203. doi: 10.3748/wjg.v25.i18.2188

30. Tiago FCP, Martins FS, Souza ELS, Pimenta PFP, Araujo HRC, Castro IM, et al. Adhesion to the yeast cell surface as a mechanism for trapping pathogenic bacteria by

Saccharomyces probiotics. *J Med Microbiol*. (2012) 61:1194–207. doi: 10.1099/jmm.0.042283-0

31. Firon N, Ashkenazi S, Mirelman D, Ofek I, Sharon N. Aromatic alpha-glycosides of mannose are powerful inhibitors of the adherence of type 1 fimbriated *Escherichia coli* to yeast and intestinal epithelial cells. *Infect Immun*. (1987) 55:472–6. doi: 10.1128/iai.55.2.472-476.1987

32. Neeser JR, Koellreutter B, Wuersch P. Oligomannoside-type glycopeptides inhibiting adhesion of *Escherichia coli* strains mediated by type 1 pili: preparation of potent inhibitors from plant glycoproteins. *Infect Immun*. (1986) 52:428–36. doi: 10.1128/iai.52.2.428-436.1986

33. Spring P, Wenk C, Connolly A, Kiers A. A review of 733 published trials on bio-Mos®, a mannan oligosaccharide, and Actigen®, a second generation mannose rich fraction, on farm and companion animals. *J Appl Anim Nutr*. (2015) 3:1–11. doi: 10.1017/jan.2015.6

34. Buts JP, Bernasconi P, Van Craynest MP, Maldague P, Meyer R. Response of human and rat small intestinal mucosa to oral administration of *Saccharomyces boulardii*. *Pediatr Res*. (1986) 20:192–6. doi: 10.1203/00006450-198602000-00020

35. Buts JP, Bernasconi P, Vaerman JP, Dive C. Stimulation of secretory IgA and secretory component of immunoglobulins in small intestine of rats treated with *Saccharomyces boulardii*. *Dig Dis Sci*. (1990) 35:251–6. doi: 10.1007/BF01536771

36. Stier H, Bischoff SC. Influence of *Saccharomyces boulardii* CNCM I-745 on the gut-associated immune system. *Clin Exp Gastroenterol*. (2016) 9:269–79. doi: 10.2147/CEG.S111003

37. Castagliuolo I, Thomas Lamont J, Nikulasson ST, Pothoulakis C. *Saccharomyces boulardii* protease inhibits *Clostridium difficile* toxin a effects in the rat ileum. *Infect Immun*. (1996) 64:5225–32. doi: 10.1128/iai.64.12.5225-5232.1996

38. Castagliuolo I, Riegler MF, Valenick L, LaMont JT, Pothoulakis C. *Saccharomyces boulardii* protease inhibits the effects of *Clostridium difficile* toxins A and B in human colonic mucosa. *Infect Immun*. (1999) 67:302–7. doi: 10.1128/IAI.67.1.302-307.1999

39. Qamar A, Aboudola S, Warny M, Michetti P, Pothoulakis C, LaMont JT, et al. *Saccharomyces boulardii* stimulates intestinal immunoglobulin A immune response to *Clostridium difficile* toxin A in mice. *Infect Immun*. (2001) 69:2762–5. doi: 10.1128/IAI.69.4.2762-2765.2001

40. Lacotte PA, Simons A, Bouttier S, Malet-Villemagne J, Nicolas V, Janoir C. Inhibition of *in vitro Clostridioides difficile* biofilm formation by the probiotic yeast *Saccharomyces boulardii* CNCM I-745 through modification of the extracellular matrix composition. *Microorganisms*. (2022) 10:1082. doi: 10.3390/microorganisms10061082

41. Izadnia F, Wong CT, Kocoshis SA. Brewer's yeast and *Saccharomyces boulardii* both attenuate *Clostridium difficile*-induced colonic secretion in the rat. *Dig Dis Sci*. (1998) 43:2055–60. doi: 10.1023/A:1018811331596

42. Martins F, Nardi R, Arantes R, Rosa C, Neves M, Nicoli J. Screening of yeasts as probiotic based on capacities to colonize the gastrointestinal tract and to protect against enteropathogen challenge in mice. *J Gen Appl Microbiol*. (2005) 51:83–92. doi: 10.2323/jgam.51.83

43. Armando M, Dogi C, Pizzolitto R, Escobar F, Peirano M, Salvano M, et al. *Saccharomyces cerevisiae* strains from animal environment with in vitro aflatoxin B1 binding ability and anti-pathogenic bacterial influence. *World Mycotoxin J*. (2010) 4:59–68. doi: 10.3920/WMJ2010.1208

44. Brandão RL, Castro IM, Bambirra EA, Amaral SC, Fietto LG, Tropia MJM, et al. Intracellular signal triggered by cholera toxin in *Saccharomyces boulardii* and *Saccharomyces cerevisiae*. *Appl Environ Microbiol*. (1998) 64:564–8. doi: 10.1128/AEM.64.2.564-568.1998

45. Roussel C, Sivignon A, de Vallée A, Garrait G, Denis S, Tsilia V, et al. Anti-infectious properties of the probiotic *Saccharomyces cerevisiae* CNCM I-3856 on enterotoxigenic *E. coli* (ETEC) strain H10407. *Appl Microbiol Biotechnol*. (2018) 102:6175–89. doi: 10.1007/s00253-018-9053-y

46. Sabbatini S, Monari C, Ballet N, Mosci P, Decherf AC, Pélerin F, et al. *Saccharomyces cerevisiae*–based probiotic as novel anti-microbial agent for therapy of bacterial vaginosis. *Virulence*. (2018) 9:954–66. doi: 10.1080/21505594.2018.1464362

47. Guo W, Gu X, Tong Y, Wang X, Wu J, Chang C. Protective effects of mannan/β-glucans from yeast cell wall on the deoxyniyalenol-induced oxidative stress and autophagy in IPEC-J2 cells. *Int J Biol Macromol*. (2019) 135:619–29. doi: 10.1016/j.ijbiomac.2019.05.180

48. Liu G, Yu L, Martínez Y, Ren W, Ni H, Abdullah Al-Dhabi N, et al. Dietary *Saccharomyces cerevisiae* Cell Wall extract supplementation alleviates oxidative stress and modulates serum amino acids profiles in weaned piglets. *Oxidative Med Cell Longev*. (2017) 2017:1–7. doi: 10.1155/2017/3967439

49. Zhang J, Fang Y, Fu Y, Jalukar S, Ma J, Liu Y, et al. Yeast polysaccharide mitigated oxidative injury in broilers induced by mixed mycotoxins via regulating intestinal mucosal oxidative stress and hepatic metabolic enzymes. *Poult Sci*. (2023) 102:102862. doi: 10.1016/j.psj.2023.102862

50. Guo H, Guo S, Liu H. Antioxidant activity and inhibition of ultraviolet radiation-induced skin damage of selenium-rich peptide fraction from selenium-rich yeast protein hydrolysate. *Bioorg Chem*. (2020) 105:104431. doi: 10.1016/j.bioorg.2020.104431

51. Schlemm DJ, Crowe MJ, McNeill RB, Stanley AE, Keller SJ. Medicinal yeast extracts. *Cell Stress Chaperones*. (1999) 4:171–6. doi: 10.1379/1466-1268(1999)004<0171:MYE>2.3.CO;2

52. Bentley JP, Hunt TK, Weiss JB, Taylor CM, Hanson AN, Davies GH, et al. Peptides from live yeast cell derivative stimulate wound healing. *Arch Surg*. (1990) 125:641–6. doi: 10.1001/archsurg.1990.01410170089019

53. Crowe MJ, McNeill RB, Schlemm DJ, Greenhalgh DG, Keller SJ. Topical application of yeast extract accelerates the wound healing of diabetic mice. *J Burn Care Rehabil*. (1999) 20:155–62. doi: 10.1097/00004630-199903000-00032

54. Goodson W, Hohn D, Hunt TK, Leung DYK. Augmentation of some aspects of wound healing by a "skin respiratory factor". *J Surg Res*. (1976) 21:125–9. doi: 10.1016/0022-4804(76)90072-X

55. Kaplan JZ. Acceleration of wound healing by a live yeast cell derivative. *Arch Surg*. (1984) 119:1005–8. doi: 10.1001/archsurg.1984.01390210009003

56. Gruenstein EI, Schlemm DJ, Bethi M, Keller SJ. The early signaling pathway of live yeast cell derivative in THP-1 monocytes. *Cell Calcium*. (2018) 73:112–20. doi: 10.1016/j.ceca.2018.04.008

57. Du B, Bian Z, Xu B. Skin health promotion effects of natural beta-glucan derived from cereals and microorganisms: a review. *Phyther Res*. (2014) 28:159–66. doi: 10.1002/ptr.4963

58. Oryan A, Jalili M, Kamali A, Nikahval B. The concurrent use of probiotic microorganism and collagen hydrogel/scaffold enhances burn wound healing: an *in vivo* evaluation. *Burns*. (2018) 44:1775–86. doi: 10.1016/j.burns.2018.05.016

59. Espinosa C, Esteban MA. Effect of dietary supplementation with yeast *Saccharomyces cerevisiae* on skin, serum and liver of gilthead seabream (*Sparus aurata* L). *J Fish Biol*. (2020) 97:869–81. doi: 10.1111/jfb.14449

60. Sheikhzadeh N, Heidarieh M, Karimi Pashaki A, Nofouzi K, Ahrab Farshbafi M, Akbari M. Hilyses®, fermented *Saccharomyces cerevisiae*, enhances the growth performance and skin non-specific immune parameters in rainbow trout (*Oncorhynchus mykiss*). *Fish Shellfish Immunol*. (2012) 32:1083–7. doi: 10.1016/j.fsi.2012.03.003

61. Gaspar LR, Camargo FB, Gianeti MD, Maia Campos PMBG. Evaluation of dermatological effects of cosmetic formulations containing *Saccharomyces cerevisiae* extract and vitamins. *Food Chem Toxicol*. (2008) 46:3493–500. doi: 10.1016/j.fct.2008.08.028

62. Lu Y, Liu M, Cao Y, Yin J, Zhou H, Yu W, et al. Hydrogel sunscreen based on yeast /gelatin demonstrates excellent UV-shielding and skin protection performance. *Colloids Surf B: Biointerfaces*. (2021) 205:111885. doi: 10.1016/j.colsurfb.2021.111885

63. Zülli F, Suter F, Biltz H, Nissen HP. Improving skin function with CM-glucan, a biological response modifier from yeast. *Int J Cosmet Sci*. (1998) 20:79–86. doi: 10.1046/j.1467-2494.1998.171740.x

64. Dhurat R, Sharma A, Surve R, McCoy J, Kovacevic M, Goren A, et al. Novel yeast extract is superior to colloidal oatmeal in providing rapid itch relief. *J Cosmet Dermatol*. (2021) 20:207–9. doi: 10.1111/jocd.13436

65. Zhang Y, Tan Y, Zou Y, Bulat V, Lugovic Mihic L, Kovacevic M, et al. Yeast extract demonstrates rapid itch relief in chronic pruritus. *J Cosmet Dermatol*. (2020) 19:2131–4. doi: 10.1111/jocd.13265

66. Hassan S, Poulos C, Bhatti J, Rangwani S, Khan Z, Mohammed T, et al. *Saccharomyces cerevisiae* as a skin physiology, pathology, and treatment model. *Dermatol Online J*. (2020) 26:1–4. doi: 10.5070/D32611048118

67. Doney E, Cadoret A, Dion-Albert L, Lebel M, Menard C. Inflammation-driven brain and gut barrier dysfunction in stress and mood disorders. *Eur J Neurosci*. (2022) 55:2851–94. doi: 10.1111/ejn.15239

68. Hadizadeh M, Hamidi GA, Salami M. Probiotic supplementation improves the cognitive function and the anxiety-like behaviors in the stressed rats. *Iran J Basic Med Sci*. (2019) 22:506–14. doi: 10.22038/ijbms.2019.33956.8078

69. Babaei F, Mirzababaei M, Mohammadi G, Dargahi L, Nassiri-Asl M. *Saccharomyces boulardii* attenuates lipopolysaccharide-induced anxiety-like behaviors in rats. *Neurosci Lett*. (2022) 778:136600. doi: 10.1016/j.neulet.2022.136600

70. Karbownik MS, Kręczyńska J, Wiktorowska-Owczarek A, Kwarta P, Cybula M, Stilinović N, et al. Decrease in salivary serotonin in response to probiotic supplementation with *Saccharomyces boulardii* in healthy volunteers under psychological stress: secondary analysis of a randomized, double-blind, placebo-controlled trial. *Front Endocrinol*. (2022) 12:1836. doi: 10.3389/fendo.2021.800023

71. Tao D, Zhong T, Pang W, Li X. *Saccharomyces boulardii* improves the behaviour and emotions of spastic cerebral palsy rats through the gut-brain axis pathway. *BMC Neurosci*. (2021) 22:76–16. doi: 10.1186/s12868-021-00679-4

72. Lee H, Jung Y, Chae J. The effects of *Saccharomyces Cerevisiae* yeast extract SCP-20 on stress response, anxiety and depression: a double-blind placebo-controlled trial. *Anxiety Mood*. (2009) 5:8–13.

73. Bampidis V, Azimonti G, Bastos ML, Christensen H, Dusemund B, Fašmon M, et al. Safety and efficacy of a feed additive consisting of *Saccharomyces cerevisiae* MUCL 39885 (Biosprint®) for cats and dogs (Prosol S.P.A.). *EFSA J*. (2021) 19:e06699. doi: 10.2903/j.efsa.2021.6699

74. Stercova E, Kumprechtova D, Auclair E, Novakova J. Effects of live yeast dietary supplementation on nutrient digestibility and fecal microflora in beagle dogs. *J Anim Sci*. (2016) 94:2909–18. doi: 10.2527/jas.2016-0584

75. Bastos TS, Souza CMM, Kaelle GCB, do Nascimento MQ, de Oliveira SG, Félix AP. Diet supplemented with *Saccharomyces cerevisiae* from different fermentation media modulates the faecal microbiota and the intestinal fermentative products in dogs. *J Anim Physiol Anim Nutr*. (2023) 107:30–40. doi: 10.1111/jpn.13824

76. Oba PM, Carroll MQ, Sieja KM, de Souza Nogueira JP, Yang X, Epp TY, et al. Effects of a *Saccharomyces cerevisiae* fermentation product on fecal characteristics, metabolite concentrations, and microbiota populations of dogs subjected to exercise challenge. *J Anim Sci*. (2023) 101, 101:1–13. doi: 10.1093/jas/skac424

77. Wilson SM, Oba PM, Applegate CC, Koziol SA, Panasevich MR, Norton SA, et al. Effects of a *Saccharomyces cerevisiae* fermentation product-supplemented diet on fecal characteristics, oxidative stress, and blood gene expression of adult dogs undergoing transport stress. *J Anim Sci*. (2023) 101:378. doi: 10.1093/jas/skac378

78. Wilson SM, Oba PM, Koziol SA, Applegate CC, Soto-Diaz K, Steelman AJ, et al. Effects of a *Saccharomyces cerevisiae* fermentation product-supplemented diet on circulating immune cells and oxidative stress markers of dogs. *J Anim Sci*. (2022) 100:1–11. doi: 10.1093/jas/skac245

79. Santos KM, Risolia LW, Rentas MF, Amaral AR, Rodrigues RBA, Urrego MIG, et al. *Saccharomyces cerevisiae* dehydrated culture modulates fecal microbiota and improves innate immunity of adult dogs. *Fermentation*. (2021) 8:1–19. doi: 10.3390/fermentation8010002

80. Varney JL, Coon CN, Norton SA. PSV-B-20 effects of *Saccharomyces Cerevisiae* fermentation product (SCFP) postbiotic in labrador retrievers during exercise and transport stress. *J Anim Sci*. (2021) 99:332–3. doi: 10.1093/jas/skab235.611

81. Lin CY, Alexander C, Steelman AJ, Warzecha CM, De Godoy MRC, Swanson KS. Effects of a *Saccharomyces cerevisiae* fermentation product on fecal characteristics, nutrient digestibility, fecal fermentative end-products, fecal microbial populations, immune function, and diet palatability in adult dogs. *J Anim Sci*. (2019) 97:1586–99. doi: 10.1093/jas/skz064

82. Strompfová V, Kubašová I, Mudroňová D, Štempelová L, Takáčová M, Gąsowski B, et al. Effect of hydrolyzed yeast administration on faecal microbiota, haematology, serum biochemistry and cellular immunity in healthy dogs. *Probiot Antimicrob Prot*. (2021) 13:1267–76. doi: 10.1007/s12602-021-09765-9

83. Martins MS, Sakomura NK, Souza DF, Filho FOR, Gomes MOS, Vasconcellos RS, et al. Brewer's yeast and sugarcane yeast as protein sources for dogs. *J Anim Physiol Anim Nutr*. (2014) 98:948–57. doi: 10.1111/jpn.12145

84. Kaelle GCB, Souza CMM, Bastos TS, Vasconcellos RS, Oliveira SG, Félix AP. Diet digestibility and palatability and intestinal fermentative products in dogs fed yeast extract. *Ital J Anim Sci*. (2022) 21:802–10. doi: 10.1080/1828051X.2022.2054733

85. Van den Abbeele P, Duysburgh C, Rakebrandt M, Marzorati M. Dried yeast cell walls high in beta-glucan and mannan-oligosaccharides positively affect microbial composition and activity in the canine gastrointestinal tract *in vitro*. *J Anim Sci*. (2020) 98:1–10. doi: 10.1093/jas/skaa173

86. Lin CY, Carroll MQ, Miller MJ, Rabot R, Swanson KS. Supplementation of yeast cell wall fraction tends to improve intestinal health in adult dogs undergoing an abrupt diet transition. *Front Vet Sci*. (2020) 7:905. doi: 10.3389/fvets.2020.597939

87. Theodoro S, Putarov TC, Tiemi C, Volpe LM, de Oliveira CAF, Mb G, et al. Effects of the solubility of yeast cell wall preparations on their potential prebiotic properties in dogs. *PLoS One*. (2019) 14:e0225659. doi: 10.1371/journal.pone.0225659

88. Beloshapka AN, Dowd SE, Suchodolski JS, Steiner JM, Duclos L, Swanson KS. Fecal microbial communities of healthy adult dogs fed raw meat-based diets with or without inulin or yeast cell wall extracts as assessed by 454 pyrosequencing. *FEMS Microbiol Ecol*. (2013) 84:532–41. doi: 10.1111/1574-6941.12081

89. Beloshapka AN, Duclos LM, Vester BM, Swanson KS. Effects of inulin or yeast cell-wall extract on nutrient digestibility, fecal fermentative end-product concentrations, and blood metabolite concentrations in adult dogs fed raw meat–based diets. *Am J Vet Res*. (2012) 73:1016–23. doi: 10.2460/ajvr.73.7.1016

90. Middelbos IS, Godoy MR, Fastinger ND, Fahey GC. A dose-response evaluation of spray-dried yeast cell wall supplementation of diets fed to adult dogs: effects on nutrient digestibility, immune indices, and fecal microbial populations. *J Anim Sci*. (2007) 85:3022–32. doi: 10.2527/jas.2007-0079

91. Kroll FSA, Putarov TC, Zaine L, Venturini KS, Aoki CG, Santos JPF, et al. Active fractions of mannoproteins derived from yeast cell wall stimulate innate and acquired immunity of adult and elderly dogs. *Anim Feed Sci Technol*. (2020) 261:114392. doi: 10.1016/j.anifeedsci.2020.114392

92. Pawar MM, Pattanaik AK, Sinha DK, Goswami TK, Sharma K. Effect of dietary mannanoligosaccharide supplementation on nutrient digestibility, hindgut fermentation, immune response and antioxidant indices in dogs. *J Anim Sci Technol*. (2017) 59:11. doi: 10.1186/s40781-017-0136-6

93. Gouveia EMMF, Silva IS, Nakazato G, Onselem VJV, Corrêa RAC, Araujo FR, et al. Action of phosphorylated mannanoligosaccharides on immune and hematological responses and fecal consistency of dogs experimentally infected with enteropathogenic *Escherichia coli* strains. *Braz J Microbiol*. (2013) 44:499–504. doi: 10.1590/S1517-83822013000200027

94. Grieshop CM, Flickinger EA, Bruce KJ, Patil AR, Czarnecki-Maulden GL, Fahey GC. Gastrointestinal and immunological responses of senior dogs to chicory and mannan-oligosaccharides. *Arch Anim Nutr*. (2004) 58:483–94. doi: 10.1080/00039420400019977

95. Swanson K, Grieshop C, Flickinger EA, Bauer LL, Healy HP, Dawson KA, et al. Supplemental Fructooligosaccharides and mannanoligosaccharides influence immune function, ileal and total tract nutrient digestibilities, microbial populations and concentrations of protein catabolites in the large bowel of dogs. *J Nutr*. (2002) 132:980–9. doi: 10.1093/jn/132.5.980

96. Strickling JA, Harmon DL, Dawson KA, Gross KL. Evaluation of oligosaccharide addition to dog diets: influences on nutrient digestion and microbial populations. *Anim Feed Sci Technol*. (2000) 86:205–19. doi: 10.1016/S0377-8401(00)00175-9

97. Rummell LM, Steele MA, Templeman JR, Yohe TT, Akhtar N, Lambie JG, et al. A proof of principle study investigating the effects of supplemental concentrated brewer's yeast on markers of gut permeability, inflammation, and fecal metabolites in healthy non-challenged adult sled dogs. *J Anim Sci*. (2022) 100:1–11. doi: 10.1093/jas/skac281

98. Ferreira CS, Vendramini THA, Amaral AR, Rentas MF, Ernandes MC, da Silva FL, et al. Metabolic variables of obese dogs with insulin resistance supplemented with yeast beta-glucan. *BMC Vet Res*. (2022) 18:14–0. doi: 10.1186/s12917-021-03106-2

99. Traughber Z, He F, de Godoy MR, Varney JL, Fowler JW, Coon CN. PSVI-24 immunological effects of a purified 1,3/1,6 β-glucan supplementation in retorted canine diets. *J Anim Sci*. (2020) 98:313–3. doi: 10.1093/jas/skaa278.558

100. Vetvicka V, Oliveira C. β(1-3)(1-6)-D-glucans modulate immune status and blood glucose levels in dogs. *J Pharm Res Int*. (2014) 4:981–91. doi: 10.9734/BJPR/2014/7862

101. Rychlik A, Nieradka R, Kander M, Nowicki M, Wdowiak M, Kołodziejska-Sawerska A. The effectiveness of natural and synthetic immunomodulators in the treatment of inflammatory bowel disease in dogs. *Acta Vet Hung*. (2013) 61:297–308. doi: 10.1556/avet.2013.015

102. Beynen AC, Saris DHJ, Paap PM, van Altena F, Visser EA, Middelkoop J, et al. Dietary beta-1,3/1,6-glucans reduce clinical signs of canine atopy. *Am J Anim Vet Sci*. (2011) 6:146–52. doi: 10.3844/ajavsp.2011.146.152

103. Beynen AC, Legerstee E. Influence of dietary beta-1,3/1,6-glucans on clinical signs of canine osteoarthritis in a double-blind, placebo-controlled trial. *Am J Anim Vet Sci*. (2010) 5:97–101. doi: 10.3844/ajavsp.2010.97.101

104. Stuyven E, Verdonck F, Van Hoek I, Daminet S, Duchateau L, Remon JP, et al. Oral administration of β-1,3/1,6-glucan to dogs temporally changes total and antigen-specific IgA and IgM. *Clin Vaccine Immunol*. (2010) 17:281–5. doi: 10.1128/CVI.00344-09

105. Reilly LM, He F, Rodriguez-Zas SL, Southey BR, Hoke JM, Davenport GM, et al. Use of legumes and yeast as novel dietary protein sources in extruded canine diets. *Front Vet Sci*. (2021) 8:667642. doi: 10.3389/fvets.2021.667642

106. Drill VA. The calorie intake and weight balance of hyperthyroid dogs in relation to vitamin B1 and yeast. *Am J Physiol Content*. (1941) 132:629–35. doi: 10.1152/ajplegacy.1941.132.3.629

107. Matheus LFO, Risolia LW, Ernandes MC, de Souza JM, Oba PM, Vendramini THA, et al. Effects of *Saccharomyces cerevisiae* cell wall addition on feed digestibility, fecal fermentation and microbiota and immunological parameters in adult cats. *BMC Vet Res*. (2021) 17:351–10. doi: 10.1186/s12917-021-03049-8

108. Lima LM, Silva JW, Ogoshi RCS, dos Reis JS, Franca J, Zangeronimo MG, et al. Evaluation of raw yeast extract (*Saccharomyces cerevisiae*) as an ingredient, additive or palatability agent in wet diet for cats. *Int J Biol*. (2015) 8:1. doi: 10.5539/ijb.v8n1p1

109. Ogoshi RCS, Zangeronimo MG, Dos Reis JS, França J, Santos JPF, Pires CP, et al. Acidifying and yeast extract in diets for adults cats. *Anim Sci J*. (2014) 85:555–61. doi: 10.1111/asj.12166

110. Aquino AA, Saad FMOB, Santos JPF, Leite CAL, Sampaio GR, Feliciano MAR. Efeitos da parede de levedura em dieta úmida na microbiota fecal, na produção de gás e na morfologia intestinal de gatos adultos. *Arq Bras Med Veterinária Zootec*. (2013) 65:1673–80. doi: 10.1590/S0102-09352013000600013

111. Aquino AA, Saad FMOB, Santos JPF, Alves MP, Ferrazza RA, Miranda MCMG. Efeitos do extrato da parede de levedura na digestibilidade, no escore fecal e na palatabilidade de dietas para gatos. *Arq Bras Med Veterinária Zootec*. (2010) 62:622–30. doi: 10.1590/S0102-09352010000300018

112. Santos J, Aquino A, Glória M, Avila-Campos M, Oba P, Santos K, et al. Effects of dietary yeast cell wall on faecal bacteria and fermentation products in adult cats. *J Anim Physiol Anim Nutr*. (2018) 102:1091–101. doi: 10.1111/jpn.12918

113. Rodrigues BM, Olivo PM, Osmari MP, Vasconcellos RS, Ribeiro LB, Bankuti FI, et al. Microencapsulation of probiotic strains by lyophilization is efficient in maintaining the viability of microorganisms and modulation of fecal microbiota in cats. *Int J Microbiol*. (2020) 2020:1–10. doi: 10.1155/2020/1293481

114. Aktas MS, Borku MK, Ozkanlar Y. Efficacy of *Saccharomyces boulardii* as a probiotic in dogs with lincomycin induced diarrhoea. *Bull Vet Inst Pulawy*. (2007) 51:365–9.

115. D'Angelo S, Fracassi F, Bresciani F, Galuppi R, Diana A, Linta N, et al. Effect of *Saccharomyces boulardii* in dogs with chronic enteropathies: double-blinded, placebo-controlled study. *Vet Rec*. (2018) 182:258–8. doi: 10.1136/vr.104241

116. Baroncello S, Candiago NT, Gelinski JMLN, Caliari V, Baratto CM. Meat pet snacks by containing encapsulated *Saccharomyces boulardii*. *Eur J Agric Food Sci*. (2020) 2:1–9. doi: 10.24018/ejfood.2020.2.4.69

117. De Oliveira RT, Haese D, Kill JL, Lima A, Malini PV, Thompson GR. Palatabilidade de rações de gatos com pirofosfato de sódio e extrato de levedura. *Cienc Rural*. (2016) 46:2202–5. doi: 10.1590/0103-8478cr20151651

frontiers | Frontiers in Veterinary Science

TYPE Original Research
PUBLISHED 03 November 2023
DOI 10.3389/fvets.2023.1294095

OPEN ACCESS

EDITED BY
Tao Wang,
Jilin Agricultural University, China

REVIEWED BY
Shugui Zheng,
Shenyang Agricultural University, China
Shuting Cao,
Guangdong Academy of Agricultural Sciences, China

*CORRESPONDENCE
Ming Qi
✉ qmcharisma@sina.com
Jing Wang
✉ jingwang023@hunau.edu.cn

†These authors have contributed equally to this work

RECEIVED 14 September 2023
ACCEPTED 23 October 2023
PUBLISHED 03 November 2023

CITATION
Wang CM, Wang N, Deng YK, Zha AD, Li JY, Tan B, Qi M, Wang J and Yin YL (2023) β-hydroxybutyrate administration improves liver injury and metabolic abnormality in postnatal growth retardation piglets. *Front. Vet. Sci.* 10:1294095. doi: 10.3389/fvets.2023.1294095

β-hydroxybutyrate administration improves liver injury and metabolic abnormality in postnatal growth retardation piglets

Chengming Wang[1,2†], Nan Wang[1,2†], Yuankun Deng[1,2], Andong Zha[1,2], Junyao Li[1,2], Bie Tan[1,2], Ming Qi[1,2*], Jing Wang[1,2*] and Yulong Yin[1,2,3]

[1]College of Animal Science and Technology, Hunan Agricultural University, Changsha, Hunan, China, [2]Yuelushan Laboratory, Changsha, Hunan, China, [3]Institute of Yunnan Circular Agricultural Industry, Puer, Yunnan, China

Abnormal hepatic energy metabolism limits the growth and development of piglets. We hypothesized that β-hydroxybutyrate (BHB) might improve the growth performance of piglets by maintaining hepatic caloric homeostasis. A total of 30 litters of newborn piglets were tracked, and 30 postnatal growth retardation (PGR) piglets and 40 healthy piglets were selected to treat with normal saline with or without BHB (25 mg/kg/days) at 7-d-old. At the age of 42 days, 8 piglets in each group were sacrificed, and serum and liver were collected. Compared with the healthy-control group piglets, PGR piglets showed lower body weight (BW) and liver weight ($p < 0.05$), and exhibited liver injury and higher inflammatory response. The contents of serum and hepatic BHB were lower ($p < 0.05$), and gene expression related to hepatic ketone body production were down-regulated in PGR piglets ($p < 0.05$). While BHB treatment increased BW and serum BHB levels, but decreased hepatic BHB levels in PGR piglets ($p < 0.05$). BHB alleviated the liver injury by inhibiting the apoptosis and inflammation in liver of PGR piglets ($p < 0.05$). Compared with the healthy-control group piglets, liver glycogen content and serum triglyceride level of PGR piglets were increased ($p < 0.05$), liver gluconeogenesis gene and lipogenesis gene expression were increased ($p < 0.05$), and liver NAD^+ level was decreased ($p < 0.05$). BHB supplementation increased the ATP levels in serum and liver ($p < 0.05$), whereas decreased the serum glucose, cholesterol, triglyceride and high-density lipoprotein cholesterol levels and glucose and lipid metabolism in liver of PGR piglets ($p < 0.05$). Therefore, BHB treatment might alleviate the liver injury and inflammation, and improve hepatic energy metabolism by regulating glucose and lipid metabolism, thereby improving the growth performance of PGR piglets.

KEYWORDS

β-hydroxybutyrate, liver injury, lipid, postnatal growth retardation, piglets

1. Introduction

Growth retardation (GR) is when an individual grows significantly slower than a healthy individual, accompanied by metabolic disorders, systemic inflammation, or intestinal dysbiosis, usually occurring in the early stages of life (1). Growth retardation can be divided into two categories: intrauterine growth retardation (IUGR) and postnatal growth retardation (PGR). In

mammals, the incidence of IUGR is high in multiple animals such as pigs, and 15–20% of newborn piglets weigh less than 1.1 kg (2). PGR piglets are defined as piglets with normal birth weight, while the body weight during postnatal growth and development is lower than 70% of the average weight of health piglets in the same period, and there is no obvious trauma (3). The incidence of PGR piglets during lactation and after weaning is about 10–30 and 5% respectively, which seriously restricts the breeding efficiency (4). Normally, IUGR is mainly caused by decreased blood flow and insufficient supply of nutrients that are induced by uterine-placental disorders during maternal pregnancy, resulting in impaired growth and development of the fetus or its organs (5). PGR is due to the impairment of intestinal barrier function and the imbalance of intestinal microorganisms after birth that triggered a decrease in the absorption capacity of intestinal nutrients, which induced the slow growth and development of neonatal piglets (6). Interestingly, our tracing test of piglets (from birth to 60 d of age) found that some IUGR piglets were able to develop catch-up growth at weaning, whose body weight exceeded the PGR piglets (unpublished data). Our previous studies showed PGR piglets have dysregulated nutrition absorption and aberrant energy metabolism (6–9), which suggested that increasing nutrient utilization and maintaining the balance of energy metabolism might improve the growth inhibition of PGR piglets.

The liver serves as a key modulator in nutrition metabolic homeostasis involving energy storage, xenobiotic metabolism, and detoxification activity (10). Studies have shown that the metabolism of nutrients in the liver of IUGR piglets is abnormal, which inevitably impairs liver function (5, 11). We previously observed a decreased peroxisome proliferator-activated receptor gamma (PPARγ) signaling pathway and an impairment in glucose utilization in the small intestine of PGR piglets compared to these in the healthy piglets (7). These considerations raised the possibility that there is a deficient hepatic energy metabolism in PGR piglets and mediating the energy metabolism might promote their growth performance. The ketone bodies, especially β-hydroxybutyrate (BHB), are synthesized in the liver from acetyl-CoA derived primarily from fatty acid oxidation. In the case of nutritional deficiency, BHB can be used as an alternative energy source to maintain caloric homeostasis (12). Meanwhile, BHB also acts as a signaling molecule for modulating lipolysis, oxidative stress, and neuroprotection. Studies have found that mice with ketogenic insufficiency and fed a high-fat diet exhibit abnormal hepatic glucose and lipid metabolism to induce mitochondrial dysfunction and liver injury (13). BHB could prevent liver ischemia–reperfusion injury and oxidative stress by up-regulating FOXO1 and HO-1, and reduce inflammatory response and apoptotic cell death by down-regulating NF-κB and NLRP3 inflammasome (14, 15). In addition, BHB ameliorates endoplasmic reticulum stress by activating AMPK and inhibits NLRP3 (16–18).

Therefore, we hypothesized that supplementing with exogenous BHB could improve the growth performance of PGR piglets by regulating liver energy metabolism. The effect of supplementation of BHB on the liver morphology, hepatic inflammatory response and energy metabolism was determined in healthy or PGR piglets.

2. Materials and methods

All animals used in this study are humanely managed in accordance with Chinese animal welfare guidelines. The experimental scheme was approved by the Animal Protection and Utilization Committee of Hunan Agricultural University (Changsha, China; 2,021,042).

2.1. Animal and experimental design

30 litters of newborn piglets with similar parity (3rd ~ 5th parity) and genetic background (about 12 piglets per litter) were recorded. All piglets are breast-fed normally. Diet of sow and piglet, drinking water and feeding environment are carried out in accordance with the operating standards of the company's farm. All newborn piglets are marked with ear defects. The birth weight of newborn piglets was recorded and defined as IUGR piglets according to the birth weight lower than 1.1 kg (2). At 7 days old, PGR piglets were defined according to the standard that whose body weight was less than 70% of the average weight of healthy piglets and there was no obvious trauma (8). Forty normal healthy piglets and thirty PGR piglets were randomly divided into 4 groups: control group, BHB group, PGR group and PGR + BHB group. The piglets of control and PGR group were fed daily with 5 mL saline (0.9%) from 7 days old, the piglets in BHB group and PGR + BHB group while fed with BHB solution dissolved in 5 mL saline (25 mg/kg/days, body weight was calculated according to the average weight per week). At the age of 42 days, 8 piglets in each group were randomly selected for slaughter sampling. The body weight of piglets is shown in Table 1.

2.2. Sample collection

After 12 h fasting, all piglets were collected the anterior vena cava blood in the blood vessels containing heparin sodium. 3,000 g was centrifuged at room temperature for 10 min and stored in the refrigerator at −80°C. After the blood was collected, the liver was taken for weighing, and then the liver sections (about 1 cm^2) and liver samples (about 5 g) were rinsed thoroughly with cold PBS (PH 7.4) to remove blood contamination. Liver slices were quickly fixed in 4% paraformaldehyde and prepared for embedding and slicing for hematoxylin–eosin (HE) and periodate-Schaefer (PAS) staining. Liver samples were rapidly frozen in liquid nitrogen and stored at −80°C for further analysis. All samples were collected within 15 min after execution.

2.3. Analysis of serum biochemical indexes

Serum biochemical indexes including triglyceride (TG), cholesterol (TC), high-density lipoprotein cholesterol (HDL-C),

TABLE 1 The body weight of healthy and post-natal growth retardation (PGR) pigs.

Item	Healthy-control	Healthy-BHB	PGR-control	PGR-BHB	*p*-value
Day 7 weight	2.83 ± 0.16^{a}	2.65 ± 0.07^{a}	1.83 ± 0.14^{b}	2.02 ± 0.05^{b}	0.000
Day 42 weight	10.44 ± 0.42^{b}	13.03 ± 0.76^{a}	6.35 ± 0.79^{c}	9.73 ± 0.66^{b}	0.000

BHB, β-hydroxybutyrate; PGR, postnatal growth retardation. $^{a, b, c}$Values within a row with different superscripts differ significantly ($p < 0.05$). $n = 8$ per treatment group.

low-density lipoprotein cholesterol (LDL-C) and glucose (GLU) were detected by automatic biochemical analyzer BS-200 (BeckmanCX4, Beckman Coulter Inc.). The specific kit was purchased from Shanghai Kehua Bio-Engineering Co., Ltd.

2.4. Determination of transaminase activity and BHB content in plasma and liver

The activities of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) in plasma and liver were determined by commercial kit (Beijing Solarbio Science & Technology Co., Ltd.). The content of BHB in serum was detected by pig enzyme-linked immunosorbent assay (Shanghai Fanke industrial Co., Ltd.), and the content of BHB in liver was detected by β-hydroxybutyrate colorimetric assay kits (Cayman Inc., United States) according to the manufacturer's instructions.

2.5. Determination of inflammatory cytokines in liver

Liver samples were homogenized in cold PBS. The liver homogenate was taken and centrifuged at 4°C for 12,000 g 10 min. The supernatant was collected for further detection. The concentrations of interleukin-1β (IL-1β), interleukin-10 (IL-10), tumor necrosis factor-α (TNF-α) and interferon-γ (IFN-γ) were detected by enzyme-linked immunosorbent assay kit (CSB-E06782p, CSB-E06779p, CSB-E16980p, CSB-E06794p, CUSABIO, https://www.cusabio.com/). The final results were normalized with the total protein concentration in each sample, and the protein concentration was detected by BCA protein concentration determination kit (Beyotime Biotech. Inc.).

2.6. Determination of energy metabolism index of liver

The content of adenosine triphosphate (ATP) (Jiangsu Meimian Industrial Co., Ltd., Yancheng, China) was detected by an enzyme-linked immunosorbent assay kit in pigs. The contents of oxidized coenzyme I (NAD^+) and reduced coenzyme I (NADH) were determined according to the operating instructions of the corresponding kits (Beyotime Biotech. Inc.). The final results were normalized with the total protein concentration in each sample.

2.7. Quantitative reverse analyzes

According to the primer design principles, primer premier 6.0 software was used to design primers, which were synthesized by Beijing Tsingke Biotech Co., Ltd. The primer sequence is shown in Table 2. TRIZOL reagent (Invitrogen, Carlsbad, CA, United States) extracted total RNA from liver. The RNA integrity, quality and purity of the samples were determined by 1% agarose gel electrophoresis and automatic nucleic acid/protein analyzer. Using the Evo M-MLV reverse transcription Premix kit (AG11728, Accurate Biotechnology (Hunan) Co., Ltd., Chang Sha, China) reverse-transcribed RNA into cDNA. Refer to the SYBR Green Pro Taq HS Premixed qPCR Kit

TABLE 2 Primers used for quantitative reverse transcription-PCR.

Gene	Gene Bank No.	Sequence (5′-3′)
β-actin	XM_0031242803	F:CTGCGGCATCCACGAAACT
		R:AGGGCCGTGATCTCCTTCTG
NLRP3	NM_001256770.2	F:CCTTCAGGCTGATTCAGGAG
		R:GACTCTTGCCGCTATCCATC
IL-1β	NM_214055.1	F:CAGCCATGGCCATAGTACCT
		R:CCACGATGACAGACACCATC
IL-4	NM_214340.1	F:CCCGAGTGTCAAGTGGCTTA
		R:TGATGATGCCGAAATAGCAG
IL-8	NM_213867.1	F:GCTCTCTGTGAGGCTGCAGTTC
		R:AAGGTGTGGAATGCGTATTTATGC
IL-10	NM_214041.1	F:GGGCTATTTGTCCTGACTGC
		R:GGGCTCCCTAGTTTCTCTTCC
Bcl-2	NM_214285.1	F:AGGGCATTCAGTGACCTGAC
		R:CGATCCGACTCACCAATACC
Bax	XM_003127290.5	F:GAAGTTGAGCGAGTGTCT
		R:AGTTGAAGTTGCCGTCAG
Caspase-3	NM_214131	F:CGTGCTTCTAAGCCATGGTG
		R:GTCCCACTGTCCGTCTCAAT
Caspase-8	XM_021074712.1	F:CGAAGACCAGAGTTTGCCCT
		R:GGATCCTCACCGTGGCAG
Caspase-9	XM_013998997.2	F:GTACCCACCACCAAGGTCTG
		R:AAGCTCACGGTTCAGCAGAG
HMGCL	XM_005656051.3	F:GTCACCACGTCGTCCCAGAG
		R:TTACTGTCGCCATCTTGCCC
HMGCS2	XM_021089968.1	F:CACGACGGGGCAACTCTC
		R:AAAACCTTTGGTGGGCTGCT
BDH1	XM_021070089.1	F:TCGGGGCAGAGTCTCCTTT
		R:GCCATAAGAGGCAGAGTGGT
PCK1	NM_001123158.1	F:TAAAGCTGGGAGGTTCTGCC
		R:CCAAGGTCGCCTACGTTTTC
PGC-1α	NM_213963.2	F:GGACTGACATCGAGTGTGCT
		R:TGAGTCCACCCAGAAAGCTG
G6PC	NM_001113445.1	F:CAGGACTCCCAGGATTGGTTC
		R:ATCACAGCTACCCAGAGGAGT
C/EBP-α	XM_003127015.4	F:GGTGGACAAGAACAGCAACGA
		R:TGGTCAGCTCCAGCACCTTC
SREBP1	NM_214157.1	F:GCAAGGCCATCGACTACATC
		R:AGGTTCTCCTGCTTGAGCTT
ACC	XM_021066238.1	F:ATGAAGGCTGTGGTGATGGA
		R:CTTGGTGACTTGAGCGTGAG
SCD1	NM_213781.1	F:TAAACAGTGCTGCCCACCTA
		R:AGGGAAAGGTGTGGTGGTAG
PPARγ	NM_214379.1	F:ATTTACACCATGCTGGCCTC
		R:GGGCTCCATAAAGTCACCAA

instructions (AG11701, Accurate Biotechnology (Hunan) Co., Ltd., Chang Sha, China), using a fluorescent quantitative PCR instrument (LightCycler®480 Real-Time PCR System, Roche, Switzerland) for real-time fluorescence quantitative PCR detection. The experiment was carried out with a reaction system of 10 μL, and each reaction was repeated three times. Refer to *Qi Ming* et al. for the reaction procedure (8). β-actin was the internal reference gene, and $2^{-\Delta\Delta Ct}$ was used to calculate the mRNA relative expression of the target gene.

2.8. Statistical analysis

All the data in this study are obtained by using Excel 2019 after preliminary finishing. All statistical analyzes were performed by one-way ANOVA using SPSS software 20.0 (SPSS Inc., Chicago, IL, United States). The differences among treatments were evaluated using Turkey's test. All data are expressed as the mean ± standard error (SEM) of the average. $p < 0.05$ indicates that there is a significant difference between the two groups.

3. Results

3.1. Effect of BHB on liver morphology and hepatic inflammation of PGR piglets

The body weight (BW) and absolute liver weight of PGR piglets was significantly lower than those of age-matched healthy piglets ($p < 0.05$) (Table 1 and Figure 1A). Administration of BHB significantly increased the BW and absolute liver weight of healthy piglets and PGR piglets ($p < 0.05$) (Table 1 and Figure 1A), but had no significant effect on relative liver weight ($p > 0.05$) (Figure 1B).

The hepatic morphology was showed in Figure 1D, the portal vein cell necrosis, nuclear fragmentation, macrophage vacuolation, hepatocyte cord disorder, inflammatory cell infiltration, and hepatocyte watery degeneration were observed in the PGR piglets. While BHB administration in PGR piglets could alleviate their hepatic injury ($p < 0.05$) (Figures 1C,D). The activities of ALT and AST in plasma and the content of ALT in liver tissue of PGR-control piglets were significantly higher than those of healthy-control piglets ($p < 0.05$) (Figures 1E–H). As compared to the PGR-control group, BHB decreased the AST and ALT levels in the plasma and liver of PGR piglets, while BHB declined the plasma AST levels of healthy piglets in comparison with the healthy-control group ($p < 0.05$) (Figures 1E–H). Meanwhile, PGR-control group showed higher IFN-γ and TNF-α concentrations, as well as *NLRP3* and *IL-8* mRNA expression levels in the liver, but lower hepatic IL-10 concentration, and *IL-4*, *IL-10* mRNA expression levels as compared to the healthy-control group ($p < 0.05$) (Figures 1I–M). BHB treatment in healthy piglets declined the hepatic IFN-γ concentration as compared to the healthy-control group ($p < 0.05$) (Figure 1K), while BHB treatment in PGR piglets significantly decreased IL-1β, IFN-γ and TNF-α concentrations, as well as the mRNA expression levels of *NLRP3*, *IL-1β* and *IL-8* in the liver ($p < 0.05$) (Figures 1I–M). Compared to the PGR-control group, BHB treatment in PGR piglets also showed higher IL-10 concentrations, and *IL-4*, *IL-10* mRNA levels in the liver ($p < 0.05$) (Figures 1I–M). In addition, the relative expressions of *Bax*, *Caspase-3*, *Caspase-8*, and *Caspase-9* mRNA in the liver of PGR-control piglets were significantly up-regulated ($p < 0.05$) (Figure 1N). Compared to the healthy-control piglets, BHB supplementation increased the relative expressions of *Bcl-2* mRNA ($p < 0.05$), while compared to the PGR-control piglets, BHB supplementation decreased the *Bax*, *Caspase-3*, *Caspase-8*, and *Caspase-9* mRNA expression levels, but increased *Bcl-2* mRNA levels in the liver ($p < 0.05$) (Figure 1N).

3.2. Changes of BHB metabolism in the liver and serum of piglets

The BHB levels in the serum and liver of PGR-control piglets were significantly lower than those of healthy-control piglets ($p < 0.05$) (Figures 2A,B). Compared with the PGR-control group, BHB supplementation increased the serum BHB level and reduced the liver BHB content of PGR piglets ($p < 0.05$), while BHB supplementation reduced the liver BHB content of healthy piglets in comparison with the healthy-control group ($p < 0.05$) (Figures 2A,B). At the same time, the expression level of *HMGCS2* mRNA in the liver of the PGR-control group was significantly lower than that of the healthy-control group ($p < 0.05$) (Figure 2C). As compared to the PGR-control group, BHB supplementation increased the expression levels of *HMGCS2* and *HMGCL* mRNA in the liver of PGR piglets, and decreased the expression level of *BDH1* mRNA ($p < 0.05$) (Figures 2C–E). While BHB supplementation decreased the expression level of *BDH1* mRNA in the liver of healthy piglets in comparison with the healthy-control group ($p < 0.05$) (Figures 2C–E).

3.3. Effect of BHB on the energy tatus of PGR piglets

Compared with the healthy-control group piglets, BHB supplementation increased the serum ATP levels of healthy piglets ($p < 0.05$) (Figure 3A), while BHB supplementation increased the serum ATP levels and liver ATP contents of PGR piglets in comparison with the PGR-control group piglets ($p < 0.05$) (Figures 3A,B). In addition, the liver NAD^+ content of piglets in PGR-control group was significantly lower than that in the healthy-control group ($p < 0.05$) (Figure 3C). Compared with the healthy-control group piglets, BHB supplementation decreased the liver NAD^+ and NADH contents of healthy piglets ($p < 0.05$), while compared with the PGR-control group piglets, BHB supplementation decreased the liver NADH contents of PGR piglets ($p < 0.05$), but had no significant effect on NAD^+/NADH ($p > 0.05$) (Figures 3C–E).

3.4. Effect of BHB on liver glucose metabolism in PGR piglets

Compared with healthy-control group piglets, liver glycogen content of piglets in PGR-control group were increased ($p < 0.05$) (Figures 4A–D). BHB supplementation significantly reduced blood glucose levels and liver glycogen content in healthy piglets and PGR piglets ($p < 0.05$) (Figures 4A–D) ($p < 0.05$) (Figures 4A–D). Compared with the healthy-control group piglets, the mRNA expression levels of gluconeogenesis-related genes *PGC-1α* and *G6PC* in liver of PGR

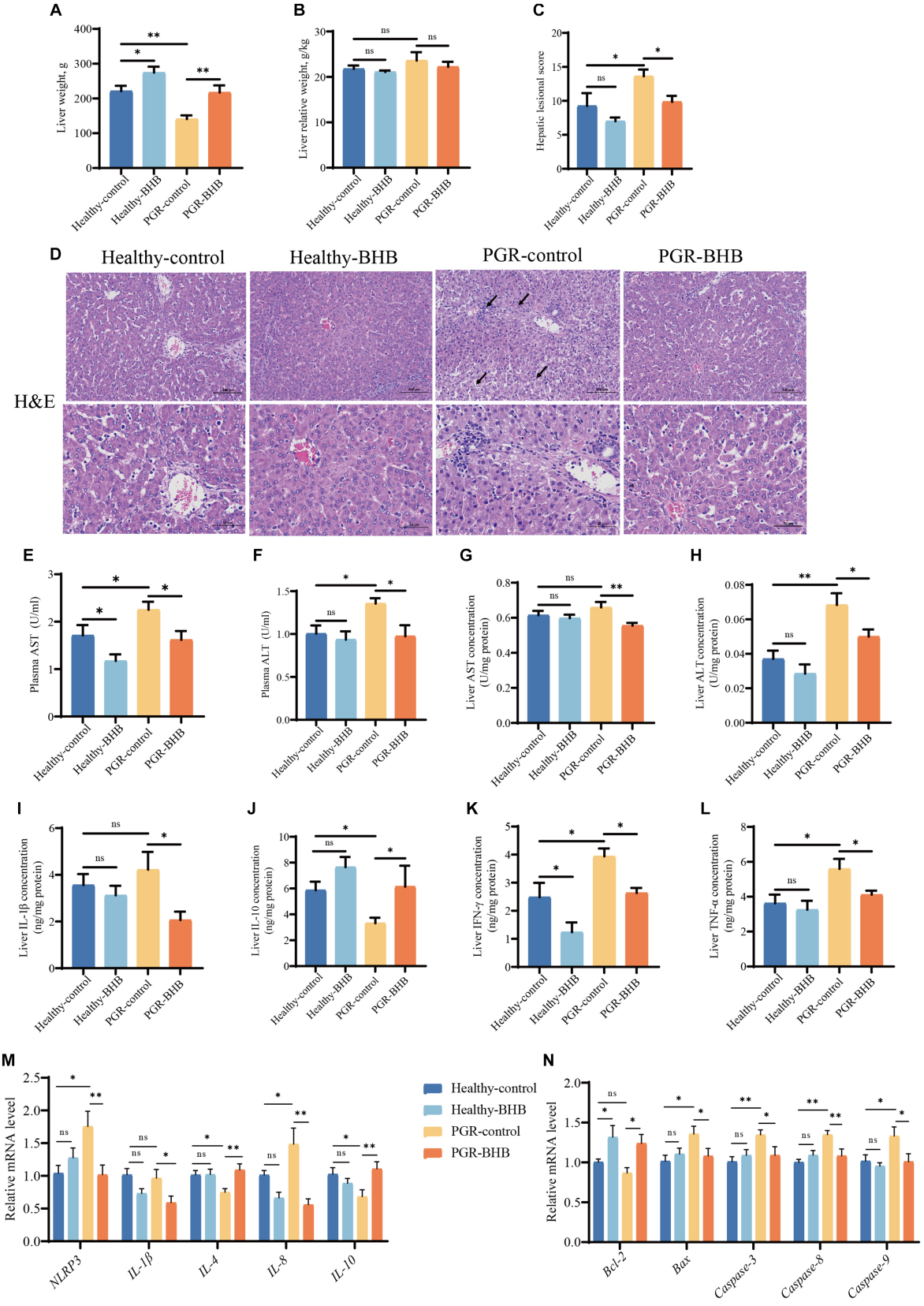


FIGURE 1
Effect of BHB on liver morphology and function of PGR piglets. **(A)** Liver weight, **(B)** liver relative weight, **(C,D)** representative images of H&E staining and lesioned score in liver, **(E,F)** plasma activity of AST and ALT, **(G,H)** liver concentration of AST and ALT, **(I–L)** liver concentration of IL-1β, IL-10, IFN-γ, and TNF-α, (**M,N**) hepatic mRNA expression of *NLRP3*, *IL-1β*, *IL-4*, *IL-8*, *IL-10*, *Bcl-2*, *Bax*, *Caspase-3*, *Caspase-8*, and *Caspase-9*. BHB, β-hydroxybutyrate; PGR, postnatal growth retardation; AST, aspartate aminotransferase; ALT, alanine aminotransferase; IL-1β, interleukin-1β; IL-10,

(Continued)

FIGURE 1 (Continued)
interleukin-10; TNF-α, tumor necrosis factor-α; IFN-γ, interferon-γ; NLRP3, NOD-like receptor pyrin domain 3; Bcl-2, B-cell lymphoma 2; Bax, Bcl-2-associated X protein; Caspase-3, cysteine-aspartic acid protease 3; Caspase-8, cysteine-aspartic acid protease 8, Caspase-9, cysteine-aspartic acid protease 9. Data were presented as mean ± SEM ($n = 8$). Different lowercase letters indicated significant differences between the compared groups ($p < 0.05$).

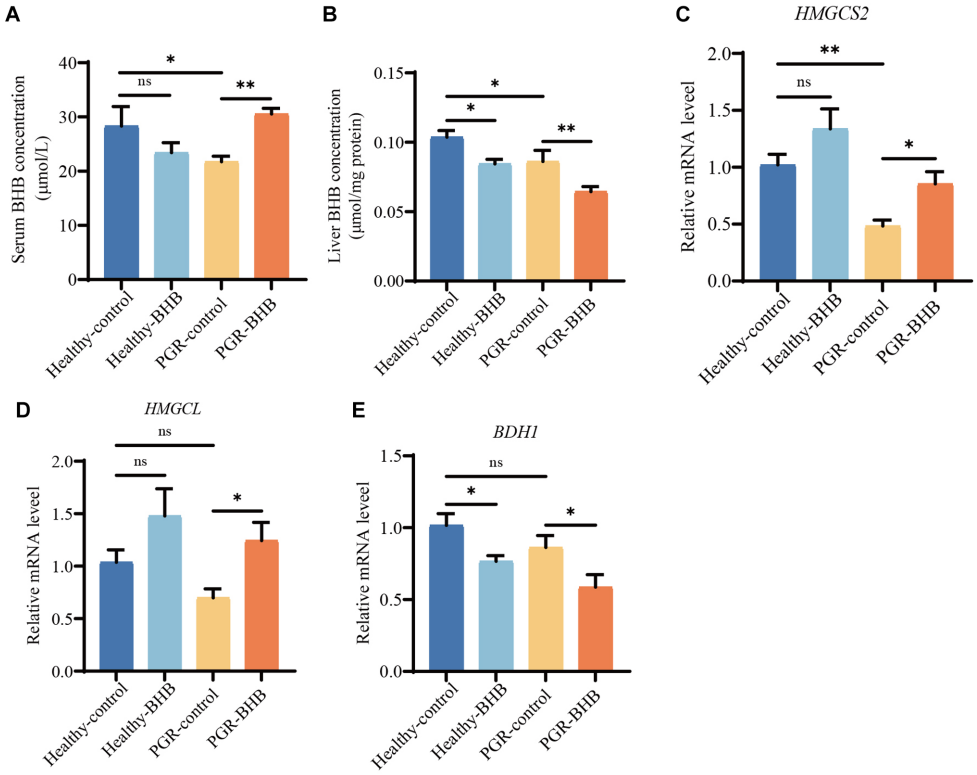


FIGURE 2
BHB metabolism of liver and serum in piglets. **(A)** Serum concentration of BHB. **(B)** Liver concentration of BHB. **(C–E)** Hepatic mRNA expression of *HMGCS2*, *HMGCL* and *BDH1*. BHB, β-hydroxybutyrate; PGR, postnatal growth retardation; HNGCS2, 3-hydroxy-3-methylglutaryl-CoA synthase 2; HMGCL, 3-hydroxy-3-methylglutaryl-CoA lyase; BDH1, 3-hydroxybutyrate dehydrogenase 1. Data were presented as mean ± SEM ($n = 8$). Different lowercase letters indicated significant differences between the compared groups ($p < 0.05$).

piglets were increased ($p < 0.05$) (Figures 4E–G). While BHB supplementation significantly up-regulated the mRNA expression levels of *PCK1* and down-regulated the mRNA expression levels of *PGC-1α* and *G6PC* in liver of PGR piglets ($p < 0.05$) (Figures 4E–G).

3.5. Effect of BHB on liver lipid metabolism in PGR piglets

The serum TG levels of piglets in the PGR-control group were significantly higher than those in the healthy-control group ($p < 0.05$) (Figures 5A–D). Compared with the PGR-control group, BHB supplementation decreased the serum TC, TG, and HDL-C concentrations of PGR piglets ($p < 0.05$) (Figures 5A–D). While BHB supplementation decreased the serum TG and HDL-C concentrations of healthy piglets in comparison with the healthy-control group ($p < 0.05$) (Figures 5A–D). Compared with healthy-control group, mRNA expression levels of *C/EPP-α*, *SCD1*, and *PPARγ* in liver of piglets in PGR-control group were increased ($p < 0.05$) (Figure 5E). Meanwhile, BHB supplementation decreased the expression levels of *C/EBP-α*, *SREBP1*, *SCD1*, and *PPARγ* mRNA in the liver of healthy and PGR piglets ($p < 0.05$) (Figure 5E).

4. Discussion

GR is the root cause of high morbidity and mortality in infants and young children. Children with GR are more likely to suffer from metabolic diseases and pathogen infections (1). PGR occurs in 10 to 30% of piglets in the pig industry, which leads to a decrease in feed utilization and an increase in mortality, which seriously affects the efficiency of the pig industry.

Some studies have found that, compared with healthy counterparts, PGR shows poor growth performance and lower organ weight (8, 19). Consistent with the results of this study, it was found that the body weight and liver weight of PGR piglets were lower than those of healthy piglets of the same age. BHB is a water-soluble organic compound derived from lipids, which is most significantly magnified

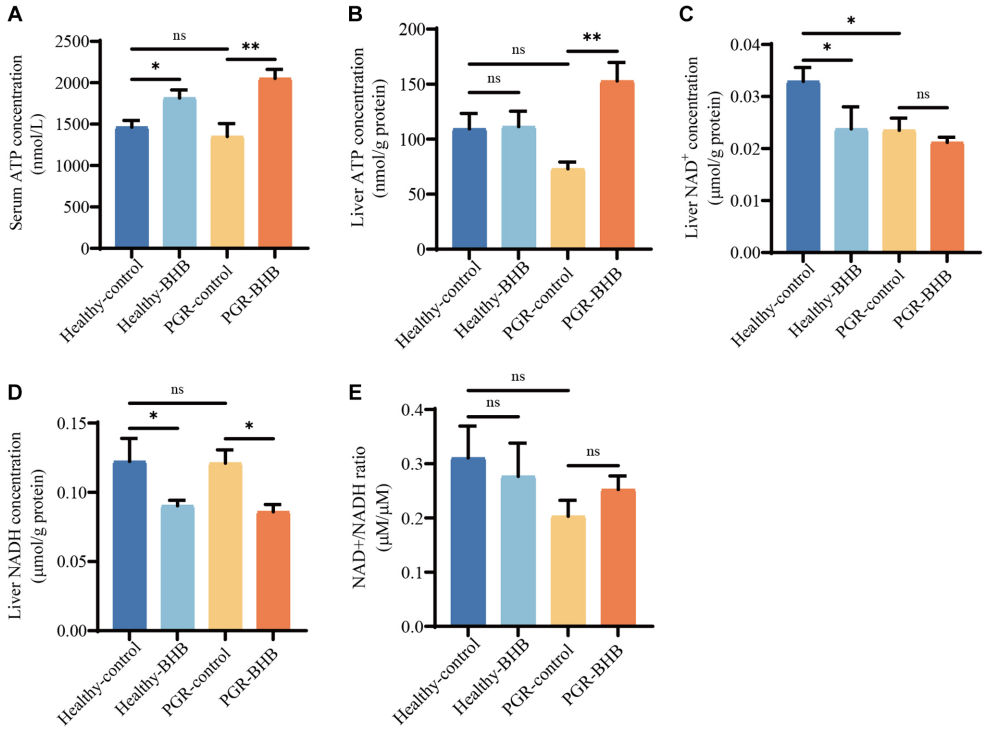


FIGURE 3
Effect of BHB on energy status of PGR piglets. **(A)** Serum concentration of ATP. **(B)** Liver concentration of ATP. **(C–E)** Liver concentration of NAD^+ and NADH. BHB, β-hydroxybutyrate; PGR, postnatal growth retardation; ATP, adenosine triphosphate, NAD^+, oxidized coenzyme I; NADH, reduced coenzyme I. Data were presented as mean ± SEM ($n = 8$). Different lowercase letters indicated significant differences between the compared groups ($p < 0.05$).

under the physiological conditions of low utilization of carbohydrates (i.e., hunger), prolonged fasting or ketogenic diet (20). Dietary supplementation of low-dose BHB can effectively improve the growth performance and organ development of early-weaned goats (21). Indeed, BHB increased the body weight of PGR piglets and improved hepatic injury and inflammatory responses in the current study. ALT and AST are considered as sensitive indicators of liver injury and are usually expressed only in hepatocytes. When the liver is damaged, they are released into the bloodstream. In this study, the activities of AST and ALT in plasma and the content of ALT in the liver of PGR piglets increased, indicating liver damage in PGR piglets, which was consistent with the results of other scholars (5, 8, 11). NLRP3 inflammatory bodies are a wide range of sensors for risk-related molecular patterns, which can be activated by many types of pathogens or danger signals (22). Pro-inflammatory cytokines (including IL-1β, IL-6, and IL-8) are necessary to initiate an inflammatory response during infection (8). The PGR piglets showed higher IFN- γ and TNF-α levels, as well as IL-8 mRNA levels in this study, suggesting that those piglets might have higher hepatic inflammation than the healthy piglets. BHB treatment could alleviate the inflammatory responses, evidenced by lower pro-inflammatory cytokines concentration and mRNA expressions, and increased anti-inflammatory cytokines levels in the liver. Consistently, *Yun-Hee Youm* et al. demonstrate that BHB can promote anti-inflammatory effects, regulate inflammation by inhibiting the activation of NLRP3 inflammatory bodies, reduce the downstream production of proinflammatory cytokines IL-1β and IL-8, and prevent NLRP3-mediated inflammatory diseases (14, 17, 18). Meanwhile, excessive inflammatory activation might be related to dysregulated apoptosis (23). It is well known that Caspase-3 and Bcl-2 are the executors and inhibitors of apoptosis, respectively. Bcl-2 is an anti-apoptotic protein that can protect cells from apoptosis, while pro-apoptotic proteins such as Bax promote programmed cell death (24, 25). Studies have shown BHB could reduce apoptosis induced by hepatic ischemia–reperfusion and paraquat challenge (14, 26). We also found that the expression of anti-apoptosis gene Bcl-2 increased and the expression of pro-apoptosis genes Bax, Caspase-3, Caspase-8, and Caspase-9 decreased in the liver of PGR piglets fed with exogenous BHB.

The ketogenic diet (KD) and exogenous ketone supplements (EKS) can increase blood BHB concentration (27–30). Increased BHB levels of serum were observed in the PGR piglets fed with BHB, while their hepatic BHB levels were decreased. This result might be explained by the feedback regulation system of ketone bodies. Ketone body synthesis in the liver mitochondrial matrix begins with fatty acid β-oxidized acetyl-CoA. Mitochondrial hydroxymethyl glutaryl-CoA synthetase (HMGCS2) condenses acetyl-CoA with acetoacetyl-CoA to form HMG-CoA, which is then released by HMG-CoA lyase (HMGCL). Most acetoacetate is further metabolized by β-hydroxybutyrate dehydrogenase (BDH1) to BHB and the activity of BDH1 is proportional to the ratio of ACAC/BHB in circulation (12, 31). The condensation of acetyl-CoA and acetoacetyl-CoA to HMG-CoA mainly depends on the mitochondrial HMGCS2, which is a rate-limiting step in ketone body synthesis (31). In the current study, the HMGCS2 and HMGCL mRNA levels were enhanced by

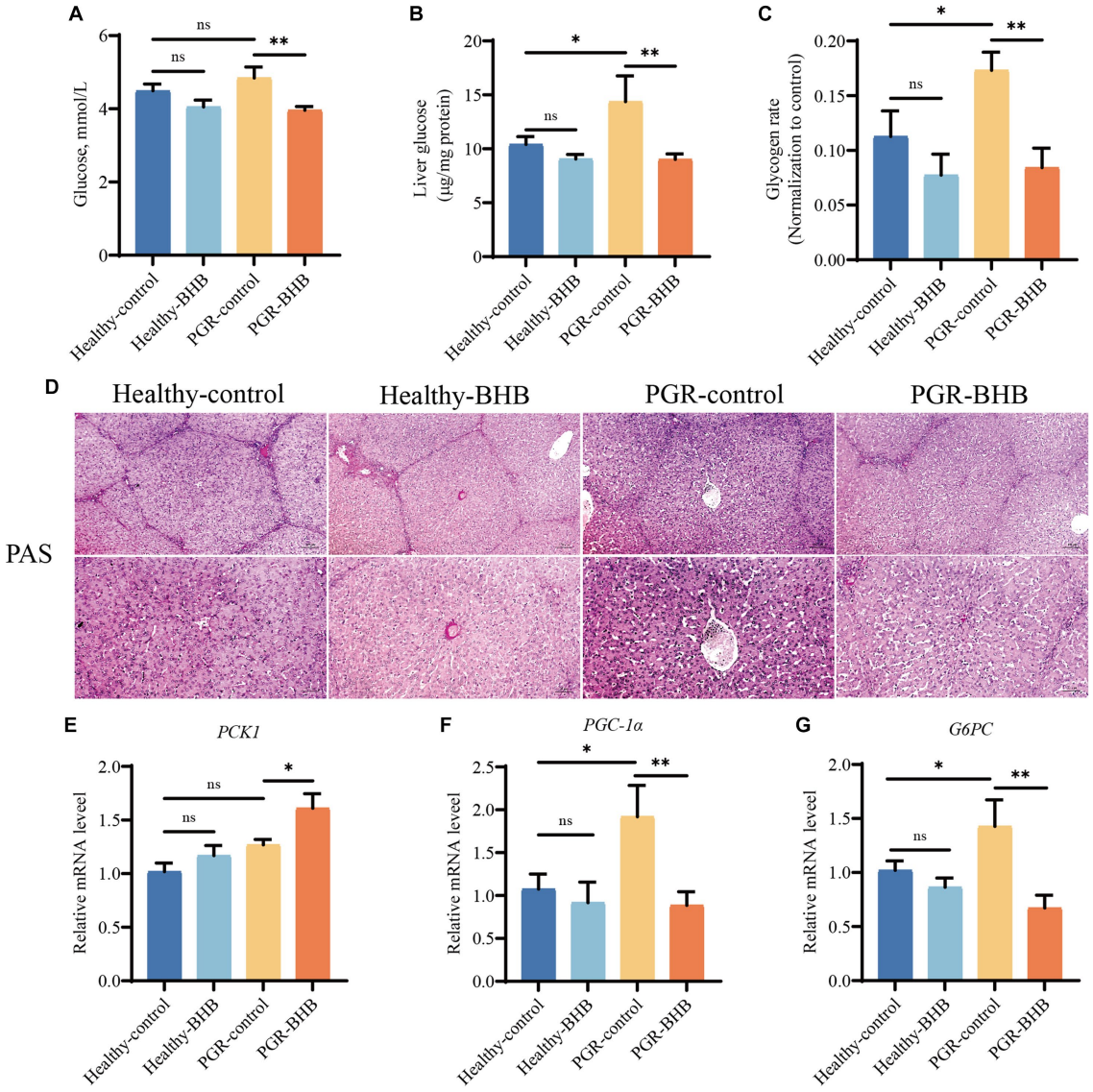


FIGURE 4
Effect of BHB on liver glucose metabolism in PGR piglets. **(A)** Blood sugar level. **(B)** Liver concentration of glucose. **(C,D)** Representative images of PAS staining and lesioned score in liver. **(E–G)** Hepatic mRNA expression of *PCK1*, *PGC-1α* and *G6PC*. BHB, β-hydroxybutyrate; PGR, postnatal growth retardation; PCK1, Phosphoenolpyruvate carboxykinase 1; PGC-1α: Peroxisome proliferator-activated receptor gamma coactivator 1-alpha; G6PC, Glucose-6-phosphatase. Data were presented as mean ± SEM ($n = 8$). Different lowercase letters indicated significant differences between the compared groups ($p < 0.05$).

BHB treatment in PGR piglets, while the BDH1 mRNA expression was inhibited. It is implied that BHB supplementation may alter the ACAC/BHB ratio in circulation, thereby inhibiting BDH1 activity and leading to a decrease in liver BHB content.

Liver ketone body level is strongly linked to glucose and lipid metabolism, which plays an important role in maintaining energy balance (32). ATP is the main energy source for maintaining cellular physiological responses (33). There is increasing evidence that ATP deficiency is associated with the development of liver glucose lipid metabolism disorders (34, 35). Clearly, restoring ATP synthesis in the liver may be a potential therapeutic strategy for the treatment of metabolic disorders. Some studies have found that ATP concentration in the liver of IUGR piglets and jejunum of LBW piglets is significantly decreased (36, 37), but we did not find a decrease in the liver of PGR piglets. This may be related to the acquired formation of PGR piglets. In addition, we found that administration of BHB increased the amount of ATP in the liver and blood of PGR piglets, which is consistent with the Motohisa Suzuki et al. (38) report that BHB can maintain high ATP levels. Glucose is the major energy substrate for fetal oxidative metabolism. In this study, liver glycogen production was increased and gluconeogenic gene expression was upregulated in PGR piglets, which was consistent with previous reports in IUGR (39–41). These abnormalities in glucose metabolism may be caused by insulin resistance (41). At the same time, insufficient production of ketogenic in the liver can lead to mild hyperglycemia and increased liver gluconeogenesis in adult mice (13), so increased liver glycogen production and upregulation of gluconeogenic gene in PGR piglets may also be caused by insufficient

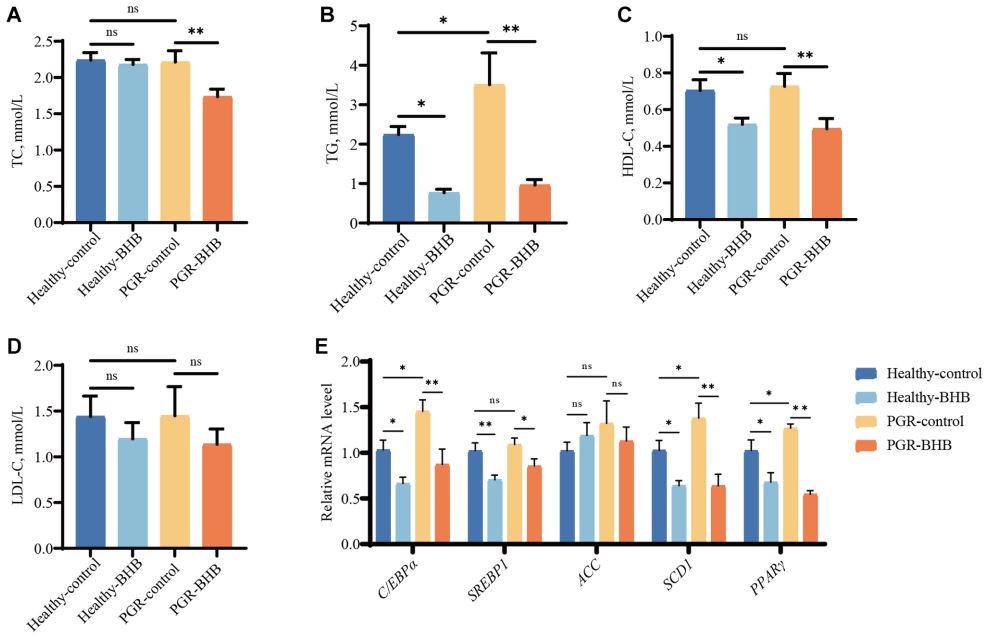


FIGURE 5
Effect of BHB on liver lipid metabolism in PGR piglets. **(A–D)** Serum biochemical parameters; **(E)** hepatic mRNA expression of *C/EBP-α*, *SREBP1*, *ACC*, *SCD1* and *PPARγ*. BHB, β-hydroxybutyrate; PGR, postnatal growth retardation; C/EBP-α, CCAAT/enhancer binding protein α; SREBP1, sterol regulatory element-binding protein 1; ACC, acetyl-CoA carboxylase; SCD1, Stearoyl-CoA desaturase 1; PPARγ, Peroxisome proliferator-activated receptor γ. Data were presented as mean ± SEM (n = 8). Different lowercase letters indicated significant differences between the compared groups ($p < 0.05$).

production of ketogenic. Supplementation of ketogenic precursors pantothenic acid and cysteine normalized liver gluconeogenesis in mice lacking ketogenic production (13). Our study also found that BHB supplementation can not only increase the ketone body level of PGR piglets, but also inhibit liver gluconeogenesis and reduce liver glycogen content and blood glucose concentration. Consistent with our study, Csilla Ari et al. also found that BHB could inhibit the signal of gluconeogenesis in the liver and reduce the utilization of hepatic glycogen so as to lower blood glucose levels (28, 30, 42–45). TC and TG are two key markers to reflect circulating blood lipids, which are the key steps in regulating fetal synthesis and lipid catabolism. Studies have shown that abnormal nutritional metabolism occurs in the liver of IUGR fetuses, such as abnormal fatty acid synthesis and lipid oxidation (46, 47). In this study, the serum TG level of PGR piglets was increased, and the expressions of key genes of liver lipogenesis C/EBP-α, SCD1 and PPARγ were up-regulated, which was consistent with the findings of Chen et al. (41) in IUGR mice. This abnormal elevated lipids and hepatic lipid accumulation may be due to hepatic insulin resistance (41). However, the intake of exogenous BHB significantly reduced the circulating lipids of PGR piglets, including TC, TG and HDL-C, which was consistent with the results of previous studies (28, 48, 49). At the same time, BHB can inhibit the expression of liver fatty acid synthetases SREBP1, ACC and SCD1, thereby regulating liver lipid accumulation (50, 51), which is consistent with the results of this study.

In summary, our results showed that PGR piglets had a liver injury and inflammation. Exogenous supplementation of BHB could increase the blood BHB level and regulate the inflammatory response, as well as the energy metabolism in the liver of PGR piglets. These findings provide a potential new intervention to improve liver damage and dysfunction in PGR piglets, while the underline mechanism should be further investigated.

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 all animals used in this study are humanely managed in accordance with Chinese animal welfare guidelines. The experimental scheme was approved by the Animal Protection and Utilization Committee of Hunan Agricultural University (Changsha, China; 2021042). 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

CW: Conceptualization, Data curation, Formal analysis, Writing – original draft. NW: Conceptualization, Formal analysis, Writing – original draft. YD: Methodology, Visualization, Writing – original draft. AZ: Methodology, Visualization, Writing – original draft. JL: Methodology, Visualization, Writing – original draft. BT:

Conceptualization, Funding acquisition, Project administration, Writing – review & editing. MQ: Conceptualization, Funding acquisition, Project administration, Writing – review & editing. JW: Conceptualization, Writing – review & editing, Funding acquisition, Funding acquisition. YY: Conceptualization, Funding acquisition, Writing – review & editing.

Funding

The author(s) declare financial support was received for the research, authorship, and/or publication of this article. This work was supported by the National Key R&D Program (2021YFD1300401), National Natural Science Foundation of China (32102571), Excellent Youth Foundation of Hunan Province (2022JJ20027), the Science and Technology Major Project of Yunnan Province (202202AE090032), China Agriculture Research System of MOF and MARA.

Conflict of interest

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

Publisher's note

References

1. Qi M, Tan B, Wang J, Liao S, Deng Y, Ji P, et al. The microbiota–gut–brain axis: a novel nutritional therapeutic target for growth retardation. *Crit Rev Food Sci Nutr*. (2021) 62:4867–92. doi: 10.1080/10408398.2021.1879004

2. Wu G, Bazer FW, Wallace JM, Spencer TE. Board-Invited Review: intrauterine growth retardation: implications for the animal sciences1. *J Anim Sci*. (2006) 84:2316–37. doi: 10.2527/jas.2006-156

3. Knauer MT, Hostetler CE. US swine industry productivity analysis, 2005 to 2010. *J Swine Health Prod*. (2013) 21:248–52.

4. Patience JF, Rossoni-Serao MC, Gutierrez NA. A review of feed efficiency in swine: biology and application. *J Anim Sci Biotechnol*. (2015) 6:33. doi: 10.1186/s40104-015-0031-2

5. Zhang H, Chen Y, Chen Y, Ji S, Jia P, Xu J, et al. Pterostilbene attenuates liver injury and oxidative stress in intrauterine growth - retarded weanling piglets. *Nutrition*. (2021) 81:110940. doi: 10.1016/j.nut.2020.110940

6. Qi M, Tan B, Wang J, Liao S, Li J, Cui Z, et al. Postnatal growth retardation is associated with deteriorated intestinal mucosal barrier function using a porcine model. *J Cell Physiol*. (2021) 236:2631–48. doi: 10.1002/jcp.30028

7. Qi M, Tan B, Wang J, Li J, Liao S, Yan J, et al. Small intestinal transcriptome analysis revealed changes of genes involved in nutrition metabolism and immune responses in growth retardation piglets. *J Anim Sci*. (2019) 97:3795–808. doi: 10.1093/jas/skz205

8. Qi M, Tan B, Wang J, Liao S, Li J, Liu Y, et al. Post-natal growth retardation associated with impaired gut hormone profiles, immune and antioxidant function in pigs. *Front Endocrinol*. (2019) 10. doi: 10.3389/fendo.2019.00660

9. Qi M, Wang J, Tan B, Liao S, Long C, Yin Y. Postnatal growth retardation is associated with intestinal mucosa mitochondrial dysfunction and aberrant energy status in piglets. *J Cell Mol Med*. (2020) 24:10100–11. doi: 10.1111/jcmm.15621

10. Robinson MW, Harmon C, O'Farrelly C. Liver immunology and its role in inflammation and homeostasis. *Cell Mol Immunol*. (2016) 13:267–76. doi: 10.1038/cmi.2016.3

11. Liu C, Lin G, Wang X, Wang T, Wu G, Li D, et al. Intrauterine growth restriction alters the hepatic proteome in fetal pigs. *J Nutr Biochem*. (2013) 24:954–9. doi: 10.1016/j.jnutbio.2012.06.016

12. Puchalska P, Crawford PA. Multi-dimensional roles of ketone bodies in fuel metabolism, signaling, and therapeutics. *Cell Metab*. (2017) 25:262–84. doi: 10.1016/j.cmet.2016.12.022

13. Cotter DG, Ercal B, Huang X, Leid JM, d'Avignon DA, Graham MJ, et al. Ketogenesis prevents diet-induced fatty liver injury and hyperglycemia. *J Clin Investig*. (2014) 124:5175–90. doi: 10.1172/jci76388

14. Miyauchi T, Uchida Y, Kadono K, Hirao H, Kawasoe J, Watanabe T, et al. Up-regulation of FOXO1 and reduced inflammation by β-hydroxybutyric acid are essential diet restriction benefits against liver injury. *Proc Natl Acad Sci*. (2019) 116:13533–42. doi: 10.1073/pnas.1820282116

15. Pawlak M, Baugé E, Lalloyer F, Lefebvre P, Staels B. Ketone body therapy protects from lipotoxicity and acute liver failure upon Pparα deficiency. *Mol Endocrinol*. (2015) 29:1134–43. doi: 10.1210/me.2014-1383

16. Bae HR, Kim DH, Park MH, Lee B, Kim MJ, Lee EK, et al. beta-Hydroxybutyrate suppresses inflammasome formation by ameliorating endoplasmic reticulum stress via AMPK activation. *Oncotarget*. (2016) 7:66444–54. doi: 10.18632/oncotarget.12119

17. Youm Y-H, Nguyen KY, Grant RW, Goldberg EL, Bodogai M, Kim D, et al. The ketone metabolite β-hydroxybutyrate blocks NLRP3 inflammasome–mediated inflammatory disease. *Nat Med*. (2015) 21:263–9. doi: 10.1038/nm.3804

18. Goldberg EL, Asher JL, Molony RD, Shaw AC, Zeiss CJ, Wang C, et al. beta-Hydroxybutyrate deactivates neutrophil NLRP3 Inflammasome to relieve gout flares. *Cell Rep*. (2017) 18:2077–87. doi: 10.1016/j.celrep.2017.02.004

19. Hu R, Wang Z, Peng Q, Zou H, Wang H, Yu X, et al. Effects of GHRP-2 and Cysteamine administration on growth performance, Somatotropic Axis hormone and muscle protein deposition in yaks (*Bos grunniens*) with growth retardation. *PLoS One*. (2016) 11. doi: 10.1371/journal.pone.0149461

20. Evans M, McClure TS, Koutnik AP, Egan B. Exogenous ketone supplements in athletic contexts: past, present, and future. *Sports Med*. (2022) 52:25–67. doi: 10.1007/s40279-022-01756-2

21. Abdelsattar MM, Vargas-Bello-Perez E, Zhuang Y, Fu Y, Zhang N. Impact of dietary supplementation of beta-hydroxybutyric acid on performance, nutrient digestibility, organ development and serum stress indicators in early-weaned goat kids. *Anim Nutr*. (2022) 9:16–22. doi: 10.1016/j.aninu.2021.11.003

22. Chakraborty S, Galla S, Cheng X, Yeo J-Y, Mell B, Singh V, et al. Salt-responsive metabolite, β-Hydroxybutyrate, attenuates hypertension. *Cell Rep*. (2018) 25:677–89.e4. doi: 10.1016/j.celrep.2018.09.058

23. Qi M, Wang N, Xiao Y, Deng Y, Zha A, Tan B, et al. Ellagic acid ameliorates paraquat-induced liver injury associated with improved gut microbial profile. *Environ Pollut*. (2022) 293:118572. doi: 10.1016/j.envpol.2021.118572

24. Chin AC, Flynn AN, Fedwick JP, Buret AG. The role of caspase-3 in lipopolysaccharide-mediated disruption of intestinal epithelial tight junctions. *Can J Physiol Pharmacol*. (2006) 84:1043–50. doi: 10.1139/y06-056

25. Schenk RL, Strasser A, Dewson G. BCL-2: Long and winding path from discovery to therapeutic target. *Biochem Biophys Res Commun*. (2017) 482:459–69. doi: 10.1016/j.bbrc.2016.10.100

26. Wei T, Tian W, Liu F, Xie G. Protective effects of exogenous β-hydroxybutyrate on paraquat toxicity in rat kidney. *Biochem Biophys Res Commun*. (2014) 447:666–71. doi: 10.1016/j.bbrc.2014.04.074

27. Kovács Z, D'Agostino DP, Diamond D, Kindy MS, Rogers C, Ari C. Therapeutic potential of exogenous ketone supplement induced ketosis in the treatment of psychiatric disorders: review of current literature. Frontiers. *Psychiatry*. (2019) 10. doi: 10.3389/fpsyt.2019.00363

28. Kesl SL, Poff AM, Ward NP, Fiorelli TN, Ari C, Van Putten AJ, et al. Effects of exogenous ketone supplementation on blood ketone, glucose, triglyceride, and lipoprotein levels in Sprague–Dawley rats. *Nutr Metab*. (2016) 13:9. doi: 10.1186/s12986-016-0069-y

29. Shaw DM, Merien F, Braakhuis A, Maunder E, Dulson DK. Exogenous ketone supplementation and keto-adaptation for endurance performance: disentangling the effects of two distinct metabolic states. *Sports Med*. (2019) 50:641–56. doi: 10.1007/s40279-019-01246-y

30. Falkenhain K, Daraei A, Forbes SC, Little JP. Effects of exogenous ketone supplementation on blood glucose: a systematic review and Meta-analysis. *Adv Nutr*. (2022) 13:1697–714. doi: 10.1093/advances/nmac036

31. Newman JC, Verdin E. Ketone bodies as signaling metabolites. *Trends Endocrinol Metab*. (2014) 25:42–52. doi: 10.1016/j.tem.2013.09.002

32. Qi M, Wang J, Be T, Li J, Liao S, Liu Y, et al. Dietary glutamine, glutamate, and aspartate supplementation improves hepatic lipid metabolism in post-weaning piglets. Animal. *Nutrition*. (2020) 6:124–9. doi: 10.1016/j.aninu.2019.12.002

33. Brad AM, Bormann CL, Swain JE, Durkin RE, Johnson AE, Clifford AL, et al. Glutathione and adenosine triphosphate content of in vivo and in vitro matured porcine oocytes. *Mol Reprod Dev*. (2003) 64:492–8. doi: 10.1002/mrd.10254

34. Veech RL. The therapeutic implications of ketone bodies: the effects of ketone bodies in pathological conditions: ketosis, ketogenic diet, redox states, insulin resistance, and mitochondrial metabolism. *Prostaglandins Leukot Essent Fatty Acids*. (2004) 70:309–19. doi: 10.1016/j.plefa.2003.09.007

35. Sato K, Kashiwaya Y, Keon CA, Tsuchiya N, King MT, Radda GK, et al. Insulin, ketone bodies, and mitochondrial energy transduction. *FASEB J*. (1995) 9:651–8. doi: 10.1096/fasebj.9.8.7768357

36. Liu J, Yu B, Mao X, He J, Yu J, Zheng P, et al. Effects of intrauterine growth retardation and maternal folic acid supplementation on hepatic mitochondrial function and gene expression in piglets. *Arch Anim Nutr*. (2012) 66:357–71. doi: 10.1080/1745039x.2012.710084

37. Zhang H, Zheng P, Chen D, Yu B, He J, Mao X, et al. Dietary arginine supplementation improves intestinal mitochondrial functions in low-birth-weight piglets but not in Normal-birth-weight piglets. *Antioxidants*. (2021) 10. doi: 10.3390/antiox10121995

38. Suzuki M, Suzuki M, Sato K, Dohi S, Sato T, Matsuura A, et al. Effect of beta-hydroxybutyrate, a cerebral function improving agent, on cerebral hypoxia, anoxia and ischemia in mice and rats. *Jpn J Pharmacol*. (2001) 87:143–50. doi: 10.1254/jjp.87.143

39. Thorn SR, Regnault TRH, Brown LD, Rozance PJ, Keng J, Roper M, et al. Intrauterine growth restriction increases fetal hepatic Gluconeogenic capacity and reduces messenger ribonucleic acid translation initiation and nutrient sensing in fetal liver and skeletal muscle. *Endocrinology*. (2009) 150:3021–30. doi: 10.1210/en.2008-1789

40. Jones AK, Brown LD, Rozance PJ, Serkova NJ, Hay WW Jr, Friedman JE, et al. Differential effects of intrauterine growth restriction and a hypersinsulinemic-isoglycemic clamp on metabolic pathways and insulin action in the fetal liver. *Am J Phys Regul Integr Comp Phys*. (2019) 316:R427–40. doi: 10.1152/ajpregu.00359.2018

41. Chen D, Wang Y-y, Li S-p, Zhao H-m, Jiang F-j, Wu Y-x, et al. Maternal propionate supplementation ameliorates glucose and lipid metabolic disturbance in hypoxia-induced fetal growth restriction. *Food Funct*. (2022) 13:10724–36. doi: 10.1039/d2fo01481e

42. Schlumbohm C, Harmeyer J. Hyperketonemia impairs glucose metabolism in pregnant and nonpregnant ewes. *J Dairy Sci*. (2004) 87:350–8. doi: 10.3168/jds.S0022-0302(04)73174-4

43. Mikkelsen KH, Seifert T, Secher NH, Grøndal T, van Hall G. Systemic, cerebral and skeletal muscle ketone body and energy metabolism during acute hyper-D-β-Hydroxybutyratemia in post-absorptive healthy males. *J Clin Endocrinol Metabol*. (2015) 100:636–43. doi: 10.1210/jc.2014-2608

44. Ari M, Koutnik G, Rogers P, et al. Exogenous ketones lower blood glucose level in rested and exercised rodent models. *Nutrients*. (2019) 11:2330. doi: 10.3390/nu11102330

45. Poff AM, Koutnik AP, Egan B. Nutritional ketosis with ketogenic diets or exogenous ketones: features, convergence, and divergence. *Curr Sports Med Rep*. (2020) 19:251–9. doi: 10.1249/jsr.0000000000000732

46. Hribal ML, He J, Dong L, Xu W, Bai K, Lu C, et al. Dietary Tributyrin supplementation attenuates insulin resistance and abnormal lipid metabolism in suckling piglets with intrauterine growth retardation. *PLoS One*. (2015) 10. doi: 10.1371/journal.pone.0136848

47. Mortensen OH, Olsen HL, Frandsen L, Nielsen PE, Nielsen FC, Grunnet N, et al. Gestational protein restriction in mice has pronounced effects on gene expression in newborn Offspring's liver and skeletal muscle. *Pediatr Res*. (2010) 67:47–53. doi: 10.1203/PDR.0b013e3181c4735c

48. Liu Y, Bharmal SH, Kimita W, Petrov MS. Effect of acute ketosis on lipid profile in prediabetes: findings from a cross-over randomized controlled trial. *Cardiovasc Diabetol*. (2022) 21. doi: 10.1186/s12933-022-01571-z

49. Stubbs BJ, Cox PJ, Evans RD, Santer P, Miller JJ, Faull OK, et al. On the metabolism of exogenous ketones in humans. *Front Physiol*. (2017) 8. doi: 10.3389/fphys.2017.00848

50. Kennedy AR, Pissios P, Otu H, Xue B, Asakura K, Furukawa N, et al. A high-fat, ketogenic diet induces a unique metabolic state in mice. *Am J Physiol Endocrinol Metab*. (2007) 292:E1724–39. doi: 10.1152/ajpendo.00717.2006

51. Badman MK, Kennedy AR, Adams AC, Pissios P, Maratos-Flier E. A very low carbohydrate ketogenic diet improves glucose tolerance in Ob/Ob mice independently of weight loss. *Am J Physiol Endocrinol Metab*. (2009) 297:E1197–204. doi: 10.1152/ajpendo.00357.2009

frontiers | Frontiers in Veterinary Science

TYPE Original Research
PUBLISHED 16 November 2023
DOI 10.3389/fvets.2023.1289010

OPEN ACCESS

EDITED BY
Tao Wang,
Jilin Agricultural University, China

REVIEWED BY
Hongbin Pan,
Yunnan Agricultural University, China
Mohammed Hamdy Farouk,
Al-Azhar University, Egypt

*CORRESPONDENCE
Weiren Yang
✉ wryang@sdau.edu.cn
Junxun Li
✉ 892686923@qq.com

RECEIVED 05 September 2023
ACCEPTED 27 October 2023
PUBLISHED 16 November 2023

CITATION
Liu H, Ren X, Li Y, Cao Q, Yang L, Jiang S, Fu J, Gao J, Yan L, Li J and Yang W (2023) Effects of different wheat bran fermentation sources on growth performance, nutrient digestibility, serum antioxidant capacity and fecal microbiota in growing pigs.
Front. Vet. Sci. 10:1289010.
doi: 10.3389/fvets.2023.1289010

Effects of different wheat bran fermentation sources on growth performance, nutrient digestibility, serum antioxidant capacity and fecal microbiota in growing pigs

Heng Liu[1], Xiaojie Ren[2], Yang Li[1], Qingjie Cao[3], Lijie Yang[1], Shuzhen Jiang[1], Jiawei Fu[1], Jie Gao[4], Lei Yan[5], Junxun Li[2]* and Weiren Yang[1]*

[1]Key Laboratory of Efficient Utilization of Non-grain Feed Resources (Co-construction by Ministry and Province), Ministry of Agriculture and Rural Affairs, Shandong Provincial Key Laboratory of Animal Biotechnology and Disease Control and Prevention, Department of Animal Science and Veterinary Medicine, Shandong Agricultural University, Tai'an, China, [2]Shandong Taishan Shengliyuan Group Co., Ltd., Tai'an, China, [3]Chambroad Holding Group, Binzhou, China, [4]College of Biology and Brewing Engineering, Mount Taishan College, Tai'an, China, [5]Shandong New Hope Liuhe Group Co., Ltd., Qingdao, China

The present study aimed to evaluate the application of different wheat bran fermentation sources in growing pigs. A total of 320 pigs (43±0.21kg), were randomly allocated to 5 groups in a 21-d trial. The control group was fed a basal diet (CON) containing raw wheat bran, and the other four treatments were fed the diets in which the raw wheat bran in the basal diet was substituted with *Aspergillus niger* (WBA), *Bacillus licheniformis* (WBB), *Candida utilis* (WBC), and *Lactobacillus plantarum* (WBL) fermented wheat bran, respectively. The results showed that compared to the CON group, the crude fiber and pH values were decreased ($p<0.05$), while the gross energy (GE), crude protein (CP), and lactic acid values were increased ($p<0.05$) in all the wheat bran fermented by different strains. Compared with other treatments, feeding *B. licheniformis* fermented wheat bran had higher final weight, average daily gain, as well as lower feed-to-gain ratio. Compared with CON group, pigs fed with fermented wheat bran diets had higher dry matter, CP, and GE availability, serum total protein, albumin and superoxide dismutase levels, and fecal *Lactobacillus* counts, as well as lower malondialdehyde level and fecal *Escherichia coli* count. Collectively, our findings suggested that feeding fermented wheat bran, especially *B. licheniformis* fermented wheat bran, showed beneficial effects on the growth performance, nutrient digestibility, serum antioxidant capacity, and the gut microbiota structure of growing pigs.

KEYWORDS

fermented wheat bran, growing pig, growth performance, antioxidant capacity, fecal microflora

Introduction

With the steady growth of animal production, feed price is increased (1, 2). Correspondingly, fermentation is recognized as a very effective approach to improve the nutrition and digestion of unconventional feed sources (3–5). Numerous studies have illustrated that the fermented feed improved growth performance, antioxidant capacity, and intestine health of pigs (6–8).

Wheat bran, a byproduct derived from flour processing, is a readily accessible material with an annual global yield of 100–150 million tons (9). Due to its high contents of energy value, crude protein (CP), and trace elements, wheat bran is extensively utilized as animal feed raw materials (10). In feedstuff production, wheat bran processing primarily involves mechanical and chemical methods (11–13). Kraler et al. (14) reported that wheat bran fermented by *Lactobacillus paracasei* and *Lactobacillus plantarum* significantly increased the apparent digestibilities of dry matter (DM), organic matter (OM), ether extract (EE), and gross energy (GE) in growing pigs. Additionally, fermentation could effectively reduce the content of insoluble dietary fiber in rice bran and corn bran (15, 16), and the enzymes produced during fermentation can enhance the absorption of minerals (17). The *Aspergillus niger*, *Bacillus licheniformis*, *Candida utilis* and *L. plantarum* are the four strains used most widely in feed fermentation. Previous studies have demonstrated that the anti-nutritional factors presenting in soybean and sorghum, specifically glycinin, β-conglycinin and tannins, can be effectively degraded by fermentation with *A. niger* or *L. plantarum* (18, 19). Ahmed et al. (20) found that fermented feed by *B. licheniformis* significantly increased growth performance of broilers. The *C. utilis* fermented rapeseed meal improved the intestinal morphology of broilers (21). However, there is little literature available on the comparative study of wheat bran fermented by *A. niger*, *B. licheniformis*, *C. utilis*, and *L. plantarum* on fermentation parameters and their application on growing pigs.

Therefore, the experiment was conducted to investigate the effects of wheat bran fermented by different strains (*A. niger*, *B. licheniformis*, *C. utilis*, and *L. plantarum*) on growth performance, nutrient digestibility, serum antioxidant capacity, and fecal microbiota of growing pigs, and select the optimal strain for wheat bran fermentation, helping to select the most effective fermentative source to improve the feed utilization.

Materials and methods

Preparation of fermented wheat bran

The starter culture of *A. niger* (CICC 2041), *B. licheniformis* (CICC 21886), *C. utilis* (CICC 31430), and *L. plantarum* (CICC 6076) used in the test were obtained from Shandong Taishan Shengliyuan Group Co., Ltd. (Tai'an, China), which obtained according to optimized cultivation methods (Supplementary Table S1). Before fermentation, the moisture content of wheat bran was adjusted to 45%. Then the WBA, WBB, WBC, and WBL were fermented according to the conditions shown in Supplementary Table S2. The WBA, WBB, WBC, and WBL products were all obtained by drying at 45°C for approximately 48 to 72 h. Samples of fermented wheat bran were collected to determine their GE, CP, EE, and crude fiber (CF) contents according to the methods described by the Association of Official Analytical Chemists (AOAC) (22). To determine pH, a sample of 5 g fermented wheat bran was dissolved in 50 mL distilled water. The pH value of the supernatant was measured with pH meter (Shanghai Russell Technology Co., Ltd., Shanghai, China) after centrifugation at 4000 × *g* for 5 min. The lactic acid content was determined with a high-performance liquid chromatography autoanalyzer (Waters PICO TAG amino acid autoanalyzer; Millipore, MA, USA) according to standard procedures.

Experimental design and management

Three hundred and twenty (Duroc × Landrace × Yorkshire) pigs (43 ± 0.21 kg) were randomly divided into 5 treatments with 8 replicates per group and 8 pigs per replicate. Control group was fed a basal diet (CON), and other 4 treatments were fed the diets in which wheat bran of the basal diet were replaced with 10% *A. niger* (WBA), *B. licheniformis* (WBB), *C. utilis* (WBC), and *L. plantarum* (WBL) fermented wheat bran (air-dry basis), respectively. The basal diet (Table 1) was formulated with reference to National Research Council (NRC, 2012). Pigs were housed in a temperature-and humidity-controlled room (26–28°C, 55–60% RH), and were fed three times daily at 0800, 1400, and 1800 h. During the period of the experiment, all pigs had free access to feed and water. Daily feed intake per replicate was recorded, and the pigs were weighted individually on the morning of day 21 of the experiment before breakfast.

Apparent nutrient digestibility

Apparent nutrient digestibility was conducted using acid insoluble ash (AIA) indicator method. Fresh feces excreted by each replicate were collected daily for four consecutive days starting on day 17 of the experiment. Daily collected feces were weighed and mixed, and 10 mL of 10% sulfuric acid per 100 g of the feces samples were added to avoid evaporation of nitrogen in the form of ammonia. Samples were stored temporarily at −20°C. All fecal samples per replicate collected consecutively during the 4 days were mixed evenly, and the nutrient contents of fecal and feed samples including AIA, GE, DM, OM, EE, and CP were analyzed according to AOAC (2012) (22), respectively. The apparent digestibilities of all parameters were calculated as indicated below:

TABLE 1 Ingredients and nutrient levels of the basal diet (air-dry basis) %.

Ingredients	Content	Nutrients	Values[2]
Corn	56.57	Metabolizable energy, MJ/kg	13.78
Soybean meal, 44.1% CP	20.65	Crude protein	16.33
Wheat bran	17.00	Calcium	0.56
Soybean oil	2.79	STTD phosphorus	0.24
Limestone, pulverized	1.20	Lysine	0.97
Calcium hydrophosphate	0.17	Methionine	0.32
Sodium chloride	0.20	Threonine	0.72
L-Lysine, 76.8%	0.20	Tryptophan	0.21
DL-methionine, 98.5%	0.07		
L-threonine, 98.0%	0.10		
Choline	0.05		
Premix[1]	1.00		
Total	100.00		

[1]The premix provided the following per kilogram of diets: vitamin A 1400 IU, vitamin B_1 1 mg, vitamin B_2 2 mg, vitamin B_5 7.5 mg, vitamin B_6 8.8 mg, vitamin B_{12} 0.008 mg, vitamin D_3 160 IU, vitamin E 11 IU, vitamin K_3 0.5 mg, biotin 0.05 mg, folic acid 0.3 mg, Fe ($FeSO_4{\cdot}H_2O$) 60 mg, Cu ($CuSO_4{\cdot}5H_2O$) 4 mg, Mn ($MnSO_4{\cdot}H_2O$) 25 mg, Zn ($ZnSO_4{\cdot}H_2O$) 60 mg, I (KIO_3) 0.14 mg, Se (Na_2SeO_3) 0.25 mg. [2]Crude protein, calcium and STTD phosphorus were analyzed values, while the other nutrient levels were calculated values.

$$\text{Apparent nutrient digestibility}(\%) = 100 - 100 \times \left[(\text{AIA} - 1 \times \text{F} - 2 / \text{AIA} - 2 \times \text{F} - 1) \right]$$

where AIA-1 was the AIA content of the diets, AIA-2 was the AIA content of the fecal, F-1 was the nutrient content of the diets, and F-2 was the nutrient content of the fecal.

Sampling procedure

Blood samples were taken from the jugular vein of 40 pigs (one pig per replicate) after being fasted for 12 h in the morning of d 21. About 5 mL of blood samples for hematological tests were collected into the routine blood tubes containing the anticoagulant (EDTA), and the other 10 mL were collected into another vacuum blood collection tube without anticoagulant for biochemical analyses. Serum samples were obtained after centrifugation at 3000 × *g* for 15 min and stored in 1.5 mL Eppendorf tubes at −20°C until analysis. Meanwhile, fresh fecal samples were collected from the 40 pigs by rectal stimulation, and then stored at −20°C until *Escherichia coli* and *Lactobacillus* analysis.

Serum biochemical parameters analysis

Serum biochemical parameters including alkaline phosphatase (ALP), aspartate amino transferase (AST), alanine aminotransferase (ALT), total protein (TP), albumin (ALB), triglyceride (TG), and total cholesterol (TCHO) were determined with commercial kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) using an automatic clinical chemistry analyzer (Roche, Cobus-MiraPlus, Roche Diagnostic System Inc., United States).

Haemato-immunological blood parameters analysis

The white blood cell count (WBC), lymphocyte ratio (LYN), red blood cell count (RBC), hemoglobin (HGB), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), and mean corpuscular hemoglobin concentration (MCHC) were detected using an Sysmex KX-21 Automated Hematological Analyzer (Abacus Junior Vet, Diatron, Vienna, Austria) with specific software.

Determination of antioxidant index

The superoxide dismutase (SOD) activity and malondialdehyde (MDA) level of serum were determined by assay kits (Nanjing Jiancheng Biotechnology Institute, China) according to the manufacturer's instructions.

Microbiota determination

The frozen fecal samples were incubated at 4°C for 10 h before enumeration. The *E. coli* and *Lactobacillus* were isolated by plating serial tenfold dilution (in sterile physiological saline) onto Luria Broth (LB) agar plates and MacConkey agar plates, respectively. All agar plates were incubated at 37°C for 36 h. The LB agar plates were aerobic incubation, while the MacConkey agar plates was incubated under the anaerobic conditions. Then the *E. coli* and *Lactobacillus* were counted according to the methods of Ganzle et al. (23) and Liu et al. (24).

Statistical analysis

The data were statistically analyzed using one-way ANOVA of SAS 9.2 (Inst. Inc., Cary, NC). Variations among the 5 treatments were compared with each other using Duncan's multiple comparisons. The mean and total standard error of the means (SEM) were used to present the results, and the differences between treatments were considered significant when $p < 0.05$.

Results

Fermentation parameters and nutrient content of wheat bran

The fermentation parameters and nutrient content of wheat bran fermented by different strains are shown in Figure 1 and Table 2, respectively. Compared to CON treatment, all fermented WB treatments had lower pH values and CF content, and higher lactic acid and CP values ($p < 0.05$). Meanwhile, WBA, WBB, and WBC treatments had higher GE value compared to CON group ($p < 0.05$).

Growth performance

The effects of wheat bran fermented by different strains on the growth performance of growing pigs are shown in Table 3. Compared to the CON, WBA, WBB, and WBC treatments had higher final weight, ADG, and lower F/G ($p < 0.05$). Meanwhile, the WBB treatments had lower F/G value compared to the WBL group ($p < 0.05$).

Apparent nutrient digestibility

The effects of wheat bran fermented by different strains on the apparent nutrient digestibility of growing pigs are shown in Table 4. Compared to CON group, WBB, WBC, and WBL treatments had higher apparent digestibility of DM, while WBA and WBB treatments had higher apparent digestibility of CP ($p < 0.05$). All pigs fed fermented wheat bran diets exhibited higher apparent digestibility of GE ($p < 0.05$). There were no significant differences in the apparent digestibilities of OM and EE among all the experimental groups ($p > 0.05$).

Serum biochemical parameters

The effects of wheat bran fermented by different strains on the serum biochemical parameters of growing pigs are shown in Table 5.

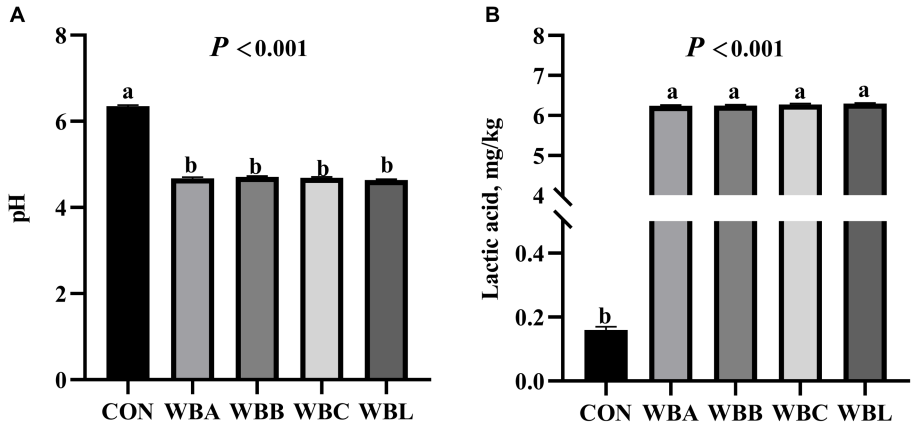


FIGURE 1
The fermentation parameters of wheat bran fermented by different strains ($n = 8$). CON is raw wheat bran, WBA, WBB, WBC, and WBL were *A. niger*, *B. licheniformis*, *C. utilis*, and *L. plantarum* fermented wheat bran, respectively. **(A)** The pH value; **(B)** The contents of lactic acid. Data are means for 8 replicates. [a,b]Means among the experimental groups with different letters are significantly different ($p < 0.05$).

TABLE 2 Nutrient content of wheat bran fermented by different strains (DM basis, %).

Items	Treatments					SEM	*p*-value
	CON	WBA	WBB	WBC	WBL		
DM	90.75[a]	86.57[ab]	87.48[ab]	87.36[ab]	88.21[ab]	0.562	0.064
CP	16.24[b]	17.85[a]	17.89[a]	17.90[a]	17.82[a]	0.407	0.026
EE	3.93	3.89	3.74	3.76	3.84	0.032	0.069
CF	8.64[a]	7.65[b]	7.31[b]	7.45[b]	7.70[b]	0.419	0.015
GE	16.73[b]	16.85[a]	16.83[a]	16.84[a]	16.78[ab]	0.012	0.025

DM, dry matter; CP, crude protein; EE, ether extract; CF, crude fiber; GE, gross energy. CON was wheat bran, WBA, WBB, WBC, and WBL were wheat bran fermented by *A. niger*, *B. licheniformis*, *C. utilis*, and *L. plantarum*, respectively. Data are means for 8 replicates. SEM, Total standard error of the means. [a,b]Means within a row with different letters are significantly different ($p < 0.05$).

TABLE 3 Effects of wheat bran fermented by different strains on the growth performance of growing pigs.

Items	Treatments					SEM	*p*-value
	CON	WBA	WBB	WBC	WBL		
Initial weight, kg	43.67	43.69	43.52	43.01	43.47	0.451	0.594
Final weight, kg	59.21[b]	60.91[a]	61.58[a]	60.23[a]	60.06[ab]	0.105	0.027
ADG, kg/d	0.74[b]	0.82[a]	0.86[a]	0.82[a]	0.79[ab]	0.014	0.035
ADFI, kg/d	1.89	1.97	1.98	1.98	1.93	0.023	0.213
F/G	2.54[a]	2.39[bc]	2.30[c]	2.41[bc]	2.43[ab]	0.010	0.023

ADG, average daily gain; ADFI, average daily feed intake; F/G, feed-to-gain ratio. Control was a basal diet (CON), WBA, WBB, WBC, and WBL were basal diet in which 10% wheat bran were replaced with *A. niger*, *B. licheniformis*, *C. utilis*, and *L. plantarum* fermented wheat bran (air-dry basis), respectively. Data are means for 8 replicates of 8 pigs per replicate. SEM, Total standard error of the means. [a,b,c]Means within a row with different letters are significantly different ($p < 0.05$).

TABLE 4 Effects of wheat bran fermented by different strains on the apparent nutrient digestibility of growing pigs %.

Items	Treatments					SEM	*p*-value
	CON	WBA	WBB	WBC	WBL		
DM	80.75[b]	82.57[ab]	85.36[a]	84.27[a]	86.14[a]	0.627	0.034
OM	83.86	84.95	85.15	85.42	85.62	0.049	0.358
CP	81.45[b]	85.19[a]	87.72[a]	84.92[ab]	84.45[ab]	0.162	0.028
EE	82.98[ab]	84.17[a]	84.25[a]	83.24[ab]	83.44[a]	0.082	0.035
GE	0.79[b]	0.81[a]	0.84[a]	0.83[a]	0.81[a]	0.125	0.02

DM, dry matter; OM, organic matter; CP, crude protein; EE, ether extract; GE, gross energy. Control was a basal diet (CON), WBA, WBB, WBC, and WBL were basal diet in which 10% wheat bran were replaced with *A. niger*, *B. licheniformis*, *C. utilis*, and *L. plantarum* fermented wheat bran (air-dry basis), respectively. Data are means for 8 replicates of 1 pig per replicate. SEM, Total standard error of the means. [a,b]Means within a row with different letters are significantly different ($p < 0.05$).

TABLE 5 Effects of wheat bran fermented by different strains on the serum biochemical parameters of growing pigs.

Items	Treatments					SEM	*p*-value
	CON	WBA	WBB	WBC	WBL		
ALT, U/L	29.89	31.01	30.85	31.97	30.98	0.324	0.432
AST, U/L	33.89	32.28	31.98	33.45	34.65	0.605	0.328
ALP, U/L	156.82^{b}	163.88^{ab}	169.38^{a}	162.10^{ab}	159.20^{ab}	1.520	0.035
TP, g/L	63.82^{c}	64.24^{ab}	65.21^{ab}	66.20^{a}	65.43^{ab}	0.517	<0.001
ALB, g/L	32.28^{b}	35.43^{a}	36.07^{a}	34.92^{a}	36.60^{a}	0.132	<0.001
TG, mmol/L	0.41	0.34	0.38	0.40	0.42	0.002	0.561
TCHO, mmol/L	2.16	2.28	2.31	2.14	2.18	0.040	0.601

ALT, alanine aminotransferase; AST, aspartate amino transferase; ALP, alkaline phosphatase; TP, total protein; ALB, albumin; TG, triglyceride; TCHO, total cholesterol. Control was a basal diet (CON), WBA, WBB, WBC, and WBL were basal diet in which 10% wheat bran were replaced with *A. niger*, *B. licheniformis*, *C. utilis*, and *L. plantarum* fermented wheat bran (air-dry basis), respectively. Data are means for 8 replicates of 1 pig per replicate. SEM, Total standard error of the means. ^{a,b,c}Means within a row with different letters are significantly different ($p < 0.05$).

Compared to CON group, WBB treatment had higher serum ALP activity ($p < 0.05$). Meanwhile, all pigs fed fermented wheat bran diets had higher values of serum ALB and TP than CON group ($p < 0.05$). There were no significant differences in serum ALT, AST, TG, and TCHO concentrations among all the experimental groups ($p > 0.05$).

Haemato-immunological blood parameters

The effects of wheat bran fermented by different strains on the haemato-immunological blood parameters of growing pigs are shown in Table 6. Compared to the CON and WBA groups, WBB and WBC treatments had higher HGB value ($p < 0.05$). The higher MCH value were observed in WBB, WBC, WBL, and WBA treatments in respective order compared to the CON group ($p < 0.05$). There were no significant differences in WBC, LYN, RBC, MCV, and MCHC among all the experimental groups ($p > 0.05$).

Antioxidant index

The effects of wheat bran fermented by different strains on antioxidant capacity of growing pigs are shown in Table 7. Compared to CON, all pigs fed fermented wheat bran diets had higher serum SOD activity and lower MDA concentration ($p < 0.05$).

Fecal microbiota

The effects of wheat bran fermented by different strains on fecal microbiota of growing pigs are shown in Table 8. Compared to the CON group, pigs fed *B. licheniformis*, *C. utilis*, and *L. plantarum* fermented wheat bran diets had lower fecal *Escherichia coli* count ($p < 0.05$); pigs fed fermented wheat bran diets exhibited higher fecal *Lactobacillus* counts ($p < 0.05$).

Discussion

Microbial fermentation is considered a highly efficient and cost-effective processing technique for improving the nutritional quality of feed (25). The pH is a crucial reference used to estimate the quality of fermented feed, and a lower pH is advantageous for the nutrient digestion and suppression of harmful bacteria growth (26). Our results showed significant lower pH values of fermented wheat brans, which might be attributed to the increase in lactic acid (27). During the fermentation, the synthesis of microbial protein and the synergistic effect of enzymes secreted by strains may lead to an increase in CP content and a decrease in CF content (28). Consistent with previous research, our data also indicated that the CP values of wheat brans after fermentation were increased, while the CF values were decreased. Additionally, the GE values of wheat bran fermented by *A. niger*, *B. licheniformis*, and *C. utilis* were significantly higher than that of unfermented wheat bran, which was similar to Liu et al. (7).

Under the action of microorganisms, complex macromolecular compounds in feed are degraded into small molecular substances, which are effectively absorbed and utilized by animals (29). Vast majority of studies have reported the positive effects of fermented feed on growth performance. For instance, Dei et al. (20) found that feed fermented by *A. niger* increased feed intake and decreased F/G of broilers. Zhao et al. (30) also proved that fermented Ginkgo-leaves by *C. utilis* and *A. niger* improved the feed conversation ratio of laying hens. Similarly, our data showed that pigs fed with WBA, WBB, and WBC diets exhibited significant increases in the final weight and ADG values, and decreases in the F/G ratio. The mechanism by which fermented feed enhanced animal growth performance of above studies might be related to its promotion of intestinal development (31). However, pigs fed with wheat bran fermented by *L. plantarum* did not exhibit significant improvement in growth performance of pigs. Consistently, Le et al. (32) also did not observe significant difference of weaned pigs fed with wheat fermented by *Lactobacillus*.

The nutrient digestibility is an important indicator associated with growth performance. In the present study, the fermented wheat bran using different strains improved the digestibility of nutrient (CP, DM, and GE) to varying degrees, which was consistent with results in previous studies related to fermented soybean meal (33, 34). It was reported that the complex macromolecular compounds of wheat bran could be degraded into small molecular nutrients after fermentation (35), and fermented wheat bran supplementation could increase the digestive enzymatic activity in the intestine. Feng et al. (36) indicated that wheat bran fermented by *Bacillus cereus* increased the activity of amylase in the duodenum of broilers. Similar results were obtained from Deng et al. (37), who demonstrated that the *Bacillus subtilis* increased the activities of amylase and lipase in the ileum of piglets. It is worth emphasizing that Cruz et al. (38) demonstrated that dietary

TABLE 6 Effects of wheat bran fermented by different strains on the haemato-immunological parameters of growing pigs.

Items	Treatments					SEM	*p*-value
	CON	WBA	WBB	WBC	WBL		
WBC, 10^9/L	20.56	21.49	21.53	22.80	20.93	1.024	0.961
LYN, %	43.86	44.38	44.35	44.20	44.61	1.273	0.528
RBC, 10^{12}/L	6.65	6.41	6.73	6.69	6.45	0.009	0.856
HGB, g/L	119.87^{b}	120.20^{b}	138.59^{a}	140.20^{a}	125.64^{ab}	7.694	0.024
MCV, fL	65.01	66.06	65.38	67.68	65.87	1.438	0.597
MCH, pg	18.67^{c}	19.60^{bc}	21.87^{a}	20.98^{a}	20.58^{ab}	1.357	0.015
MCHC, g/L	300.07	298.35	297.28	295.92	301.58	1.286	0.489

WBC, white blood cell count; LYN, lymphocyte ratio; RBC, red blood cell count; HGB, hemoglobin; MCV, mean corpuscular volume; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration. Control was a basal diet (CON), WBA, WBB, WBC, and WBL were basal diet in which 10% wheat bran were replaced with *A. niger*, *B. licheniformis*, *C. utilis*, and *L. plantarum* fermented wheat bran (air-dry basis), respectively. Data are means for 8 replicates of 1 pig per replicate. SEM, Total standard error of the means. ^{a,b,c}Means within a row with different letters are significantly different ($p < 0.05$).

TABLE 7 Effects of wheat bran fermented by different strains on antioxidant capacity in serum of growing pigs.

Items	Treatments					SEM	*p*-value
	CON	WBA	WBB	WBC	WBL		
SOD, U/mL	59.46^{c}	63.41^{ab}	65.62^{a}	64.85^{a}	62.78^{ab}	1.864	<0.001
MDA, nmol/mL	2.47^{a}	2.25^{b}	2.17^{b}	2.08^{b}	2.34^{b}	0.031	<0.001

SOD, superoxide dismutase; MDA, malondialdehyde. Control was a basal diet (CON), WBA, WBB, WBC, and WBL were basal diet in which 10% wheat bran were replaced with *A. niger*, *B. licheniformis*, *C. utilis*, and *L. plantarum* fermented wheat bran (air-dry basis), respectively. Data are means for 8 replicates of 1 pig per replicate. SEM, Total standard error of the means. ^{a,b,c} Means within a row with different letters are significantly different ($p < 0.05$).

TABLE 8 Effects of wheat bran fermented by different strains on fecal microbiota of growing pigs (log^{10}CFU/g).

Items	Treatments					SEM	*p*-value
	CON	WBA	WBB	WBC	WBL		
Escherichia coli	5.85^{a}	5.76^{ab}	5.68^{b}	5.60^{b}	5.54^{bc}	0.072	0.024
Lactobacillus	8.30^{b}	8.64^{a}	8.87^{a}	8.71^{a}	8.86^{a}	0.069	0.034

Control was a basal diet (CON), WBA, WBB, WBC, and WBL were basal diet in which 10% wheat bran were replaced with *A. niger*, *B. licheniformis*, *C. utilis*, and *L. plantarum* fermented wheat bran (air-dry basis), respectively. Data are means for 8 replicates of 1 pig per replicate. SEM, Total standard error of the means. ^{a,b,c}Means within a row with different letters are significantly different ($p < 0.05$).

supplementation with *C. utilis* promoted the gene expression related to nutrient transporters in jejunum of broilers. Nevertheless, intestinal morphology analysis is necessary in the further study to determine the crucial mechanism of fermented wheat bran on nutrient digestibility.

The haemato-parameters are of great significance in evaluating physical health status and predicting disease occurrence (39). Our data showed the significant increase in HGB of the WBB and WBC diets, as well as MCH of the WBB, WBC, and WBL diets. These results suggested an improvement in the body's iron absorption, which might be associated with a reduction in anti-nutritional factors (40). Dietary fiber, due to its high phytate content, has been reported to reduce the non-heme iron absorption through chelation (41). Therefore, the improvement of HGB and MCH in pigs fed fermented wheat bran might be attributed to the degradation of CF in wheat bran after fermentation.

Serum biochemical parameters reflect the nutritional metabolism and comprehensive functions of body organs, indirectly predicting the occurrence of diseases in pigs (42, 43). The ALP not only benefits the formation of hard tissue, especially bones, but also has anti-inflammatory functions (44). We found that the wheat bran fermented by *B. licheniformis* significantly increased serum ALP activity of pigs, which was consistent with previous studies in piglets (45) and tilapia fish (46). This might be due to the presences of abundant enzyme activities (phytase and enzymes that hydrolyze non starch polysaccharide components) in fermented feed, leading to an increase in mineral intake during the ossification process (47). Serum TP and ALB levels are related to body protein metabolism (48). Higher TP levels benefit to promote tissue development, while ALB is responsible for scavenging free radicals and transporting nutrients (49). In the present study, pigs fed the fermented wheat bran sources had higher serum TP and ALB concentration, indicating an improvement in body nutritional metabolism. The changes in serum TP and ALB concentration might be linked to the alteration of quantity and quality of proteins intake after wheat bran fermentation (50). Zhao et al. (35) reported that the high molecular weight proteins were degraded into low molecular weight peptides in wheat bran fermented by *yeast*.

MDA is an advanced oxidation product and a recognized biomarker of oxidative stress, whose presence have a negative impact on growth performance of pigs (51, 52). Serum SOD is a well-known antioxidant enzyme responsible for scavenging superoxide anion radicals and converting them into hydrogen peroxide and molecular oxygen (53, 54). In the present study, pigs fed with fermented wheat

bran diets showed decreased MDA level and increased SOD activity in the serum, indicating an improvement in antioxidant capacity of the body. Zhao et al. (35) reported that yeast and *Lactobacillus bulgaricus* fermentation increased the total phenols contents in wheat bran, which contributed to the improvement of antioxidant capacity of the body. Additionally, Kumari et al. (55) found that *B. licheniformis* fermentation increased antioxidant activity of soybean meal hydrolysates. In summary, our findings suggested the fermented wheat bran had potential to alter the blood profiles of growing pigs, and the mechanism behind these associations needs further research.

The fecal microbiota plays a pivotal role in safeguarding the hosts against disease resulting from bacterial colonization, promoting nutrient absorption, and maintaining intestinal morphology (56, 57). The *E. coli* is a harmful pathogen known to cause poor growth performance, diarrhea and mortality in pigs (58). On the other hand, it has been reported that *Lactobacillus casei* has the ability to regulate intestinal immune function, maintain microbial balance and reduce inflammatory reactions (59). Multiple studies have demonstrated that fermented feed has the potential to decrease the abundance of *Enterobacteriaceae* and increase the abundance of *Lactobacilli* in the gastrointestinal tract of animals (60, 61). Consistent with previous studies, our data showed a significant decrease in fecal *E. coli* counts in pigs fed the WBB, WBC, and WBL diets, while a significant increase in fecal *Lactobacillus* counts in pigs fed the fermented wheat bran diets, which might associate with the increased lactic acid content in fermented wheat bran in our study. The presences of numerous organic acids in fermented feed not only establish an optimal environment for beneficial bacteria to adhere (4), but also compete against pathogenic bacteria by freely traversing membrane, resulting in enzyme breakdown and proton movement (62, 63). In summary, our findings indicated the beneficial effects of fermented wheat bran on fecal microbial composition of growing pigs.

Conclusion

In conclusion, our findings suggested that replacing 10% wheat bran with *B. licheniformis* fermented wheat bran could improve the growth performance, nutrient digestibility, serum antioxidant capacity, and gut microbiota composition in growing pigs. This study provides new ideas for the application of wheat bran in pig production.

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 Animal Care and Use Committee of Shandong Agricultural University (Approval Number: SDAUA-2017-0318). The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

HL: Data curation, Formal analysis, Methodology, Project administration, Resources, Software, Writing – original draft. XR: Methodology, Writing – review & editing, Data curation, Formal analysis, Project administration, Resources. YL: Methodology, Validation, Writing – review & editing. QC: Writing – review & editing. LiY: Formal analysis, Methodology, Validation, Writing – review & editing. SJ: Conceptualization, Investigation, Methodology, Software, Validation, Writing – review & editing. JF: Data curation, Project administration, Resources, Writing – review & editing. JG: Conceptualization, Funding acquisition, Investigation, Supervision, Writing – review & editing. LeY: Writing – review & editing. JL: Conceptualization, Investigation, Visualization, Writing – review & editing. WY: Conceptualization, Funding acquisition, Investigation, Supervision, Visualization, Writing – review & editing.

Funding

The author(s) declare financial support was received for the research, authorship, and/or publication of this article. This research was supported by the University-Industry Collaborative Education Program (KGRCYJY2021004), Natural Science Foundation of Shandong Province (ZR2022QC084), Shandong Science and Technology-Based Small and Medium-Sized Enterprises Innovation Capacity Improvement Project (grant number: 2022TSGC1275), the Shandong Province Pig Industry Technology System (SDAIT-08-04), and Major Innovative Projects in Shandong Province of research and application of environmentally friendly feed and the critical technologies for pigs and poultry without antibiotic (2019JZZY020609).

Conflict of interest

XR and JL were employed by Shandong Taishan Shengliyuan Group Co., Ltd., QC was employed by Chambroad Holding Group, and LeY was employed by Shandong New Hope Liuhe Group Co., Ltd.

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

Publisher's note

Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2023.1289010/full#supplementary-material

References

1. Kim TI, Mayakrishnan V, Lim DH, Yeon JH, Baek KS. Effect of fermented total mixed rations on the growth performance, carcass and meat quality characteristics of Hanwoo steers. *Anim Sci J*. (2018) 89:606–15. doi: 10.1111/asj.12958

2. Martin NP, Russelle MP, Powell JM, Sniffen CJ, Smith SI, Tricarico JM, et al. Invited review: sustainable forage and grain crop production for the US dairy industry. *J Dairy Sci*. (2017) 100:9479–94. doi: 10.3168/jds.2017-13080

3. Dai Z, Cui L, Li J, Wang B, Guo L, Wu Z, et al. Fermentation techniques in feed production. *Anim Agric*. (2020) 2020:407–29. doi: 10.1016/B978-0-12-817052-6.00024-0

4. Sugiharto S, Ranjitkar S. Recent advances in fermented feeds towards improved broiler chicken performance, gastrointestinal tract microecology and immune responses: a review. *Anim Nutr*. (2019) 5:1–10. doi: 10.1016/j.aninu.2018.11.001

5. Yeh RH, Hsieh CW, Chen KL. Screening lactic acid bacteria to manufacture two-stage fermented feed and pelleting to investigate the feeding effect on broilers. *Poult Sci*. (2018) 97:236–46. doi: 10.3382/ps/pex300

6. Hao L, Su W, Zhang Y, Wang C, Xu B, Jiang Z, et al. Effects of supplementing with fermented mixed feed on the performance and meat quality in finishing pigs. *Anim Feed Sci Technol*. (2020) 266:114501. doi: 10.1016/j.anifeedsci.2020.114501

7. Liu S, Xiao H, Xiong Y, Chen J, Wu Q, Wen X, et al. Effects of fermented feed on the growth performance, intestinal function, and microbiota of piglets weaned at different age. *Front Vet Sci*. (2022) 9:841762. doi: 10.3389/fvets.2022.841762

8. van Winsen RL, Urlings BAP, Lipman LJA, Snijders JMA, Keuzenkamp D, Verheijden Jos HM, et al. Effect of fermented feed on the microbial population of the gastrointestinal tracts of pigs. *Am Soc Microbiol*. (2001) 67:3071–6. doi: 10.1128/aem.67.7.3071-3076.2001

9. Apprich S, Tirpanalan Ö, Hell J, Reisinger M, Böhmdorfer S, Siebenhandl-Ehn S, et al. Wheat bran-based biorefinery 2: valorization of products. *LWT Food Sci Technol*. (2014) 56:222–31. doi: 10.1016/j.lwt.2013.12.003

10. Mateo AN, Hemery YM, Bast A, Haenen GR. Optimizing the bioactive potential of wheat bran by processing. *Food Funct*. (2012) 3:362–75. doi: 10.1039/c2fo10241b

11. Coda R, Rizzello CG, Curiel JA, Poutanen K, Katina K. Effect of bioprocessing and particle size on the nutritional properties of wheat bran fractions. *Innovative Food Sci Emerg Technol*. (2014) 25:19–27. doi: 10.1016/j.ifset.2013.11.012

12. Du B, Meenu M, Xu B. Insights into improvement of physiochemical and biological properties of dietary fibers from different sources via Micron Technology. *Food Rev Int*. (2019) 36:367–83. doi: 10.1080/87559129.2019.1649690

13. Zhang M, Liao A, Thakur K, Huang J, Zhang J, Wei Z. Modification of wheat bran insoluble dietary fiber with carboxymethylation, complex enzymatic hydrolysis and ultrafine comminution. *Food Chem*. (2019) 297:124983. doi: 10.1016/j.foodchem.2019.124983

14. Kraler M, Schedle K, Domig KJ, Heine D, Michlmayr H, Kneifel W. Effects of fermented and extruded wheat bran on total tract apparent digestibility of nutrients, minerals and energy in growing pigs. *Anim Feed Sci Technol*. (2014) 197:121–9. doi: 10.1016/j.anifeedsci.2014.07.010

15. Chu J, Zhao H, Lu Z, Lu F, Bie X, Zhang C. Improved physicochemical and functional properties of dietary fiber from millet bran fermented by Bacillus natto. *Food Chem*. (2019) 294:79–86. doi: 10.1016/j.foodchem.2019.05.035

16. Liu P, Zhao J, Guo P, Lu W, Geng Z, Levesque CL, et al. Dietary corn bran fermented by *Bacillus subtilis* MA139 decreased gut cellulolytic bacteria and microbiota diversity in finishing pigs. *Front Cell Infect Microbiol*. (2017) 7:526. doi: 10.3389/fcimb.2017.00526

17. Haefner S, Knietsch A, Scholten E, Braun J, Lohscheidt M, Zelder O. Biotechnological production and applications of phytases. *Appl Microbiol Biotechnol*. (2005) 68:588–97. doi: 10.1007/s00253-005-0005-y

18. Chen L, Zhao Z, Yu W, Zheng L, Li L, Gu W, et al. Nutritional quality improvement of soybean meal by *Bacillus velezensis* and *Lactobacillus plantarum* during two-stage solid-state fermentation. *AMB Express*. (2021) 11:23. doi: 10.1186/s13568-021-01184-x

19. Espitia-Hernández P, Ruelas-Chacón X, Chávez-González ML, Ascacio-Valdés JA, Flores-Naveda A, Sepúlveda-Torre L. Solid-state fermentation of Sorghum by *Aspergillus oryzae* and *Aspergillus niger*: effects on tannin content, phenolic profile, and antioxidant activity. *Foods*. (2022) 11:3121. doi: 10.3390/foods11193121

20. Saleh AA, Shukry M, Farrag F, Soliman MM, Abdel-Moneim AME. Effect of feeding wet feed or wet feed fermented by *Bacillus licheniformis* on growth performance, histopathology and growth and lipid metabolism marker genes in broiler chickens. *Animals*. (2021) 11:83. doi: 10.3390/ani11010083

21. Hu Y, Wang Y, Li A, Wang Z, Zhang X, Yun T, et al. Effects of fermented rapeseed meal on antioxidant functions, serum biochemical parameters and intestinal morphology in broilers. *Food Agric Immunol*. (2016) 27:182–93. doi: 10.1080/09540105.2015.1079592

22. Association of Official Analytical Chemists (AOAC). *Official methods of analysis, association of official analytical chemist. 19th* ed. (Washington D.C: Association of Official Analytical Chemists (AOAC)) (2012).

23. Gänzle MG, Hertel C, van der Vossen JM, Hammes WP. Effect of bacteriocin-producing lactobacilli on the survival of *Escherichia coli* and Listeria in a dynamic model of the stomach and the small intestine. *Int J Food Microbiol*. (1999) 48:21–35. doi: 10.1016/s0168-1605(99)00025-2

24. Liu P, Piao X, Kim SW, Wang L, Shen Y, Lee HS, et al. Effects of chito-oligosaccharide supplementation on the growth performance, nutrient digestibility, intestinal morphology, and fecal shedding of *Escherichia coli* and Lactobacillus in weaning pigs. *J Anim Sci*. (2008) 86:2609–18. doi: 10.2527/jas.2007-0668

25. Yang L, Zeng X, Qiao S. Advances in research on solid-state fermented feed and its utilization: the pioneer of private customization for intestinal microorganisms. *Anim Nutr*. (2021) 7:905–16. doi: 10.1016/j.aninu.2021.06.002

26. Varsha KK, Priya S, Devendra L, Nampoothiri KM. Control of spoilage fungi by protective lactic acid bacteria displaying probiotic properties. *Appl Biochem Biotechnol*. (2014) 172:3402–13. doi: 10.1007/s12010-014-0779-4

27. Shi C, Zhang Y, Lu Z, Wang Y. Solid-state fermentation of corn-soybean meal mixed feed with *Bacillus subtilis* and *Enterococcus faecium* for degrading antinutritional factors and enhancing nutritional value. *J Anim Sci Biotechnol*. (2017) 8:50. doi: 10.1186/s40104-017-0184-2

28. Liu Y, Feng J, Wang Y, Lv J, Li J, Guo L, et al. Fermented corn–soybean meal mixed feed modulates intestinal morphology, barrier functions and cecal microbiota in laying hens. *Animals*. (2021) 11:3059. doi: 10.3390/ani11113059

29. He W, Gao Y, Guo Z, Yang Z, Wang X, Liu H, et al. Effects of fermented wheat bran and yeast culture on growth performance, immunity, and intestinal microflora in growing-finishing pigs. *J Anim Sci*. (2021) 99:skab308. doi: 10.1093/jas/skab308

30. Zhao L, Zhang X, Cao F, Sun D, Wang T, Wang G. Effect of dietary supplementation with fermented Ginkgo-leaves on performance, egg quality, lipid metabolism and egg-yolk fatty acids composition in laying hens. *Livest Sci*. (2013) 155:77–85. doi: 10.1016/j.livsci.2013.03.024

31. He W, Yang Z, Ding H, Shi B. Dietary supplementation with fermented wheat bran improves the inflammatory state and intestinal health in pigs. *Livest Sci*. (2023) 274:105286. doi: 10.1016/j.livsci.2023.105286

32. Le MHA, Galle S, Yang Y, Landero JL, Beltranena E, Gänzle MG, et al. Effects of feeding fermented wheat with *Lactobacillus reuteri* on gut morphology, intestinal fermentation, nutrient digestibility, and growth performance in weaned pigs. *J Anim Sci*. (2016) 94:4677–87. doi: 10.2527/jas.2016-0693

33. Torres-Pitarch A, Gardiner GE, Cormican P, Mary R, Fiona C, Doherty JV, et al. Effect of cereal fermentation and carbohydrase supplementation on growth, nutrient digestibility and intestinal microbiota in liquid-fed grow-finishing pigs. *Sci Rep*. (2020) 10:13716. doi: 10.1038/s41598-020-70443-x

34. Yuan L, Chang J, Yin Q, Lu M, Di Y, Wang P, et al. Fermented soybean meal improves the growth performance, nutrient digestibility, and microbial flora in piglets. *Anim Nutr*. (2017) 3:19–24. doi: 10.1016/j.aninu.2016.11.003

35. Zhao H, Guo X, Zhu K. Impact of solid state fermentation on nutritional, physical and flavor properties of wheat bran. *Food Chem*. (2017) 217:28–36. doi: 10.1016/j.foodchem.2016.08.062

36. Feng Y, Wang L, Khan A, Zhao R, Wei S, Jing X. Fermented wheat bran by xylanase-producing *Bacillus cereus* boosts the intestinal microflora of broiler chickens. *Poult Sci*. (2020) 99:263–71. doi: 10.3382/ps/pez482

37. Deng B, Wu J, Li X, Zhang C, Men X, Xu Z. Effects of *Bacillus subtilis* on growth performance, serum parameters, digestive enzyme, intestinal morphology, and colonic microbiota in piglets. *AMB Express*. (2020) 10:212–0. doi: 10.1186/s13568-020-01150-z

38. Cruz A, Håkenåsen IM, Skugor A, Mydland LT, Åkesson CP, Hellestveit SS, et al. *Candida utilis* yeast as a protein source for weaned piglets: effects on growth performance and digestive function. *Livest Sci*. (2019) 226:31–9. doi: 10.1016/j.livsci.2019.06.003

39. Huyut MT, Ilkbahar F. The effectiveness of blood routine parameters and some biomarkers as a potential diagnostic tool in the diagnosis and prognosis of COVID-19 disease. *Int Immunopharmacol*. (2021) 98:107838. doi: 10.1016/j.intimp.2021.107838

40. Soltan MA. Potential of using raw and processed canola seed meal as an alternative fish meal protein source in diets for Nile tilapia, (*Oreochromis niloticus*). *Nutr Feeds*. (2005) 8:1111–28.

41. Baye K, Guyot JP, Mouquet-Rivier C. The unresolved role of dietary fibers on mineral absorption. *Crit Rev Food Sci Nutr*. (2017) 57:949–57. doi: 10.1080/10408398.2014.953030

42. He L, Li H, Huang N, Zhou X, Tian J, Li T, et al. Alpha-ketoglutarate suppresses the NF-κB-mediated inflammatory pathway and enhances the PXR-regulated detoxification pathway. *Oncotarget*. (2017) 8:102974:–102988. doi: 10.18632/oncotarget.16875

43. Xu X, Li L, Li B, Guo W, Ding X, Xu F. Effect of fermented biogas residue on growth performance, serum biochemical parameters, and meat quality in pigs. *Asian Australas J Anim Sci*. (2017) 30:1464–70. doi: 10.5713/ajas.16.0777

44. Fernandez NJ, Kidney BA. Alkaline phosphatase: beyond the liver. *Vet Clin Pathol*. (2007) 36:223–33. doi: 10.1111/j.1939-165x.2007.tb00216.x

45. Wlazło Ł, Nowakowicz-Dębek B, Ossowski M, Łukaszewicz M, Czech A. Effect of fermented rapeseed meal in diets for piglets on blood biochemical parameters and the microbial composition of the feed and faeces. *Animals*. (2022) 12:2972. doi: 10.3390/ani12212972

46. Gobi N, Vaseeharan B, Chen J, Rekha R, Vijayakumar S, Anjugam M, et al. Dietary supplementation of probiotic *Bacillus licheniformis* Dahb 1 improves growth

performance, mucus and serum immune parameters, antioxidant enzyme activity as well as resistance against *Aeromonas hydrophila* in tilapia *Oreochromis mossambicus*. *Fish Shellfish Immunol*. (2018) 74:501–8. doi: 10.1016/j.fsi.2017.12.066

47. Bosco R, Leeuwenburgh SCG, Jansen JA, van den Beucken JJJP. Configurational effects of collagen/ALP coatings on enzyme immobilization and surface mineralization. *Appl Surf Sci*. (2014) 311:292–9. doi: 10.1016/j.apsusc.2014.05.057

48. Yang Z, Wang Z, Yang H, Xu L, Gong D. Effects of dietary methionine and betaine on slaughter performance, biochemical and enzymatic parameters in goose liver and hepatic composition. *Anim Feed Sci Technol*. (2017) 228:48–58. doi: 10.1016/j.anifeedsci.2017.04.003

49. Su Y, Chen G, Chen L, Li J, Wang G, He J, et al. Effects of antimicrobial peptides on serum biochemical parameters, antioxidant activity and non-specific immune responses in *Epinephelus coioides*. *Fish Shellfish Immunol*. (2019) 86:1081–7. doi: 10.1016/j.fsi.2018.12.056

50. Walkowiak J, Mądry E, Lisowska A, Szaflarska-Popławska A, Grzymisławski M, Stankowiak-Kulpa H, et al. Adaptive changes of pancreatic protease secretion to a short-term vegan diet: influence of reduced intake and modification of protein. *Br J Nutr*. (2012) 107:272–6. doi: 10.1017/s0007114511002923

51. Chang PL, Boyd RD, Zier-Rush C, Rosero DS, van Heugten E. Lipid peroxidation impairs growth and viability of nursery pigs reared under commercial conditions. *J Anim Sci*. (2019) 97:3379–89. doi: 10.1093/jas/skz183

52. Yonny ME, García EM, López A, Arroquy JI, Nazareno MA. Measurement of malondialdehyde as oxidative stress biomarker in goat plasma by HPLC-DAD. *Microchem J*. (2016) 129:281–5. doi: 10.1016/j.microc.2016.07.010

53. Savas HA, Gergerlioglu HS, Armutcu F, Herken H, Yilmaz HR, Kocoglu E, et al. Elevated serum nitric oxide and superoxide dismutase in euthymic bipolar patients: impact of past episodes. *World J Biol Psych*. (2006) 7:51–5. doi: 10.1080/15622970510029993

54. Xu J, Su X, Burley SK, Zheng XFS. Nuclear SOD1 in growth control, oxidative stress response, amyotrophic lateral sclerosis, and cancer. *Antioxidants*. (2022) 11:427. doi: 10.3390/antiox11020427

55. Kumari R, Sharma N, Sharma S, Samurailatpam S, Padhi S, Singh SP, et al. Production and characterization of bioactive peptides in fermented soybean meal produced using proteolytic Bacillus species isolated from kinema. *Food Chem*. (2023) 421:136130. doi: 10.1016/j.foodchem.2023.136130

56. Patterson JA, Burkholder KM. Application of prebiotics and probiotics in poultry production. *Poult Sci*. (2003) 82:627–31. doi: 10.1093/ps/82.4.627

57. von Martels JZH, Sadaghian SM, Bourgonje AR, Blokzijl T, Dijkstra G, Faber KN, et al. The role of gut microbiota in health and disease: *in vitro* modeling of host-microbe interactions at the aerobe-anaerobe interphase of the human gut. *Anaerobe*. (2017) 44:3–12. doi: 10.1016/j.anaerobe.2017.01.001

58. Wang J, Yoo JS, Kim HJ, Lee JH, Kim IH. Nutrient digestibility, blood profiles and fecal microbiota are influenced by chitooligosaccharide supplementation of growing pigs. *Livest Sci*. (2009) 125:298–303. doi: 10.1016/j.livsci.2009.05.011

59. Eun CS, Kim YS, Han D, Choi JH, Lee AR, Park YK. *Lactobacillus casei* prevents impaired barrier function in intestinal epithelial cells. *J Apmis*. (2011) 119:49–56. doi: 10.1111/j.1600-0463.2010.02691.x

60. Jin W, Ye H, Zhao J, Zhou Z, Huan F. Consuming fermented distillers' dried grains with solubles (DDGS) feed reveals a shift in the faecal microbiota of growing and fattening pigs using 454 pyrosequencing. *J Integr Agric*. (2017) 16:900–10. doi: 10.1016/s2095-3119(16)61523-x

61. Vadopalas L, Ruzauskas M, Lele V, Starkute V, Zavistanaviciute P, Zokaityte E, et al. Pigs' feed fermentation model with antimicrobial lactic acid bacteria strains combination by changing extruded soya to biomodified local feed stock. *Animals*. (2020) 10:783. doi: 10.3390/ani10050783

62. Engberg RM, Hammersh JM, Johansen NF, Abousekken MS, Steenfeldt S, Jensen BB. Fermented feed for laying hens: effects on egg production, egg quality, plumage condition and composition and activity of the intestinal microflora. *Br Poult Sci*. (2009) 50:228–39. doi: 10.1080/00071660902736722

63. Mathew AG, Chattin SE, Robbins CM, Golden DA. Effects of a direct-fed yeast culture on enteric microbial populations, fermentation acids, and performance of weanling pigs. *J Anim Sci*. (1998) 76:2138–45. doi: 10.2527/1998.7682138x

frontiers | Frontiers in Veterinary Science

TYPE Original Research
PUBLISHED 05 January 2024
DOI 10.3389/fvets.2023.1342747

OPEN ACCESS

EDITED BY
Tao Wang,
Jilin Agricultural University, China

REVIEWED BY
Yonggang Liu,
Beijing University of Chinese Medicine, China
Shuwen Guan,
Jilin University, China
Liu Jieqing,
Huaqiao University, China

*CORRESPONDENCE
Shumin Ge
✉ geshumin2013@163.com
Shuhua Zhang
✉ zhangsh1018@126.com

RECEIVED 22 November 2023
ACCEPTED 14 December 2023
PUBLISHED 05 January 2024

CITATION
Wang J, Zhang K, Zhang Y, Ge S and Zhang S (2024) Defense against oxidative stress in *Caenorhabditis elegans* by dark tea. *Front. Vet. Sci.* 10:1342747. doi: 10.3389/fvets.2023.1342747

Defense against oxidative stress in *Caenorhabditis elegans* by dark tea

Jianxiu Wang, Kaiheng Zhang, Yaya Zhang, Shumin Ge* and Shuhua Zhang*

School of Life Science and Technology, Changchun University of Science and Technology, Changchun, China

Dark tea, rich in nutricines including tea polyphenols and free amino acids, is a kind of post-fermented tea. The potential application of nutricines against oxidative damage and senescence, which drives animal health maintenance and disease prevention, has attracted considerable interest. In this study, the effect of dark tea and its effects on longevity and defense against oxidative stress was investigated in the *Caenorhabditis elegans* (*C. elegans*) model. Under normal conditions, dark tea extended the lifespan without significant impairment of propagation. It also improved the motility, alleviated the fat accumulation and apoptosis. Additionally, orally administered dark tea could significantly decrease the level of reactive oxygen species (ROS) and resulted in a superior lifespan in H_2O_2-induced oxidative stressed *C. elegans*. In antioxidant assays *in vitro*, dark tea was found to be rich in strong hydroxyl, DPPH and ABTS+ free radical scavenging capacity. Interestingly, mRNA sequence analyses further revealed that dark tea may catalyze intracellular relevant oxidative substrates and synthesize antioxidants through synthetic and metabolic pathways. These results suggest that dark tea is worth further exploration as a potential dietary supplement for the maintenance of animal health and the prevention of related diseases.

KEYWORDS

dark tea, oxidative stress, longevity, mRNA sequencing analysis, *Caenorhabditis elegans*

1 Introduction

The health of farm animals is critical to production and profitability. Alterations in energy metabolism can adversely affect their health. Elevated metabolic demands can lead to a significantly increased oxygen demand which can result in an upsurge in reactive oxygen species (ROS) (1). This, in turn, disrupts the equilibrium between the production and elimination of free radicals, contributing to a heightened inflammatory response (2). Disruptions in the redox balance have been associated with common diseases such as enteritis and mastitis in pigs and cattle, and recurrent airway obstruction in horses (3). However, the currently used chemically synthesized antioxidants have been under suspicion for their association with organic damage (4, 5). The efficient production of foods at a low cost is of great importance for good health. Therefore, researchers have developed a keen interest on the potential application of natural nutrients against oxidative damage and for longevity.

Tea is a commonly consumed functional beverage. It has high levels of safety and significant therapeutic effects, together with low toxicity and minimal side effects. According to the different production methods, tea can be classified into white tea, green tea, yellow tea, oolong tea, black tea, and dark tea. The quality characteristics of tea are associated with a combination of various active substances, including polyphenolic compounds for astringency, free amino acids for the sense of newness, and volatile substances for aromas (6, 7). Tea has a variety of health properties, such as antioxidant, anti-inflammatory, immunomodulatory, anti-cancer, cardio-protective, anti-diabetic, weight loss and hepatoprotective effects (8). The available data suggest that green tea polyphenol supplementation at postpartum improved the milk yield and health status in cows with hyperketonemia during early lactation (9). Green tea extracts could improve intestinal microflora balance, contributing to the prevention of digestive and respiratory organ diseases in calves (10). The catechins components in green tea have been found to relieve oxidative stress and fatty liver disease in dairy cows during the periparturient phase and transition period (11, 12). Hence, tea can be used as a functional substance in livestock feed.

Dark tea is a tree belonging to the *Camellia sinensis* family, distinguished by the oily black or black-brown color of its leaves (13). The process of production of dark tea involves microorganisms; hence, dark tea is the only post-fermented tea among the six major tea types (14). Compared to the widely studied green, black, and oolong teas, dark tea is often overlooked as a unique post-fermented tea. Dark tea contains phytochemicals and macronutrients, also believed to be beneficial to animals (15). As a natural and harmless nutraceutical, it can be used as a nutritional supplement, food additive and medicinal ingredient (16, 17).

Caenorhabditis elegans (*C. elegans*) is the first multicellular organism to have its genome sequenced fully. The conservation of illnesses-related pathways between *C. elegans* and higher organisms, along with the advantages of its short life cycle, simple culture and high reproduction capacity (18, 19), has made *C. elegans* a favorable *in vivo* non-rodent model organism for mechanistic explanations and high-throughput screening of drug candidates, being screened for a range of oxidative stress, toxicity and related conditions or diseases (20).

Therefore, in this study, three types of Chinese dark tea, all of which are commonly available in the market and have high sales volumes, were selected as raw materials, namely Brick tea, Pu'er tea, and Liubao tea, to investigate the effects of dark tea on the lifespan, propagation, motility, fat deposition, apoptosis, and resistance to oxidative stress in *C. elegans*. Additionally, the differences in the expression of genes were explored by the mRNA sequence analyses.

2 Materials and methods

2.1 Preparation of dark tea extract

Brick tea (Anhua, China), Pu'er tea (Xishuangbanna, China), or Liubao tea (Wuzhou, China), were mixed with water in a 1:15 ratio of tea to water, and heated in a water bath at 85°C for 1 h, cooled and filtered, and then subjected to rotary evaporation at 58°C for 1 h, respectively (21).

2.2 Active ingredients in dark tea extract

The total polyphenol contents of dark tea were determined through the iron tartrate colorimetric method (22). Free amino acids were detected through ninhydrin colorimetry (23). The active ingredients in dark tea extract are listed in Supplementary Table 1.

2.3 Free radical scavenging ability of dark tea

Determination of the free radical scavenging ability of Brick, Pu'er, and Liubao teas at concentrations of 200, 400, 600 and 800 μg/mL, respectively.

2.3.1 Hydroxyl radical scavenging ability of dark tea

A sample solution (0.5 mL) of the dark tea extracts was added to 0.5 mL of salicylic acid ethanol solution (9 mmol/L; LABGO, China), 0.5 mL of H_2O_2 (9 mmol/L; Guangfu, China) solution, and 0.5 mL of $FeSO_4$ (9 mmol/L; BEIJINGSHIJI, China) solution. The mixtures were allowed to react for 30 min in a 37°C water bath, and the absorbance of the resulting solution was measured at 510 nm, and the values were assessed against a blank (24). The hydroxyl radical scavenging ability was calculated by the following formula:

$$Hydroxyl\ radical\ scavenging\ activity\,(\%) = \left(1 - \frac{A1 - A2}{A0}\right) \times 100\%$$

where A1 is the absorbance of the reaction solution with the sample, A2 is the absorbance of the solution without salicylic acid and A0 is the control group where the sample was replaced with distilled water.

2.3.2 DPPH free radical scavenging ability of dark tea

Dark tea extracts (2 mL) were added to 2 mL of DPPH (Aladdn, China) ethanol solution (5 mg/mL). The mixtures were allowed to react for 30 min in a dark place, and the absorbance of the resulting solution was measured at 517 nm, and the values were calculated against a blank (25). The DPPH radical scavenging ability was calculated by the following formula:

$$DPPH\ radical\ scavenging\ activity\,(\%) = \left(1 - \frac{A1 - A2}{A0}\right) \times 100\%$$

where A1 is the absorbance of the reaction solution with the sample, A2 is the absorbance of anhydrous ethanol in place of DPPH and A0 is the absorbance of anhydrous ethanol in place of sample.

2.3.3 ABTS+ free radical scavenging ability of dark tea

Dark tea extracts (3 mL) were added to 1 mL of 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulphonate) (ABTS; Rhawn, Chian) solution. The mixtures were allowed to react for 6 min in a 30°C water bath, and the absorbance of the resulting solution was measured at 734 nm, and the values were assessed against a blank

(26). The ABTS+ radical scavenging ability was calculated by the following formula:

$$ABTS + radical\ scavenging\ activity\,(\%) = \left(1 - \frac{A1}{A2}\right) \times 100\%$$

where A1 is the absorbance of the reaction solution with the sample and A2 is the control group where the sample was replaced with distilled water.

2.4 *Caenorhabditis elegans* strains and maintenance

In this research, the wild-type N2 strain of *C. elegans* was used. All *C. elegans* strains were grown in the nematode growth medium (NGM) at 20°C, and all *C. elegans* feed on *Escherichia coli OP50*. All *C. elegans* strains and the *Escherichia coli OP50* were purchased from the Caenorhabditis Genetics Centre, University of Minnesota (Minneapolis, MN, USA). *C. elegans* was transferred by cutting a small portion of the nematode-containing NGM using a sterile scalpel, placing it upside down in OP50-coated NGM, and incubating it at 20°C to allow *C. elegans* to crawl onto the new NGM. At the time of synchronization during the spawning period, *C. elegans* were washed down with M9 buffer, the lysate was added, and the precipitate was taken by centrifugation at low speed.

2.5 Lifespan assay

The synchronized N2 *C. elegans* were placed on NGM containing Brick tea extract (600, 700, 800 μg/mL), Pu'er tea extract (600, 700, 800 μg/mL), and Liubao tea extract (600, 700, 800 μg/mL) for incubation, which was set up as the experimental group, and the one with no dark tea extract as the control group. To block *C. elegans* reproduction, 5-Fluorouracil (FUDR; Rhawn, China) was added. For each strain, at least 100 egg-synchronized *C. elegans* were grown at 20°C and transferred daily to a fresh plate (27). The number of *C. elegans* surviving, dying and lost was recorded every day until all *C. elegans* were dead.

2.6 Lifespan assay in the oxidative damage state

The synchronized N2 *C. elegans* were placed on NGM containing 600 μg/mL Brick tea extract, 700 μg/mL Pu'er tea extract, and 700 μg/mL Liubao tea extract for cultivation. They were allowed to grow until reaching the L4 stage after which cultivation was continued for an additional 4 days and compared with the control group. Oxidative damage to *C. elegans* was induced by adding 200 μL of H_2O_2 (0.5 mmol/L, Rhawn, China) solution to a 96-well plate (28). Then 15 *C. elegans* were placed into each well, and the number of *C. elegans* that died was observed at hourly intervals until all of the *C. elegans* were dead, and the survival rate was estimated.

2.7 Progeny assay

The method was the same as mentioned in Section 2.6. During the reproductive period (approximately days 1–6), *C. elegans* were transferred every day to new NGM plates and allowed to deposit progeny. Record the number of eggs laid per day (29).

2.8 Motility assay

The same method as mentioned in Section 2.6 was followed. FUDR (Rhawn, China) was added to block *C. elegans* reproduction and then incubated at 20°C until days 2, 4, 8, 12, and 14. *C. elegans* were then picked and placed onto agar spacers coated with OP50. The frequency of head bobbing in 1 min and the number of body bending within 20 s were observed using a body microscope (30).

2.9 Fat deposition assay

For this, the protocol mentioned in Section 2.6 was followed. Synchronized *C. elegans* were grown for 4 days after which they were washed with M9 buffer, and the precipitate was collected after low-speed centrifugation. Subsequently, 100 μL of 1% paraformaldehyde (Skyho, China) was added, and the sample was kept at 4°C for 15 min before being snap-frozen and stored at −80°C. After 1 h, the samples were removed, thawed, centrifuged, and rinsed with M9 buffer. Next, a mixture of equal volumes of 2% Triton X-100 (Applygen, China) and 1% Oil Red O (Solarbio, China) was prepared, and 100 μL of this mixture was added for precipitation. The mixture was then incubated in a 37°C temperature-controlled shaker for 30 min (31). Finally, *C. elegans* were picked and placed on an agar spacer using a picker, and images were captured under a fluorescence microscope.

2.10 Reactive oxygen species (ROS) accumulation assay

The DCFH-DA method (32) was used to detect the level of ROS *in vivo*. ROS levels in *C. elegans* were measured under both normal conditions and following induction of oxidative damage by H_2O_2. Same method as Section 2.6. Subsequently, the *C. elegans* were exposed to H_2O_2 (0.5 mmol/L) for 2 h at 20°C. The OP50 around *C. elegans* was washed using M9 buffer; from this, 100 μL of precipitate was taken and 1 μL of 10 mM H2DCF-DA (Chemstan, China) was added, and placed in a constant temperature shaker at 37°C in dark for 30 min. *C. elegans* was then transferred to a 2% agar spacer, and observed and photographed using a fluorescence microscope at the excitation wavelength of 485 nm and the emission wavelength of 528 nm.

2.11 Apoptosis assay

Apoptosis was experimentally detected *in vivo* using acridine orange staining (33), following the same method mentioned in Section 2.6. After three rinses *C. elegans* with M9 buffer, the supernatant was

aspirated, and 100 µL of acridine orange (25 µg/mL, Klamar, China) staining solution was added and placed on a constant temperature shaker at 37°C, in the dark for 2 h. Subsequently, *C. elegans* were placed in blank NGM for 10 min to allow recovery from the staining. Finally, they were transferred to 2% agar pads and observed under a fluorescence microscope at an excitation wavelength of 488 nm and an emission wavelength of 515 nm.

2.12 Transcriptomics analysis

After 4 days of feeding on dark tea, *C. elegans* was rinsed, placed in microfuge tubes, and left to settle, after which the precipitate was taken. RNA-seq was performed on a sequencing platform at Beijing BMK Biotechnology Co., Ltd. (Beijing, China), to obtain insights into aggregate gene transcription in subject *C. elegans* cells.

2.13 Statistical analysis

Statistical analysis was performed using GraphPad Prism 8, and expressed as mean ± standard deviation (SD). One-way ANOVA was used for multiple group comparisons. Values with $p < 0.05$ were considered statistically significant. The intensity of quantified fluorograms was analyzed using ImageJ software. Each experiment was repeated three times.

3 Results

3.1 Dark tea extends the lifespan of *Caenorhabditis elegans*

The *C. elegans* were successively treated with Brick, Pu'er, and Liubao teas under normal culture conditions. As shown in Figure 1 and Table 1, all these treatments affected the normal *C. elegans* lifespan. The mean lifespan of control *C. elegans* was 14 days. Treatment with the different teas was found to prolong the lifespan of *C. elegans*, resulting in increased average lifespan of 21.4, 14.3, and 14.3% after treatment with Brick tea at concentrations of 600, 700, and 800 µg/mL, respectively, 7.1, 21.4, and 14.3% after exposure to the same concentrations of Pu'er tea, and 21.4, 28.4, and 21.4% for Liubao tea, respectively, all relative to the control group. In addition, 600 µg/mL Brick tea, 700 µg/mL Pu'er tea, and 700 µg/mL Liubao tea had the most significant effect on the increase in average and maximum lifespan (Table 1). Therefore, the above-mentioned concentrations were selected for subsequent experiments.

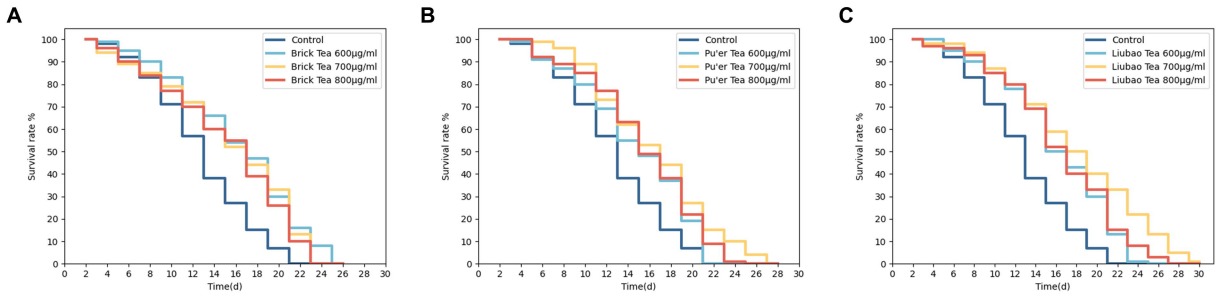


FIGURE 1
Effects of various concentrations of dark tea (**A**, Brick Tea; **B**, Pu'er Tea; **C**, Liubao Tea) on the lifespan in *C. elegans*.

TABLE 1 Mean and maximum lifespan in *C. elegans* (Mean ± SD).

Group	Mean lifespan (d)	Maximum lifespan (d)	Lifespan improvement rate%[a]
Control	13.76 ± 1.67	20 ± 2.05	
600 µg/ml Brick Tea	17.2 ± 1.70	22 ± 2.48	20
700 µg/ml Brick Tea	16.24 ± 1.77	22 ± 1.7	15.3
800 µg/ml Brick Tea	16.14 ± 1.60	22 ± 2.05	14.7
600 µg/ml Pu'er Tea	15.7 ± 1.85	20 ± 1.63	12.4
700 µg/ml Pu'er Tea	17.44 ± 1.05	26 ± 2.05	21.1
800 µg/ml Pu'er Tea	16.5 ± 1.62	24 ± 1.63	16.6
600 µg/ml Liubao Tea	17.08 ± 1.37	24 ± 2.24	19.4
700 µg/ml Liubao Tea	18.7 ± 1.51	30 ± 1.63	26.4
800 µg/ml Liubao Tea	17.42 ± 1.57	26 ± 1.64	21

[a]Lifespan improvement rate%: Maximum life increase/Maximum lifespan of the control.

10.3389/fvets.2023.1342747

3.2 Dark tea enhances the oxidative stress resistance in *Caenorhabditis elegans*

3.2.1 Dark tea extends the lifespan of *Caenorhabditis elegans* under oxidative damage conditions

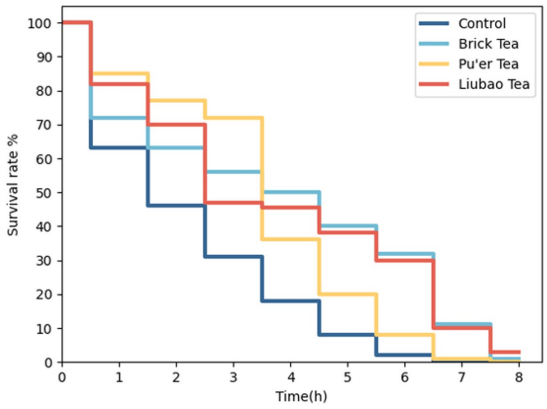


FIGURE 2
Effects of dark tea on the lifespan in *C. elegans* under oxidative damage conditions.

Lifespan involves the process of gradual aging, and the oxidative damage pathway accelerates aging (34). Under oxidative stress, the lifespan of dark tea-treated *C. elegans* increased significantly, especially in those treated with Brick tea or Liubao tea (Figure 2).

3.2.2 Dark tea decreases the ROS level of *Caenorhabditis elegans*

Under both normal conditions and oxidative stress, the control group had the highest ROS level and this effect was minimized by dark tea treatments (Figure 3). The fluorescence intensity decreased by 41, 68, and 37% after treatment with Brick, Pu'er, and Liubao teas, respectively (Figure 3C), without H_2O_2 treatment, and decreased by 28.4, 35.4, and 22.2%, respectively (Figure 3D), in the presence of H_2O_2 compared with the control group. These results demonstrated that dark tea markedly reduced the levels of ROS in *C. elegans*, indicating that dark tea has strong antioxidant activity *in vivo*.

3.2.3 Dark tea scavenges hydroxyl, DPPH, and ABTS+ free radical *in vitro*

To further assess the effects of dark tea on rendering stress resistance in *C. elegans*, the free radicals scavenging capacity of dark tea was investigated. As shown in Figure 4, the scavenging capacity of 600 μg/mL of Brick tea for hydroxyl, DPPH, and ABTS+ radicals was maximized. The scavenging ability of Pu'er tea and Liubao tea for DPPH, and ABTS+ radicals increased with increasing concentration; however, the scavenging

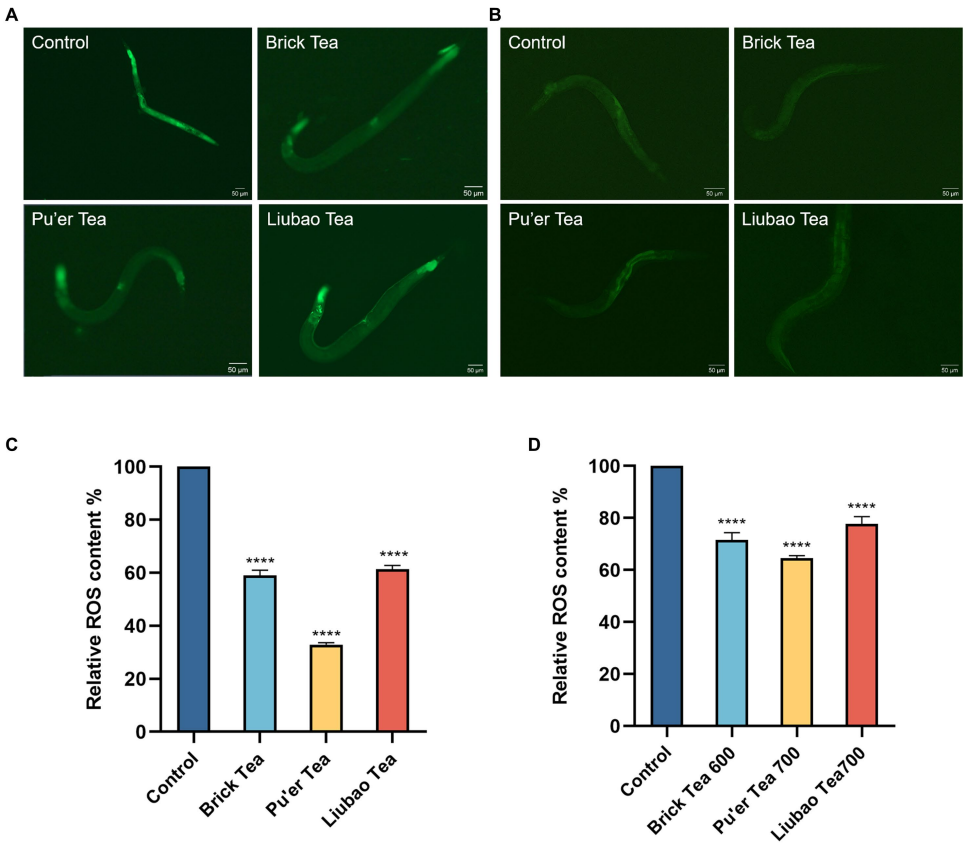


FIGURE 3
Effects of dark tea on the ROS levels in *C. elegans*. **(A)** ROS fluorescence plot in the normal state. **(B)** ROS fluorescence plot in the oxidative damage state. **(C)** Quantitative analysis plot of ROS fluorescence intensity in the normal state. **(D)** Quantitative analysis plot of ROS fluorescence intensity in the oxidative damage state. **** indicates $p < 0.0001$.

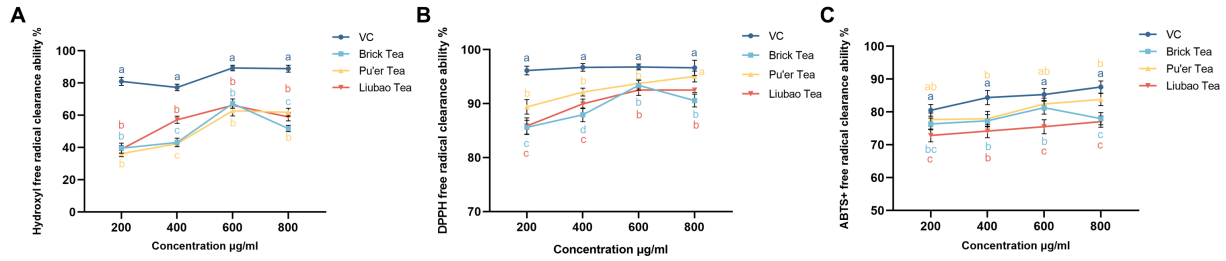


FIGURE 4
Effects of dark tea on the free radicals scavenging capacity. **(A)** -OH. **(B)** DPPH. **(C)** ABTS+.

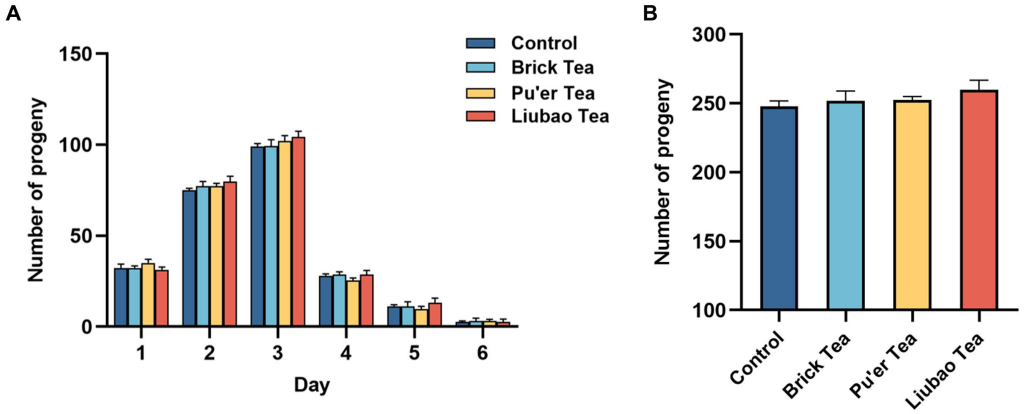


FIGURE 5
Effects of dark tea on the reproductive capacity in *C. elegans*. **(A)** The number of offspring daily in each groups in the pawning period. **(B)** The total of number of offspring in each group in the pawning period.

ability for hydroxyl radicals reached a maximum at 600 μg/mL. These results suggest the strong free radicals scavenging capacity of dark tea.

3.3 Dark tea exerts no toxicity on the fertility of *Caenorhabditis elegans*

The spawning period of *C. elegans* was about 6–7 days, and mainly concentrated in the first 3 days (Figure 5A). There was no significant difference between the experimental groups in terms of the total number of offspring (Figure 5B). The results indicated that the reproductive capacity of *C. elegans* is not impaired by dark tea and provided evidence for the safety of dark tea.

3.4 Dark tea promotes the motility of *Caenorhabditis elegans*

With aging, muscle function gradually declines, which in turn slows down mobility. The effect of dark tea on motility was recorded on days 2, 4, 8, 12 and 14 post-dosing, and the results are presented in Figure 6. On day 12, the motility of *C. elegans* decreased substantially. The dark tea significantly affected the frequency of head bobbing in *C. elegans* in the pre-preliminary stage, among these, Liubao tea had the most significant effect (Figure 6A). Dark tea substantially enhanced the body bending frequency of *C. elegans*, whereas the effect of Pu'er tea on *C. elegans* in the later stages of the process was not significant (Figure 6B). Thus, dark tea can alleviate the aging-related decrease in the energy transportation capacity of *C. elegans*.

3.5 Dark tea alleviates the accumulation of fat of *Caenorhabditis elegans*

Fat is the root cause of inflammation due to aging (35). Staining with Oil Red O (Solarbio, China) showed a significantly lower number of fat cells in the experimental group than those in the control group (Figure 7A), and *C. elegans* fed Brick tea had the lowest body fat content. Further analysis revealed that the body fat content of *C. elegans* fed with Brick tea, Pu'er tea, or Liubao tea was reduced by 9, 4, and 8%, respectively (Figure 7B). These results suggest that dark tea significantly ameliorated age-associated physiological characteristics by reducing fat deposition in *C. elegans* by reducing fat accumulation.

3.6 Dark tea reduced the apoptosis of *Caenorhabditis elegans*

Caenorhabditis elegans treated with Brick tea, Pu'er tea, and Liubao tea had significantly lower apoptosis than the control group

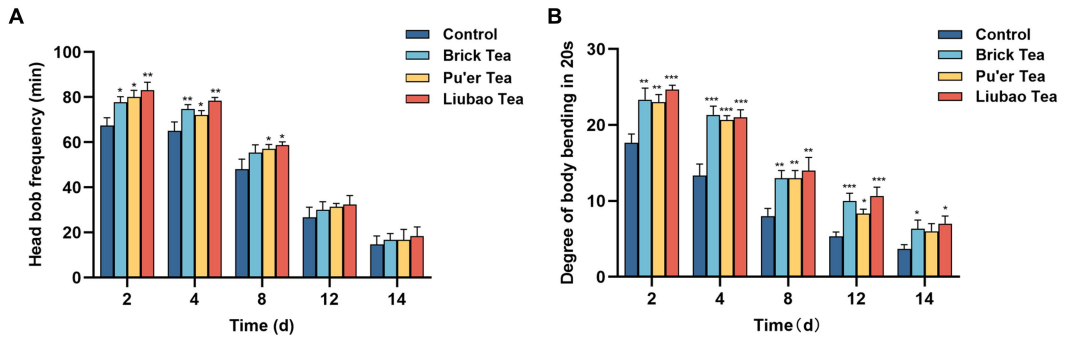


FIGURE 6
Effects of dark tea on the motility in *C. elegans*. **(A)** Frequency of head bobbing in 1 min. **(B)** Degree of body bending in 20 s. * indicates $p < 0.05$, ** indicates $p < 0.01$, *** indicates $p < 0.001$.

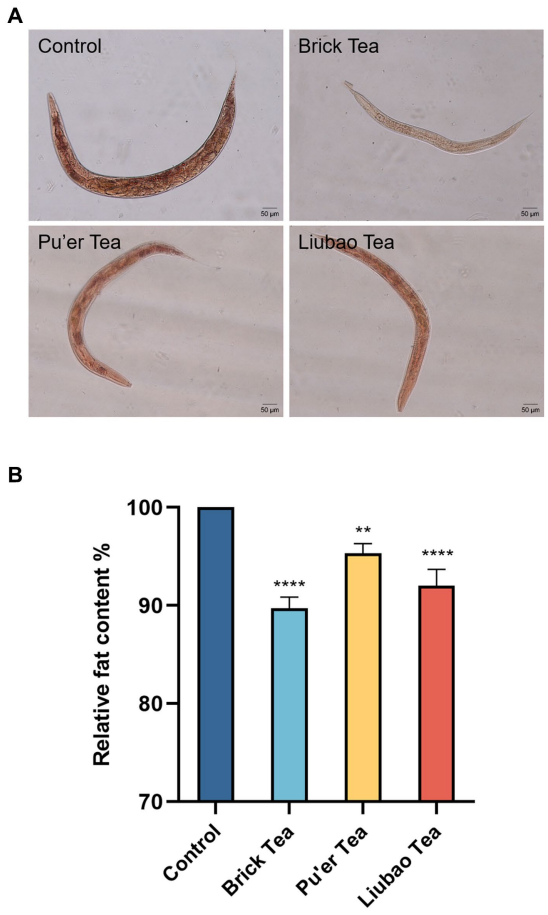


FIGURE 7
Effects of dark tea on the fat content in *C. elegans*. **(A)** Plot of fat staining. **(B)** Plot of quantitative analysis of staining intensity. ** indicates $p < 0.01$, **** indicates $p < 0.0001$.

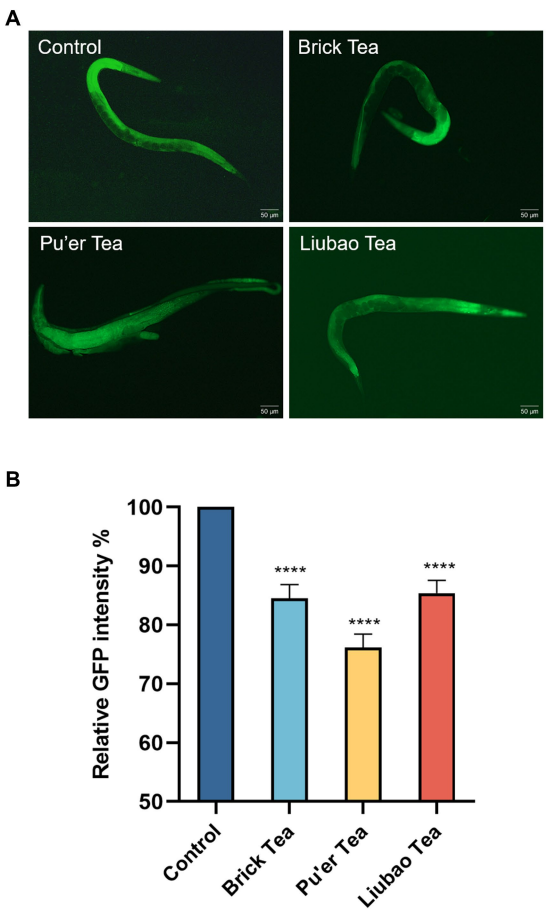


FIGURE 8
Effects of dark tea on the apoptosis in *C. elegans*. **(A)** Apoptosis fluorescence graph. **(B)** Quantitative analysis graph of apoptosis fluorescence intensity. **** indicates $p < 0.0001$.

(Figure 8A). Quantitative analysis of the fluorescence intensity in *C. elegans* fed Brick tea, Pu'er tea, and Liubao tea decreased by 15, 24, and 14%, respectively (Figure 8B). The above results show that dark tea could achieve anti-aging effects by inhibiting the degree of apoptosis.

3.7 Transcriptomics analysis

3.7.1 Gene expression differences

Genetic data of dark tea-treated three groups are shown in Figure 9. The volcano plot (red, up-regulated; blue, down-regulated)

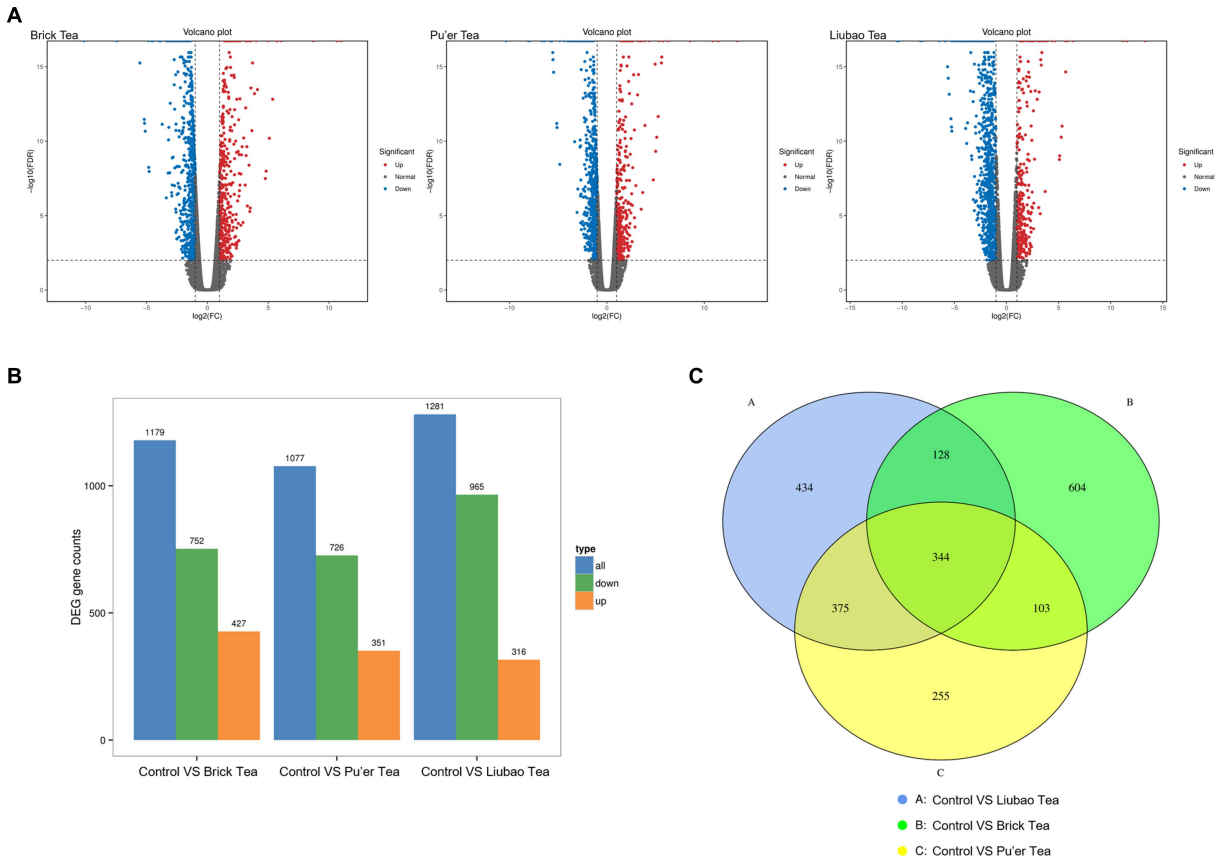


FIGURE 9
Distribution of genes identified in dark tea. **(A)** Volcano plot of differentially expressed genes. The horizontal coordinate indicates the logarithmic value of the fold difference in expression of a gene in the two samples and the vertical coordinate indicates the negative logarithmic value of the statistical significance of the change in gene expression. Red dots indicate up-regulation and blue dots indicate down-regulation. **(B)** Up-regulated and down-regulated genes. **(C)** Venn diagram of the set of differential genes (fold change≥2).

revealed that the number of down-regulated or up-regulated genes in the Brick tea, Pu'er tea, and Liubao tea groups overlapped, while each group also exhibited unique genes (Figure 9A). Interestingly, the number of down-regulated genes was the highest in the Liubao tea group, while the number of up-regulated genes was the most in the Brick tea group was the most (Figure 9B). Compared to the control group, the same 344 genes were identified in the Brick tea, Pu'er tea, and Liubao tea groups (Figure 9C).

3.7.2 GO enrichment of differentially quantified genes

The gene products were analyzed for GO enrichment in terms of biological process (BP), cellular component (CC), and molecular function (MF), as shown in Figure 10. A few of the same up-regulated and down-regulated genes with functional annotations were identified in the Brick tea, Pu'er tea, and Liubao tea groups, which included cellular process and metabolic process in BP, cellular anatomical entity in CC, and catalytic activity and binding in MF. The alterations in the above-mentioned biological functions have all been shown to affect lifespan (36). Thus, dark tea mainly affects endocrine system-related pathways, especially catalysis-related bio-metabolism within cells.

3.7.3 Specific regulation pathways of dark tea

To further explore the regulation pathways of dark tea, a KEGG pathway enrichment analysis was conducted (Figure 11). In the Brick tea, Pu'er tea, and Liubao tea groups, the ubiquitin mediated proteolysis, spliceosome and mannose type O-glycan biosynthesis pathways were significantly up-regulated, while fatty acid metabolism and fatty acid degradation pathways were significantly down-regulated. Among the annotated pathways, a majority were closely linked to glucose and lipid metabolism. These pathways suggested the metabolism and biosynthesis are significantly involved in the redox reaction, which were important to improve antioxidant defense system.

A total of 2, 1, and 2 genes, respectively, were significantly up-regulated ($p < 0.05$) in the ubiquitin mediated proteolysis, mannose type O-glycan biosynthesis, and fatty acid metabolism and degradation pathways, respectively (Table 2). Specifically, the expression of PRKN and SKP1 in the ubiquitin mediated proteolysis was significantly up-regulated ($p < 0.05$). After dark tea treatment, the expression of K12H6.3 in the mannose type O-glycan biosynthesis was significantly up-regulated ($p < 0.05$); in addition, the expression of ACADM, CPT1 in the fatty acid metabolism and degradation pathways was significantly up-regulated ($p < 0.05$).

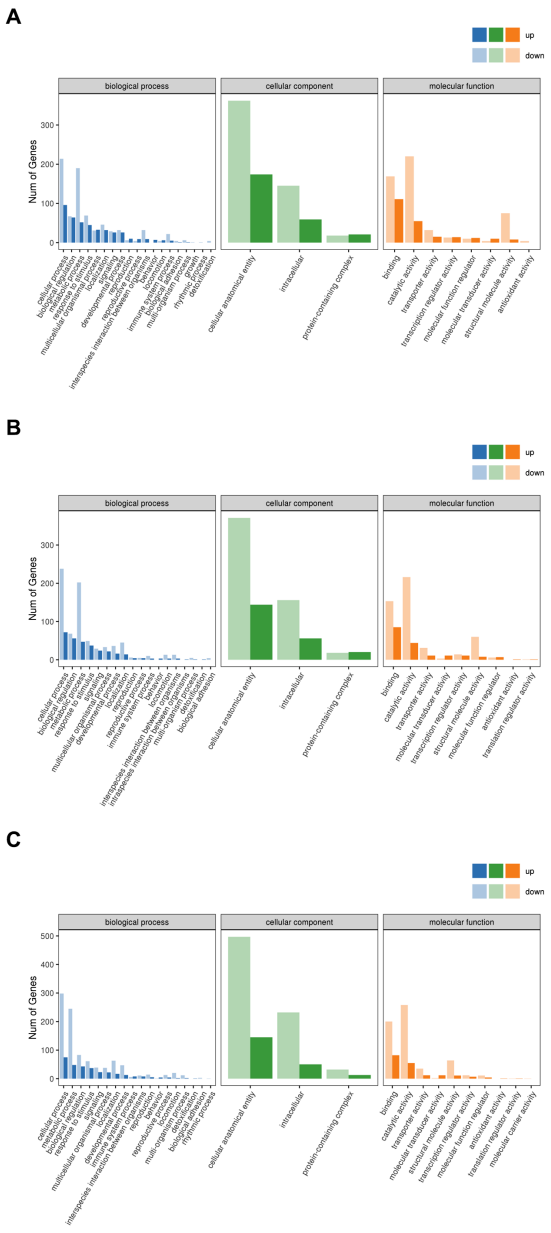


FIGURE 10
GO annotation categorization statistical chart. **(A)** Brick Tea. **(B)** Pu'er Tea **(C)** Liubao Tea. The horizontal coordinate is the GO classification, the vertical coordinate is the number of genes, and different colors represent the different primary classifications to which they belong.

4 Discussion

As the only post-fermented tea, many biochemical reactions of the fermentation process in the production of dark tea result in differences that set dark tea and other five types of tea. Twenty-two macronutrient chemical composition indicators in 65 Chinese dark teas have been reported, with the richer ones being polyphenols and free amino acids (9). In the present study, we first determined the content of the main two nutrients in the three represented dark teas. The contents of tea polyphenols and L-theanine in dark tea were maintained at around 10 and 1.5% (Supplementary Table 1).

Free radical production is essential in normal metabolism, and excessive free radical formation may lead to oxidative stress and other related diseases (37). Xu et al. (38) found that tea polyphenols increased SOD, CAT, T-AOC and GSH-Px contents along with a reduction in MAD and ROS in crows. In this study, we found that orally supplied dark tea could significantly decrease the ROS levels and exhibited a superior lifespan in H_2O_2-induced oxidative stress *C. elegans*. Similarly, we observed an improvement in-OH, DPPH, and ABTS+ free radicals scavenging capacity in antioxidant assays *in vitro*, indicating the scavenging ability of dark tea.

The relationship between the activity of antioxidants and longevity promotion has long been noticed. Although many chemically synthesized antioxidants are available, long-term use may have potential side effects (2, 3). We found that dark tea extended the lifespan and motility without significantly impairing propagation in *C. elegans*. Obesity due to excessive fat accumulation is associated with accelerated onset of diseases occurring in old age, while fat ablation increases life span (39). Apoptosis is also considered an effective immune defense mechanism when the body is subjected to various harmful stimuli (40, 41). Our results indicated that the lipid oxidation products and apoptosis in *C. elegans* could be reduced after dark tea treatment. Therefore, several conclusions and speculations can be drawn, which suggest that the longevity extension of dark tea might be attributed to the inhibition of lipid oxidation and the extent of generation of apoptosis-related products to protect the health of the body. Noticeably, no significant toxic effects of dark tea were observed in our study.

GO analysis allows for a standardized description of the gene products in terms of the biological pathways involved and cellular localization (42). To elucidate the roles of the identified differentially expressed genes, GO analysis revealed significant enrichment of BP, CC, and MF, namely, cellular process, metabolic process, catalytic activity, and binding, which play independent roles in antioxidant and anti-aging traits. According to KEGG enrichment analysis the number of genes annotated to a metabolic pathway in a differentially expressed gene is significantly greater than the proportion of background genes among all such genes (43). Among the annotated pathways, most are related to glucose and lipid metabolism. Four common enriched signaling pathways identified in three dark tea groups included mannose type O-glycan biosynthesis, fatty acid metabolism, fatty acid degradation, ubiquitin mediated proteolysis, and spliceosome. Further comparison of the differentially expressed genes with the functional annotations of the genes in the database, revealed that most of the genes with more significant changes in gene up-regulation and down-regulation because of dark tea treatment were regulated by SKP1, K12H6.3, ACADM, and CPT1. SKP1 was up-regulated in the ubiquitin mediated proteolysis pathway, which connects cell cycle regulators to the ubiquitin proteolysis machinery to thus improve cellular growth (44). K12H6.3 was found to be involved in the mannose type O-glycan biosynthesis pathway; the gene encodes the relevant core α1,3-fucosyltransferase, whose expression is promoted during

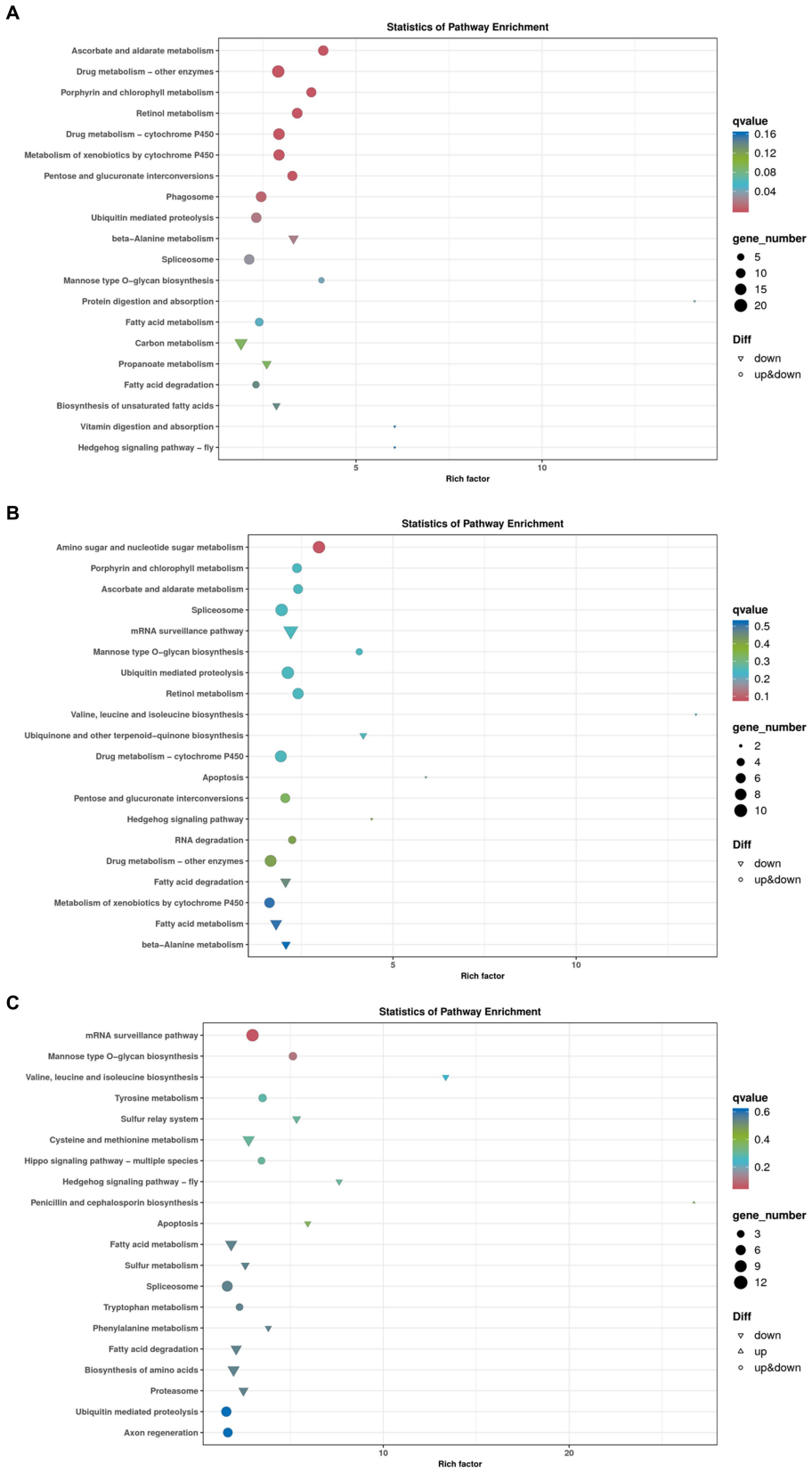


FIGURE 11
Bubble chart of genes enriched in KEGG pathways. **(A)** Brick Tea. **(B)** Pu'er Tea **(C)** Liubao Tea. The bubble size represents the number of genes in the enriched pathway terms, and the bubble color represents the *p* value.

TABLE 2 Genes after dark tea intervention in the ubiquitin mediated proteolysis, mannose type O-glycan biosynthesis, fatty acid metabolism and fatty acid degradation pathways in *C. elegans*.

Pathways	No	Gene name	Regulated	Log2FC[a]
Ubiquitin mediated proteolysis	1	PRKN	Up	1.43
	2	SKP1	Up	2.63
Mannose type O-glycan biosynthesis	1	K12H6.3	Up	3.32
Fatty acid metabolism and degradation	1	ACADM	Down	−1.17
	2	CPT1	Down	−1.48

[a]Log2FC indicates differences in gene expression in the comparison of two samples.

development (45). ACADM and CPT1 are key genes catalyzing mitochondrial fatty acid metabolism and degradation. They catalyze the rate-limiting step of the conversion of acyl-coenzyme. As into acyl-carnitines, which can cross membranes to enter the mitochondria, and elevate triglyceride, phospholipid, and droplet levels of cellular lipids to regulate genetic, epigenetic, physiological, and nutritional modulators (46).

Overall, it may be safely concluded that dark tea can independently reduce oxidative stress, thus preventing oxidative injury. As a natural and non-toxic antioxidant, dark tea can be used as a nutritional supplement for livestock and as a functional component of livestock feed, thus acting as a defense against various diseases caused by oxidative damage. It thus has great promise for animal health and disease prevention. Further exploration of dark tea as a potential dietary supplement for animal health maintenance and disease prevention is worth exploring.

5 Conclusion

In conclusion, the safety of dark tea was demonstrated in *C. elegans* and dark tea was found to mitigate oxidative stress-induced damage by promoting the clearance of free radicals. Additionally, it was observed that dark tea modulated metabolic processes, including carbohydrates, lipids, and proteins, catalyzing the synthesis of antioxidants from specific oxidative substrates within the cell.

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://figshare.com/, 10.6084/m9.figshare.24609774.

Ethics statement

The animal study was approved by Animal Care and Use Committee, Changchun University of Science and Technology. The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

JW: Writing – original draft, Writing – review & editing. KZ: Writing – original draft. YZ: Writing – original draft. SG: Writing – review & editing. SZ: Writing – review & editing.

Funding

The author(s) declare financial support was received for the research, authorship, and/or publication of this article. This research was supported by the Science and Technology Development Program Project of Jilin Provincial Science and Technology Department (grant number 20220204127YY).

Acknowledgments

We would like to acknowledge all study participants for their time, effort, and contributions.

Conflict of interest

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

Publisher's note

Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2023.1342747/full#supplementary-material

References

1. Wu S, Liu X, Cheng L, Wang D, Qin G, Zhang X, et al. Protective mechanism of leucine and isoleucine against H_2O_2-induced oxidative damage in bovine mammary epithelial cells. *Oxidative Med Cell Longev*. (2022) 2022:1–22. doi: 10.1155/2022/4013575

2. Erdemli Z, Erdemli ME, Gul M, Altinoz E, Gul S, Kocaman G, et al. Ameliorative effects of crocin on the inflammation and oxidative stress-induced kidney damages by experimental periodontitis in rat. *Iran J Basic Med Sci*. (2021) 24:825–32. doi: 10.22038/ijbms.2021.55875.12499

3. Lykkesfeldt J, Svendsen O. Oxidants and antioxidants in disease: oxidative stress in farm animals. *Vet J*. (2007) 173:502–11. doi: 10.1016/j.tvjl.2006.06.005

4. Poljsak B, Šuput D, Milisav I. Achieving the balance between ROS and antioxidants: when to use the synthetic antioxidants. *Oxidative Med Cell Longev*. (2013) 2013:1–11. doi: 10.1155/2013/956792

5. Gianeti MD, Mercurio DG, Maia Campos PM. The use of green tea extract in cosmetic formulations: not only an antioxidant active ingredient. *Dermatol Ther*. (2013) 26:267–71. doi: 10.1111/j.1529-8019.2013.01552.x

6. Zhang L, Cao QQ, Granato D, Xu YQ, Ho CT. Association between chemistry and taste of tea: a review. *Trends Food Sci Technol*. (2020) 101:139–49. doi: 10.1016/j.tifs.2020.05.015

7. Chen GH, Yang CY, Lee SJ, Wu CC, Tzen JT. Catechin content and the degree of its galloylation in oolong tea are inversely correlated with cultivation altitude. *J Food Drug Anal*. (2014) 22:303–9. doi: 10.1016/j.jfda.2013.12.001

8. Tang GY, Meng X, Gan RY, Zhao CN, Liu Q, Feng YB, et al. Health functions and related molecular mechanisms of tea components: an update review. *Int J Mol Sci*. (2019) 20:6196. doi: 10.3390/ijms20246196

9. Ma Y, Feng Y, Song L, Li M, Dai H, Bao H, et al. Green tea polyphenols supplementation alters immunometabolism and oxidative stress in dairy cows with hyperketonemia. *Anim Nutr*. (2021) 7:206–15. doi: 10.1016/j.aninu.2020.06.005

10. Ishihara N, Chu DC, Akachi S, Juneja LR. Improvement of intestinal microflora balance and prevention of digestive and respiratory organ diseases in calves by green tea extracts. *Livest Prod Sci*. (2001) 68:217–29. doi: 10.1016/S0301-6226(00)00233-5

11. Wein S, Beyer B, Gohlke A, Blank R, Metges CC, Wolffram S. Systemic absorption of catechins after intraruminal or intraduodenal application of a green tea extract in cows. *PLoS One*. (2016) 11:e0159428. doi: 10.1371/journal.pone.0159428

12. Vizzotto EF, Stivanin SC, de Paris M, Passos LT, Werncke D, Klein CP, et al. Supplementation with green tea and oregano extracts on productive characteristics, blood metabolites, and antioxidant status of Jersey cows during the transition period. *Animal*. (2021) 15:100032. doi: 10.1016/j.animal.2020.100032

13. Kong YS, Ren HY, Liu R, da Silva RR, Aksenov AA, Melnik AV, et al. Microbial and nonvolatile chemical diversities of Chinese dark teas are differed by latitude and pile fermentation. *J Agric Food Chem*. (2022) 70:5701–14. doi: 10.1021/acs.jafc.2c01005

14. Yue Y, Chu GX, Liu XS, Tang X, Wang W, Liu GJ, et al. TMDB: a literature-curated database for small molecular compounds found from tea. *BMC Plant Biol*. (2014) 14:243–8. doi: 10.1186/s12870-014-0243-1

15. Hamer M. The beneficial effects of tea on immune function and inflammation: a review of evidence from in vitro, animal, and human research. *Nutr Res*. (2007) 27:373–9. doi: 10.1016/j.nutres.2007.05.008

16. Lin FJ, Wei XL, Liu HY, Li H, Xia Y, Wu DT, et al. State-of-the-art review of dark tea: from chemistry to health benefits. *Trends Food Sci Technol*. (2021) 109:126–38. doi: 10.1016/j.tifs.2021.01.030

17. Deng H, Liu J, Xiao Y, Wu JL, Jiao R. Possible mechanisms of dark tea in cancer prevention and management: a comprehensive review. *Nutrients*. (2023) 15:3903. doi: 10.3390/nu15183903

18. Hunt PR. The *C. elegans* model in toxicity testing. *J Appl Toxicol*. (2017) 37:50–9. doi: 10.1002/jat.3357

19. Lin C, Zhang X, Su Z, Xiao J, Lv M, Cao Y, et al. Carnosol improved lifespan and healthspan by promoting antioxidant capacity in *Caenorhabditis elegans*. *Oxidative Med Cell Longev*. (2019) 2019:1–13. doi: 10.1155/2019/5958043

20. Shen P, Yue Y, Zheng J, Park Y. *Caenorhabditis elegans*: a convenient in vivo model for assessing the impact of food bioactive compounds on obesity, aging, and Alzheimer's disease. *Annu Rev Food Sci Technol*. (2018) 9:1–22. doi: 10.1146/annurev-food-030117-012709

21. Wang BS, Yu HM, Chang LW, Yen WJ, Duh PD. Protective effects of pu-erh tea on LDL oxidation and nitric oxide generation in macrophage cells. *LWT Food Sci Technol*. (2008) 41:1122–32. doi: 10.1016/j.lwt.2007.07.002

22. Qadir M, Muhammad T, Bakri M, Gao F. Determination of total polyphenols in tea by a flow injection-fiber optic spectrophotometric system. *Instrument Sci Technol*. (2018) 46:185–93. doi: 10.1080/10739149.2017.1346515

23. Sui H, Chen L, Han XX, Zhang X, Wang X, Zhao B. Quantitative determination of total amino acids based on surface-enhanced raman scattering and ninhydrin derivatization. *Anal Sci*. (2017) 33:53–7. doi: 10.2116/analsci.33.53

24. Romulo A. The principle of some in vitro antioxidant activity methods. *InIOP Conf Ser Earth Environ Sci*. (2020) 426:012177. doi: 10.1088/1755-1315/426/1/012177

25. Zhang L, Wang D, Chen W, Tan X, Wang P. Impact of fermentation degree on the antioxidant activity of pu-erh tea in vitro. *J Food Biochem*. (2012) 36:262–7. doi: 10.1111/j.1745-4514.2010.00536.x

26. Kına E, Uysal İ, Mohammed FS, Doğan M, Sevindik M. In-vitro antioxidant and oxidant properties of Centaurea rigida. *Turkish J Agric Food Sci Technol*. (2021) 9:1905–7. doi: 10.24925/turjaf.v9i10.1905-1907.4603

27. Reigada I, Moliner C, Valero MS, Weinkove D, Langa E, Gómez RC. Antioxidant and antiaging effects of licorice on the *Caenorhabditis elegans* model. *J Med Food*. (2020) 23:72–8. doi: 10.1089/jmf.2019.0081

28. Lin Q, Song B, Zhong Y, Yin H, Li Z, Wang Z, et al. Effect of sodium hyaluronate on antioxidant and anti-ageing activities in *Caenorhabditis elegans*. *Foods*. (2023) 12:1400. doi: 10.3390/foods12071400

29. Wang X, Zhang Z, Zhou H, Sun X, Chen X, Xu N. The anti-aging effects of *Gracilaria lemaneiformis* polysaccharide in *Caenorhabditis elegans*. *Int J Biol Macromol*. (2019) 140:600–4. doi: 10.1016/j.ijbiomac.2019.08.186

30. Lin C, Xiao J, Xi Y, Zhang X, Zhong Q, Zheng H, et al. Rosmarinic acid improved antioxidant properties and healthspan via the IIS and MAPK pathways in *Caenorhabditis elegans*. *Biofactors*. (2019) 45:774–87. doi: 10.1002/biof.1536

31. Qin X, Wang W, Chu W. Antioxidant and reducing lipid accumulation effects of rutin in *Caenorhabditis elegans*. *Biofactors*. (2021) 47:686–93. doi: 10.1002/biof.1755

32. Huang RT, Huang Q, Wu GL, Chen CG, Li ZJ. Evaluation of the antioxidant property and effects in *Caenorhabditis elegans* of Xiangxi flavor vinegar, a Hunan local traditional vinegar. *J Zhejiang Univ Sci B*. (2017) 18:324–33. doi: 10.1631/jzus.B1600088

33. Lawen A. Apoptosis—an introduction. *BioEssays*. (2003) 25:888–96. doi: 10.1002/bies.10329

34. Argyropoulou A, Aligiannis N, Trougakos IP, Skaltsounis AL. Natural compounds with anti-ageing activity. *Nat Prod Rep*. (2013) 30:1412–37. doi: 10.1039/c3np70031c

35. Bhuyan LP, Sabhapondit S, Baruah BD, Bordoloi C, Gogoi R, Bhattacharyya P. Polyphenolic compounds and antioxidant activity of CTC black tea of North-East India. *Food Chem*. (2013) 141:3744–51. doi: 10.1016/j.foodchem.2013.06.086

36. Lam WY, Jash A, Yao CH, D'Souza L, Wong R, Nunley RM, et al. Metabolic and transcriptional modules independently diversify plasma cell lifespan and function. *Cell Rep*. (2018) 24:2479–2492.e6. doi: 10.1016/j.celrep.2018.07.084

37. Wu JQ, Kosten TR, Zhang XY. Free radicals, antioxidant defense systems, and schizophrenia. *Prog Neuro Psychopharmacol Biol Psychiatry*. (2013) 46:200–6. doi: 10.1016/j.pnpbp.2013.02.015

38. Xu R, Zhu M, Cao J, Guo M. Tea polyphenols protect the mammary gland of dairy cows by enhancing antioxidant capacity and regulating the TGF-β1/p38/JNK pathway. *Meta*. (2022) 12:1009. doi: 10.3390/metabo12111009

39. Long W, Zhang G, Dong Y, Li D. Dark tea extract mitigates hematopoietic radiation injury with antioxidative activity. *J Radiat Res*. (2018) 59:387–94. doi: 10.1093/jrr/rrx072

40. Pfeffer CM, Singh AT. Apoptosis: a target for anticancer therapy. *Int J Mol Sci*. (2018) 19:448. doi: 10.3390/ijms19020448

41. Salminen A, Ojala J, Kaarniranta K. Apoptosis and aging: increased resistance to apoptosis enhances the aging process. *Cell Mol Life Sci*. (2011) 68:1021–31. doi: 10.1007/s00018-010-0597-y

42. Ashburner M, Ball CA, Blake JA, Botstein D, Butler H, Cherry JM, et al. Gene ontology: tool for the unification of biology. *Nat Genet*. (2000) 25:25–9. doi: 10.1038/75556

43. Kanehisa M, Furumichi M, Sato Y, Kawashima M, Ishiguro-Watanabe M. KEGG for taxonomy-based analysis of pathways and genomes. *Nucleic Acids Res*. (2023) 51:D587–92. doi: 10.1093/nar/gkac963

44. Guan Y, Leu NA, Ma J, Chmátal L, Ruthel G, Bloom JC, et al. SKP1 drives the prophase I to metaphase I transition during male meiosis. *Sci Adv*. (2020) 6:eaaz2129. doi: 10.1126/sciadv.aaz2129

45. Wilson IB. Glycosylation of proteins in plants and invertebrates. *Curr Opin Struct Biol*. (2002) 12:569–77. doi: 10.1016/S0959-440X(02)00367-6

46. Schlaepfer IR, Joshi M. CPT1A-mediated fat oxidation, mechanisms, and therapeutic potential. *Endocrinology*. (2020) 161:bqz046. doi: 10.1210/endocr/bqz046

frontiers | Frontiers in Veterinary Science

TYPE Original Research
PUBLISHED 15 January 2024
DOI 10.3389/fvets.2023.1300518

OPEN ACCESS

EDITED BY
Arda Yıldırım,
Gaziosmanpaşa University, Türkiye

REVIEWED BY
Maharach Matra,
Khon Kaen University, Thailand
Zhe Sun,
Jilin Agricultural University, China

*CORRESPONDENCE
Hong-Gu Lee
✉ hglee66@konkuk.ac.kr

RECEIVED 23 September 2023
ACCEPTED 01 December 2023
PUBLISHED 15 January 2024

CITATION
Ju M-S, Jo Y-H, Kim Y-R, Ghassemi Nejad J, Lee J-G and Lee H-G (2024) Supplementation of complex natural feed additive containing (*C. militaris*, probiotics and red ginseng by-product) on rumen-fermentation, growth performance and carcass characteristics in Korean native steers.
Front. Vet. Sci. 10:1300518.
doi: 10.3389/fvets.2023.1300518

Supplementation of complex natural feed additive containing (*C. militaris*, probiotics and red ginseng by-product) on rumen-fermentation, growth performance and carcass characteristics in Korean native steers

Mun-Su Ju[1], Yong-Ho Jo[1], Yoo-Rae Kim[1], Jalil Ghassemi Nejad[1], Jang-Gu Lee[2] and Hong-Gu Lee[1]*

[1]Laboratory of Animal Nutrition, Physiology and Proteomics, Department of Animal Science and Technology, Konkuk University, Seoul, Republic of Korea, [2]DM Bio Co., Ltd., Jellonam-do, Republic of Korea

This study evaluated the effects of a complex natural feed additive on rumen fermentation, carcass characteristics and growth performance in Korean-native steers. In this study, *in vitro* and *in vivo* experiment were conducted. Seven different levels of complex natural feed additive (CA) were added to the buffered rumen fluid using Ankom^{RF} gas production system for 12, 24 and 48 h. All experimental data were analyzed by mixed procedure of SAS. Total gas production increased in the CA groups, with the highest response observed in the 0.06% group at 48 h of incubation (linear, $p = 0.02$; quadratic, $p < 0.01$). Regarding rumen fermentation parameters, the total volatile fatty acid (TVFA) tended to increase in all the CA groups ($p = 0.07$). The concentrations of butyrate, iso-butyrate, and iso-valerate significantly increased in all treatment groups ($p < 0.05$). In the *in vivo* experiment, 23 Korean-native steers were allocated to two groups: (1) Control and (2) Treatment; control +0.07% CA (DM basis), in a randomized complete-block design and blocked by body weight (ave. body weight = 641.96 kg ± 62.51 kg, $p = 0.80$) and feed intake (ave. feed intake = 13.96 kg ± 0.74 kg, $p = 0.08$) lasted for 252 days. Average daily gain decreased in the treatment group ($p < 0.01$). Backfat thickness significantly decreased in the CA group ($p = 0.03$), whereas meat color tended to increase ($p = 0.07$). In conclusion, in the *in vitro* experiment, the inclusion of complex natural feed additive decreased methane proportion and tended to increase TVFA production, but supplementation to Korean native steers decreased average daily gain and backfat thickness.

KEYWORDS

***C. militaris*, probiotics, rumen, Korean-native steers, feed additive**

1 Introduction

With the increase in the global population, growth of the global economy, and urbanization, there has been an increased demand for livestock products, including meat, milk, and eggs. These phenomena put pressure on the livestock industry to meet global expectations (1). Recently, animal producers have shown interest in natural compounds to improve animal physiological state and health (2). There are numerous natural compounds that can serve as alternatives to growth-promoting antibiotics to enhance animal health and well-being (3). One of these alternatives is probiotics, referred to as 'direct-fed microbial' (DFM). DFM refers to living microorganisms utilized to enhance livestock performance by modifying the microbial composition within the animal intestinal tract (4). Probiotics are employed in the ruminant industry to modulate the microflora and fermentation activities for the benefit of the host (5). Lactic acid-producing bacteria, lactic acid-utilizing bacteria, yeast, and fungi are commonly utilized as probiotics in ruminants (5). DFMs act in both the intestinal tract and post-gastrointestinal region. Several studies have reported that these microorganisms not only enhance performance but also modulate the immune response of animals (6–8).

Cordyceps militaris (*C. militaris*) is entomopathogenic fungus that has been used for the last 300 years in East Asia due to its adaptogenic and tonic effects (9). *Cordyceps* spp. contain several bioactive compounds such as cordycepic acid (D-mannitol), cordycepin, ergosterol and adenosine (10, 11). *C. militaris* has been shown to improve *in-vitro* rumen fermentation, leading to an increase in volatile fatty acids (VFA), cellulose digestion, and cellulolytic enzyme activities (12, 13). *Panax ginseng* belongs to the Araliaceae family, which is an herbaceous plant and one of the most precious plant products in Northeast Asia (14). Red ginseng is produced by steaming fresh ginseng, known to contain pharmaceutical efficacy due to its major bioactive constituent (triterpene saponin), also called ginsenosides (15). This manufacturing process is known to enhance the saponin content in ginseng (16). Due to the medicinal efficacy of red ginseng, there has been an increase in the production of red ginseng, leading to the production of about 8,000 tons of by-products (17). Regardless of extracting its bioactive compounds using solvents like water or, alcohol, are known to contain bioactive compounds such as ginsenosides (15). By-products of red ginseng continue to be produced as the market is increasing, and several studies have indicated that these could be used as additives in the animal feed industry (17, 18).

Recently, phytogenic feed additives have been recognized for their ability to influence feed quality, animal health, as well as animal products. Several studies have shown that the individual components of natural feed additives can have various positive effects on animals (19–21). It is expected that a combination of these components will work synergistically to enhance outcomes when included in animal diets. To our knowledge, no previous work has been conducted to evaluate the performances of cattle using a mixture of these compounds. Therefore, this study aimed to evaluate the effects of combination of probiotics and natural feed additives on the physiological aspects and growth performance of Korean-native cattle.

2 Materials and methods

2.1 Preparing complex natural feed additive (CA)

The complex natural feed additive, containing (probiotics, red ginseng by-product and *C. militaris*) was obtained from DM bio-Co., LTd (Jeollanam-do, Korea). Probiotics were inoculated into a sterilized medium composed of red ginseng by-product, soy-bean meal, distillers' grain, wheat bran and condensed molasses soluble (CMS). The probiotics used in this additive included *Bacillus subtilis* (1.0×10^{11} CFU/g), *Aspergillus oryzae* (1.0×10^{10} CFU/g), *Saccharomyces cerevisiae* (1.0×10^{11} CFU/g), *Lactobacillus acidophillus* (1.0×10^{10} CFU/g), and *Streptococcus thermophilus* (1.0×10^{11} CFU/g). After inoculating the probiotics, the mixture was dried at low temperature and *C. militaris* (20%) was added to the total volume of the complex natural feed additive (Korea. Patent No. 10–1137893).

2.2 Chemical analysis

Experimental diets were finely milled through a 250 ~ 500 μm screen and analyzed for chemical composition (Table 1). All samples were dried in an air-drying oven at 60°C for 48 h to determine the chemical composition. The chemical analysis for ether extract (EE; Method 920.39), crude protein (CP; Method 954.01), and crude ash (Ash; Method 942.05) of diets were followed methods of Association of Official Analytical Chemists (AOAC, 1990). The aNDF and ADF were analyzed using the Van Soest method (22).

2.3 *In vitro* experiment

The basal diets used in the *in-vivo* study were milled through a 1 mm screen and used as a substrate in this experiment. Before collecting rumen fluid, the experimental diet was given to cannulated cows for adaptation for 7 days. After adaptation period, rumen fluid was obtained from 2 cannulated Holstein cows, and an equal amount of rumen fluid was obtained from each animal. It was then filtered through a 250 μm pore-sized nylon filter (Shanghai Bolting Cloth Manufacturing Co., Ltd., Shanghai, China). The filtered fluid was mixed with Menke's buffer in a 1:3 ratio (23). For the *in-vitro* experiment, we used a wireless gas pressure monitoring system, the Ankom^{RF} gas production system (ANKOM Technology, Macedon, NY, USA) (24). Each bottle was filled with 125 mL of buffered solution and added ANKOM bags (R510; Ankom Technology, USA). A substrate (1.25 g) was placed in each nylon bag and mixed with CA at levels of 0, 0.015, 0.03, 0.06, 0.12, 0.24, and 0.48% of dry matter basis. Maintaining an anaerobic state in buffered rumen fluid, ultra-purity argon gas (99.999%) was used in this experiment (25). Incubation was carried out in a shaking incubator (JSSI-300C, JSR, Gongju, Korea) at 39°C for 48 h at 85 rpm. A total of 3 experiments were conducted, and each experiment consisted of duplicated treatment bottles and two blank bottles, totaling 54. The blank bottles were used to correct data for Total gas production (TGP), *In-vitro*, dry matter digestibility (IVDMD), and total volatile fatty acids (TVFA).

The cumulative pressure was set to be recorded every 20 min using the official manufacturer's software (Gas Pressure Monitor, Ankom

TABLE 1 Chemical composition of experimental diet.

Items[1]	Basal diet[2]	Complex natural feed additive
Ingredients (%)		
Basal concentration	24.03	-
Soybean meal	3.03	15.00
Corn flake	30.41	21.00
DDGS	-	9.00
Wheat bran	-	15.00
Cordyceps militaris	-	20.00
Red ginseng by-product	-	20.00
Rice straw	12.00	-
Water	29.91	-
Vitamin and mineral mix	0.01	-
Probiotics, (cfu/g)	0.14	-
Saccharomyces cerevisiae	-	1.0×10^{11}
Bacillus subtilis	-	1.0×10^{11}
Lactobacillus acidophilus	-	1.0×10^{10}
Streptococcus thermophilus	-	1.0×10^{11}
Limestone	0.20	-
Salt	0.27	-
Total	100.00	100.00
Nutrient composition (% of DM)		
DM (%)	77.26	44.06
CP	14.07	21.87
EE	4.56	2.77
NDF	51.75	36.74
ADF	23.02	12.28
NDICP	10.71	18.84
ADICP	6.67	19.62
Macro minerals (% of DM)		
Ca	0.81	
P	0.47	

[1] DDGS, Dried distiller's grain with solubles; CP, Crude Protein; EE, Ether Extract; NDF, Neutral detergent fiber; ADF, Acid detergent fiber; NDICP, Neutral detergent insoluble protein; ADICP, Acid detergent insoluble protein; Ca, Calcium; P, Phosphorus. [2] Basal diet, Diet was provided as a total mixed fermented ration.

Technology, Macedon, NY, USA) until the end of the incubation. The kinetics of gas production were analyzed using a single-pool and single-lag exponential model (26).

$$V_t = 0 \left(0 \le T \le L\right).$$

$$V_t = V_{\max} * (1 - \exp\left(-K * \left(T - L\right)\right)\left(T \ge L\right).$$

Where V_t is the gas produced at a specific time T (mL), V_{max} is the asymptotic gas production (h^{-1}), Exp is the exponential function, K is the fractional rate of gas production (h^{-1}), L is the discrete lag time (h), and t is the time after the initiation of incubation (h). For CH_4 analysis, 3 mL of gas was sampled at 12, 24, and 48 h into a vacuum container (Labco Ltd., High Wycombe, UK). CH_4 analysis was conducted using a gas chromatography system (HP 6890 series GC system; Agilent Technologies Inc., Santa Clara, CA, USA). A thermal conductivity detector and capillary column (HP-PLOT/Q; Agilent Technologies Inc., Santa Clara, CA, USA) were used in the gas chromatography system, and a standard gas mixture (H_2 1.0%, CO_2 20.1%, CH_4 10.1%, and N_2 19.9% in He) was used to quantify CH_4 concentration. After the experiment completed, the nylon bags were removed to measure IVDMD by calculating the weight loss of the substrate. Ruminal pH was measured using a pH meter (S20 SevenEasy pH, Mettler Toledo, Greifensee, Switzerland). For the measurement of volatile fatty acid (VFA) and NH_3-N, rumen fluids were collected into 50 mL tube and then centrifuged at 2,000 g at 4°C for 10 min to deposit feed particles. The 5 mL of the supernatant rumen fluid were collected in 15 mL tubes and mixed with 1 mL of a 2% $HgCl_2$ (wt/vol) and 25% meta-phosphoric acid (wt/vol) solution for NH_3-N and VFA analysis. For VFA analysis, 1.4 mL of supernatant was collected to 2 mL tube and centrifuged at 20,000 g at 4°C for 20 min. After that a volume of 25 µL of 1% (wt/vol) pivalic acid was added to sample for using an internal standard and stored at brown vial until analysis. Profiles of VFA were analyzed using the gas chromatography (HP 6890 series GC system; Agilent Technologies Inc., Santa Clara, CA, USA) equipped with a flame ionization detector and a capillary column (DB-FFAP; Agilent Technologies Inc., Santa Clara, CA, USA).

For NH_3-N analysis, previously centrifuged 600 µL samples was transferred to a 1.5 mL tubes and centrifuged again at 20,000 g at 4°C for 20 min. Then, 500 µL of supernatant were collected into new 1.5 mL tubes. NH_3-N concentration was measured using the catalyzed indophenol reaction (27) using spectrophotometry (Synergy2; Biotek Instruments, Inc., Winooski, VT, USA).

2.4 Animals and experimental condition (*In-vivo*, experiment)

All the animal experimental procedure were approved by the Institute of Animal Care and Use Committee of Konkuk University (approval no. KU22079). 23-Korean native steers (Initial body weight 641.96 ± 62.51 kg; age 24.22 ± 0.93 months) were used for the experiment. Before starting the experiment, a total of 5 days, feed intake was checked daily for each pen by weighing orts to measure dry matter intake (DMI). Initial Body Weight (IBW) was measured using a digital scale (CAS Co. Ltd., Seoul, Korea). The animals were allocated to 2 dietary treatments, control (without CA) and treatment (0.07% of CA supplementation on a dry matter basis), using a randomized complete block design considering factors such as feed intake, age, and IBW. After a 7 days adaptation period, the total experiment lasted for 252 days, during which animals were fed twice daily (07,00 and 16,00). Mineral blocks and water were provided *ad libitum*. The offered diets and refusals were measured for three consecutive days each month to calculate the pen's DMI. CA was provided to the experimental animals by top-dressing onto basal diet at every 16:00 feeding time.

2.5 Blood sampling and metabolic profiles analysis

Blood samples were taken from each cow through the jugular vein on day 144. For complete blood cell count test, blood was collected into EDTA-containing tubes (Becton Dickinson, Franklin Lakes, NJ, USA) and analyzed using the HM2 analyzer (Abaxis, Union City, CA, USA). For serum analysis, blood was collected into heparin-containing tubes (Becton Dickinson, Franklin Lakes, NJ, USA) and centrifuged at 3,000 rpm for 15 min to extract serum. Serum samples were stored at −80°C until analysis. Fuji dry-chem slides (DRI-CHEM 7000i, Fuji Film, Tokyo, Japan) was used to determine serum metabolite concentration.

2.6 Carcass traits

Slaughtering procedure of experimental animals were conducted at a commercial slaughterhouse in Chungcheongnam-do, Korea. In each group, the slaughter of four animals was carried out sequentially on days 223, 244, and 255, respectively. After slaughter and a 24-h chilling process, the evaluation was conducted, including yield grade (carcass weight, back fat thickness, and ribeye area) and carcass quality grade (marbling score, meat color, texture, and maturity). Carcass traits scores were assessed according to the Korean Carcass Grading System of the Korea Institute of Animal Products Quality Evaluation (KAPE, 2019). The quality grade was assessed on the surface of the longissimus thoracis muscles in the 13th rib section.

2.7 Statistical analysis

All the data from the experiment were statistically analyzed using SAS University Studio (SAS Institute, Inc., Cary, NC, USA). The rumen fermentation data were analyzed using the PROC MIXED procedure of SAS, and the statistical model was as follows:

$$Y_{ij} = \mu + T_i + R_j + e_{ij}.$$

Where Y_{ij} is the observation from the experimental unit, μ is the overall mean, T_i is the fixed effect of the dose of the CA ($i = 1, 2, \ldots, 7$), R_j is the random effect of the experiment (experiment), and e_{ij} is the residual effect. An orthogonal polynomial test was conducted using the SAS PROC IML procedure to determine the linear and quadratic effects of *CA*. Data from gas production kinetics and broken-line regression were analyzed using the SAS PROC NLIN procedure. For analyzing the broken line regressions, TVFA was used as the criterion of the model. Graphical depiction of the broken-line regression results was done using the GPLOT procedure in SAS (Figure 1).

The experiment statistical model for the *in vivo* experiment were analyzed using the MIXED procedure of SAS and the statistical models was as follow equation.

$$Y_{ij} = \mu + T_i + R_j + e_{ij}.$$

Where Y_{ij} is the observation from the experimental unit, μ is the overall mean, T_i is the fixed effect of the CA, R_j is the random effect of the animal ($i = 1, 2 \ldots 12$), e_{ij} is the residual effect. All the data differences between the treatments were compared with Tukey's test. The significance level was declared at $p < 0.05$, and tendency was declared at $0.05 \leq p < 0.1$.

3 Results

3.1 *In vitro* rumen gas and CH_4 production

We investigated *in vitro* rumen gas production and CH_4 production at various levels of CA inclusion concerning incubation time and dosage (Table 2 and Figure 2). There were no significant differences in the fitted parameters of gas production profiles. However, the inclusion of CA tended to increase V_{max} ($p = 0.05$).

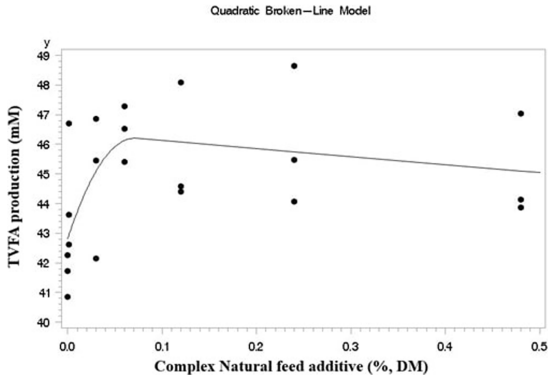


FIGURE 1
Broken-line regression models fitted to data of TVFA production expressed as (y), in response to CA inclusion level %DM as (x) $Y = 46.2021 - 684.7\ (x - 0.073)^2$ ($x > 0.073$), $Y = 46.2021 - 2.6937x$ ($x < 0.073$) ($r^2 = 0.37$; $p = 0.045$).

TABLE 2 The fitted parameters of gas production profiles.

Parameters[1]	Complex natural feed additive (% of DM)							SEM	*p*-value	Contrast[3]	
	0	0.0015	0.03	0.06	0.12	0.24	0.48			L	Q
V_{max} (mL/g DM)	188.90^{b}	200.40^{ab}	197.60^{ab}	213.90^{a}	206.43^{ab}	211.20^{ab}	205.60^{ab}	2.83	0.05	0.11	0.03
K (h^{-1})	0.057	0.054	0.057	0.057	0.055	0.057	0.058	<0.01	0.64	0.21	0.94
L (h)	0.93	1.24	1.30	1.33	1.22	1.09	0.89	0.06	0.08	0.04	0.19

Means in a row with different superscript letters are significantly different ($p < 0.05$). [1]V_{max}, the asymptotic gas production; K, fractional gas production rate; L, lag time. [2]SEM, standard error of the mean ($n = 3$). [3]L, linear; Q, quadratic.

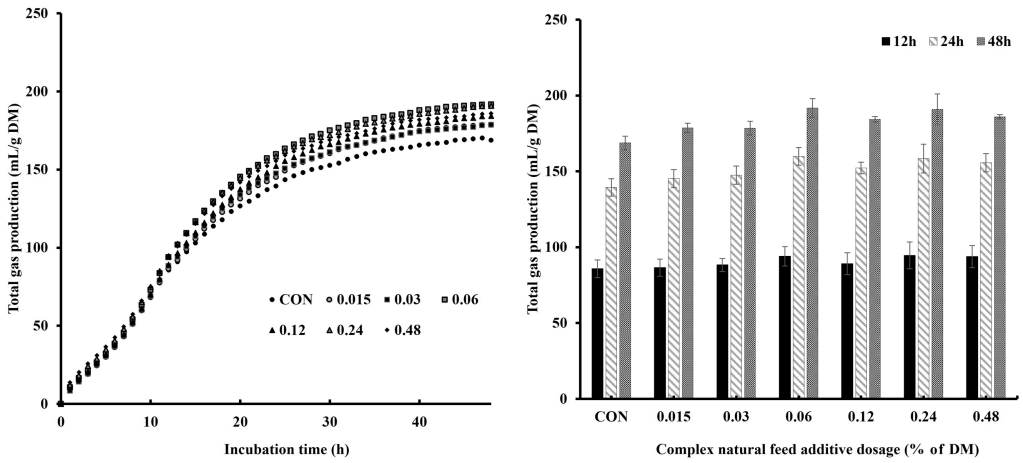


FIGURE 2
Effects of complex natural feed additives on gas production profiles during *in vitro* rumen fermentation based on incubation time and dosage.

TABLE 3 Time-dependent effects of complex natural feed additive on *in vitro* rumen gas and methane production.

Parameters[1]		Complex natural feed additive (% of DM)							SEM[2]	*p*-value	Contrast[3]	
		0	0.0015	0.03	0.06	0.12	0.24	0.48			L	Q
TGP (mL/g DM)	12 h	85.77^{b}	86.46^{ab}	88.27^{ab}	93.92^{ab}	89.10^{ab}	94.55^{a}	93.76^{b}	1.67	0.02	<0.01	0.06
	24 h	139.4^{b}	145.24^{ab}	147.47^{ab}	159.83^{a}	152.2^{ab}	158.42^{ab}	155.62^{ab}	2.10	0.03	0.02	0.03
	48 h	168.76^{b}	178.68^{ab}	178.52^{ab}	191.73^{a}	184.28^{ab}	190.86^{a}	185.99^{ab}	2.05	<0.01	0.02	<0.01
CH_4 (mL/g DM)	12 h	20.01	19.55	17.62	19.20	18.91	19.45	19.46	0.40	0.07	0.53	0.53
	24 h	30.41	30.25	33.75	33.86	33.94	33.79	32.47	0.69	0.17	0.40	0.04
	48 h	43.47	45.07	47.40	46.88	46.90	48.77	48.86	0.63	0.22	0.03	0.21
CH_4 proportion (%)	12 h	23.4^{a}	22.71^{ab}	19.94^{b}	20.62^{ab}	21.23^{ab}	20.59^{ab}	20.77^{ab}	0.43	0.02	0.07	0.05
	24 h	21.75	20.77	22.88	21.18	22.30	21.34	20.78	0.34	0.27	0.26	0.44
	48 h	25.71	25.17	26.58	24.49	25.74	25.58	26.27	0.27	0.54	0.44	0.63

Means in a row with different superscript letters are significantly different ($p < 0.05$). [1]TGP, Total gas production; CH_4 proportion, methane to total gas production ratio. [2]SEM, standard error of the mean ($n = 3$). [3]L, linear; Q, quadratic.

Time-dependent effects of *in vitro* rumen gas production and methane emission were shown in Table 3. The inclusion of CA increased TGP at all incubation times ($p < 0.05$), with the highest observed at the 0.06% level. There were no significant results in methane production, but it tended to decrease when supplemented with CA, especially with the most significant reduction observed at 0.03% of DM inclusion ($p = 0.07$). Methane proportion was found to be lower with CA inclusion at 12 h of incubation ($p = 0.02$).

3.2 *In vitro* rumen fermentation parameters

After 48 h of incubation, we investigated the rumen fermentation characteristics with various levels of CA added (Table 4). Ruminal pH was not significantly affected by the inclusion of CA in the ruminal fluid. TVFA production tended to increase in the treatment groups ($p = 0.07$), especially the molar proportion of butyrate, which increased in all the treatment groups ($p < 0.01$). Iso-butyrate and iso-valerate also increased in the treatment groups ($p = 0.01$). Correlating with these results, branched-chain fatty acids (BCFA) were also increased in the treatment group ($p = 0.01$).

3.3 Broken-line regression analysis

A quadratic effect was observed in the CA inclusion group regarding TVFA production ($p = 0.03$). The TVFA was used as the criterion for broken-line regression analysis. Quadratic broken-line regression analysis estimated that the maximum TVFA was produced at 0.0703% DM inclusion ($Y = 46.2021–684.7\ (x - 0.073)^2\ (x > 0.073)$, $Y = 46.2021–2.6937x\ (x < 0.073)$; $r^2 = 0.37$; $p = 0.045$) (Figure 1).

3.4 Growth performance

The effects of CA supplementation on the growth performance of Korean-native steers are shown in Table 5. The average daily gain (ADG) was decreased in treatment group compared to control group ($p = 0.01$). Additionally, the feed gain ratio tended to decrease in treatment group ($p = 0.07$).

3.5 Serum metabolites

The effects of CA supplementation on serum metabolites of Korean native steers are shown in Table 6. No significant differences were observed in serum metabolites between control and treatment group ($p > 0.05$).

3.6 Complete blood cell count (CBC)

The effects of CA supplementation on complete blood cell count (CBC) of Korean native steers are shown in Table 7. No significant differences were observed in CBC between control and treatment group ($p > 0.05$).

TABLE 4 Effects of complex natural feed additive on rumen fermentation characteristics (48 h).

Parameters[1]	Complex natural feed additive (% of DM)							SEM[2]	*p*-value	Contrast[3]	
	0	0.0015	0.03	0.06	0.12	0.24	0.48			L	Q
pH	6.74	6.69	6.70	6.67	6.68	6.68	6.70	0.01	0.05	0.59	0.03
IVDMD (%)	79.57	80.18	80.30	82.98	82.01	80.97	80.23	0.38	0.12	0.88	0.06
NH_3-N (mg/dL)	117.27	112.85	106.49	116.63	110.37	112.24	106.10	1.98	0.34	0.15	0.79
TVFA (mM)	41.61	44.31	44.82	46.40	45.69	46.06	45.01	0.48	0.07	0.20	0.03
Acetate (mM)	24.99	25.64	25.99	26.35	25.92	26.69	26.10	0.19	0.36	0.22	0.11
Propionate (mM)	9.13	9.84	10.35	10.15	10.16	10.14	9.83	0.17	0.34	0.60	0.10
Iso-butyrate (mM)	0.34^{b}	0.38^{ab}	0.37^{ab}	0.39^{ab}	0.41^{a}	0.39^{ab}	0.40^{a}	0.01	0.02	0.01	0.03
Butyrate (mM)	5.05^{b}	5.49^{ab}	5.65^{ab}	6.06^{a}	5.91^{a}	5.96^{a}	5.91^{a}	0.09	<0.01	0.01	0.01
Iso-valerate (mM)	1.43^{b}	1.86^{a}	1.67^{ab}	1.83^{a}	1.85^{a}	1.76^{ab}	1.73^{ab}	0.04	0.01	0.50	0.06
Valerate (mM)	1.02	1.15	1.10	1.16	1.16	1.15	1.10	0.02	0.05	0.74	0.04
BCFA (mM)	1.76^{b}	2.18^{a}	2.04^{ab}	2.22^{a}	2.26^{a}	2.17^{a}	2.13^{ab}	0.05	0.01	0.19	0.02
A:P ratio	2.73	2.61	2.61	2.65	2.59	2.65	2.63	0.03	0.77	0.83	0.70

[ab] Differing superscripts within a row indicate treatment differences ($p < 0.05$).
[1]IVDMD, in vitro dry matter degradability; NH3-N, ammonia nitrogen; BCFA, iso-butyrate + iso valerate; A:P ratio, acetate to propionate ratio. [2]SEM, standard error of the mean ($n = 3$). [3] L, linear; Q, quadratic.

TABLE 5 Growth performances of Korean-native steers supplemented complex natural feed additive.

Parameters[1]	Control	Treatment (CA)[2]	SEM[3]	*p*-value
Initial body weight, kg	638.76	645.04	13.33	0.85
Final body weight, kg	787.27	754.92	16.44	0.35
ADG, kg/d	0.62^{a}	0.46^{b}	0.03	0.01
Dry matter intake (kg/d)	10.92	10.41	0.03	0.49
Feed:gain	18.25	24.40	1.78	0.07
Carcass month	32.19	32.25	0.20	0.92

[ab] Differing superscripts within a row indicate treatment differences ($p < 0.05$). [1] ADG, Average daily gain. [2] Treatment, complex natural feed additive containing (Probiotics, Red ginseng by-product and C. militaris) supplementation at 0.07% DM. [3] SEM, Standard error of means ($n = 23$).

TABLE 6 Serum metabolites of Korean-native steers supplemented complex natural feed additive.

Parameters[1]	Control	Treatment (CA)[2]	SEM[3]	*p*-value
Total protein (g/dl)	7.53	7.85	0.19	0.41
Albumin (g/dl)	3.92	3.87	0.05	0.58
TCHO (mg/dl)	213.00	214.27	0.09	0.34
Uric Acid (mg/dl)	0.80	0.82	0.02	0.58
Total glyceride (mg/dl)	19.73	20.82	1.94	0.79
NEFA (μEq/L)	191.17	208.83	13.25	0.48
BUN (mg/dl)	16.46	14.39	1.04	0.32
Glucose (mg/dl)	82.72	85.80	4.51	0.73

[1] TCHO, Total cholesterol; NEFA, Non-esterified fatty acids; BUN, Blood urea nitrogen. [2] Treatment, complex natural feed additive containing (Probiotics, Red ginseng by-product and C. militaris) supplementation at 0.07% DM. [3]SEM, Standard error of means ($n = 23$).

TABLE 7 Complete blood cell count of Korean-native steers supplemented complex natural feed additive.

Parameters[1]	Control	Treatment (CA)[2]	SEM[3]	*p*-value
WBCs 10^9 /L	8.57	8.00	0.56	0.82
RBCs, 10^{12} /L	10.82	10.56	0.33	0.70
MCH, pg	17.16	17.35	0.17	0.60
MCHC, g/dL	35.32	35.04	0.17	0.34
HGB, g/dL	18.64	18.22	0.50	0.75
HCT, %	52.79	52.56	1.85	0.95
PLT, 10^9 /L	220.07	257.55	15.79	0.07
LYM, %	22.10	28.37	3.59	0.33
MO, %	5.83	6.18	0.37	0.62
GR, %	72.04	65.44	3.89	0.35

[1] WBC, white blood cell; RBC, red blood cell; HGB, hemoglobin; HCT, hematocrit; MCH, mean corpuscular hemoglobin; PLT, platelet; MCHC, mean corpusucular hemoglobin concentration; LYM, lymphocyte; GR, granulocyte.
[2] Treatment, complex natural feed additive containing (Probiotics, Red ginseng by-product and C. militaris) supplementation at 0.07% DM. [3] SEM, Standard error of means ($n = 23$).

TABLE 8 Carcass characteristics of Korean-native steers supplemented complex natural feed additive.

Parameters[1]	Control	Treatment (CA)[2]	SEM[3]	*p*-value
Carcass weight, kg	473.00	451.75	10.54	0.34
Loin muscle area, cm^2	99.91	95.50	2.54	0.41
Backfat thickness, cm	15.55	11.83	0.93	0.03
Marbling score	5.78	6.25	0.33	0.44
Yield index	60.91	61.17	0.55	0.81
Yield grade	19.35	20.83	1.26	0.50
Quality grade	40.00	40.00	1.66	1.00
Meat color	4.46	4.83	0.12	0.08
Fat color	3.00	3.00	0.00	1.00
Texture	2.08	2.00	0.17	0.81
Maturity	2.54	2.58	0.11	0.86

[1] Yield index = 68.184-[0.625 × back fat thickness (mm)] + [0.130 × rib eye area (cm2)] -[0.024 × carcass weight (kg)]; Yield grade, Converted into numeric value as grade A (high yield; > 67.5) = 30, B (62.0 < yields, < 67.5) = 20, C (low yield; < 67.5) = 10; Quality grade, 1++ (highest) = 50, 1+ = 40, 1 = 30, 2 = 20, 3 (lowest) = 10, Marbling score = No. 1 ~ No. 9 (1 = devoid, 9 = abundant), Meat color, No. 1 ~ No. 7 (1 = bright cherry red, 7 = dark red), Fat color = No. 1 ~ No. 7 (1 = white, 7 = dark yellow), Maturity = 1 ~ 3 (1 = youthful, 3 = mature). [2] Treatment, complex natural feed additive containing (Probiotics, Red ginseng by-product and C. militaris) supplementation at 0.07% DM. [3] SEM, Standard error of means ($n = 23$).

3.7 Carcass characteristics

The effects of CA supplementation on the carcass characteristics of Korean native steers are shown in Table 8. Backfat thickness was significantly decreased in the treatment group compared to the control group ($p = 0.03$). Meat color tended to increase in treatment group ($p = 0.08$). There were no significant differences in other parameters related to carcass characteristics ($p > 0.05$) between the control and treatment groups.

4 Discussions

4.1 *In vitro* experiment

The aim of the *in vitro* study was to assess the effects of CA on the rumen environment. Rumen gas production is an indirect measurement of rumen microbial fermentation and activity. Recently, rumen gas production has been applied in the field of ruminant research to estimate microbial fermentation processes (23) and evaluate the effect of anti-nutritional factors on microbial activity (28, 29). Gas production primarily results from the fermentation of carbohydrates in the rumen, yielding acetate, propionate, and butyrate, and releasing final catabolites, mostly carbon dioxide and methane (23).

In this study, total gas production significantly increased in the treatment group at every time-period. This increase in total gas production was observed alongside a tendency for increased VFA production. According to a previous study by Getachew et al. (30), gas production has positive correlation with butyrate production. In this study, butyrate production was positively correlated with total gas production in treatment group in linear regression analysis (not shown in table), which may be attributed to the increased TGP. Consistent with our findings, another study reported that *C. militaris* increased rumen cellulolytic bacteria, specifically *Ruminococcus flavefaciens* in rumen *in vitro* fermentation (13, 31). Rumen bacteria are the most abundant microbes in rumen and approximately 10^{10}–10^{11} cells/ml and over 200 species inhabit in the rumen (32). The plant fiber matrix is complex and composed of polymer of carbohydrate, breakdown of fiber requires the coordination of a hydrolytic enzymes (33). Cellulose-degrading bacteria reside in the rumen, the most effective cellulose degraders in the rumen due to their ability to digest cellulose, facilitated by the presence of numerous genes encoding for fiber degradation (33). A series of fermentation processes by cellulolytic bacteria produce VFA in rumen. The reasons for the increased bacterial population in rumen are not known but, it could guess that polysaccharide from *C. militaris* plays energy sources to rumen microbes by manipulating the composition in rumen. Several studies reported that galactomannan provide nutrients for beneficial microbes in the gastrointestinal microbiota (34) and D-mannitol can also become energy sources for bacteria growth in the rumen (35). Also, Cappellozza et al. (36), reported that the inclusion of *Bacillus*-based probiotics can increase rumen *in vitro* gas production due to the production of various enzymes by *Bacillus* sp. The protease produced from *Bacillus. Subtilis* (*B. subtilis*), called subtilisin, may degrade proteins in the rumen, providing peptides and amino acids for microbial crude protein synthesis (37). Not only does *B. subtilis* play a role, but *Saccharomyces. Cerevisiae* (*S. cerevisiae*) also contributes to an increased fermentation status in the rumen. The mechanisms behind the higher VFA concentration in *S. cerevisiae* are not fully known, but it is associated with anaerobic microflora in the rumen (35). *S. cerevisiae* has been reported to act as an oxygen scavenger in the rumen, creating favorable conditions for the activity of various microorganisms (38). Additionally, exogenous enzymes such as xylanase from *S. cerevisiae* have been reported to have positive effects on ruminal fibrolytic bacteria by providing readily usable carbohydrates (39). Therefore, the inclusion of CA could enhance the status of rumen microorganisms through the synergistic effects of

bioactive compounds in CA, promoting the production of diverse enzymes and making the substrate more efficient to break down.

Methane production results from the fermentation of digestible structural carbohydrates in the rumen (40). Ruminal methane is produced by methanogens in a reduction pathway of carbon dioxide (CO_2) and hydrogen (H_2) (41). In this study, methane proportion tended to decrease at 12 h of incubation. Kim et al. (31) reported that *C. militaris* had an adverse effect on ciliate protozoan populations in rumen *in vitro* experiments during early incubation. They reported that methanogen archaea were significantly decreased at 12 h incubation with low concentrations of *C. militaris* inclusion (0.10 g/L). Additionally, red ginseng contains triterpene saponin and pharmacological compounds (42). Saponins have a chemical structure that can act like soap, interacting with cell membrane components such as cholesterol and phospholipids (43). Especially in the rumen saponins can form complexes within protozoal cell membrane, leading to cell rupture and lysis (44). Rumen protozoa have a symbiotic relationship with methanogen archaea and are known to provide hydrogen as a substrate for methanogens (45). This interaction may affect microbial diversity and fermentation, thus inhibiting methanogenesis in ruminants. Consistent with the results of Hamid et al. (18), who evaluated the red ginseng byproduct (RGP) as a protein source for the rumen and found that RGP significantly decreased methanogen archaea compared to another conventional ingredient. Thus, the decreased methane production in our study may be attributed to the bioactive compounds of the CA acting synergistically to inhibit the growth of protozoa, ultimately leading to lower the methane formation.

BCFA results from the oxidative deamination and decarboxylation steps of branched-chain amino acids (BCAA) during fermentation (46). Cellulolytic bacteria utilize BCFA for the synthesis of BCAA (47). In this study, branched-chain fatty acids significantly increased with CA inclusion, which was associated with increased protein digestibility. Sun et al. (48) reported that *B. subtilis* natto, used in rumen *in vitro* fermentation at a concentration of 1.0×10^{11} CFU/g, significantly increased BCFA production. *B. subtilis* could produce proteolytic enzymes, including subtilisin, which promote protein digestion (37). It can be speculated that *B. subtilis* may accelerate the deamination and increase the utilization of isoacid for the synthesis of BCAA.

4.2 *In vivo* experiment

Based on the results from the *in vitro* experiment, 0.07% of CA (on a dry matter basis) was selected for *in vivo* dosage because it resulted in the highest production of VFA, and there were no harmful effects on ruminal fermentation. However, contrary to the results of the *in vitro* experiment, there were no significant results in final body weight and DMI between the control and treatment groups. Consistent with our results, previous studies on the same probiotic species did not show a significant improvement in the DMI of ruminants (49–51), while other studies reported an increased DMI of ruminants (52, 53). Discrepancies in results may be due to the variations in the amounts of probiotics fed, the species of animals, or the age of the animals.

In this experiment, we used red ginseng by-product as a component of the feed additive. RGP is known to contain compounds that can reduce feed intake due to its astringent and irritating taste (54). Also, triterpenoid saponins have been reported to inhibit pancreatic lipase activity (1). Yoshikawa et al. (55) reported that triterpenoid saponins from *Camellia sinensis* inhibited pancreatic lipase activity in mice. Furthermore, the glucuronic acid unit at C3 of the aglycone of triterpenoid saponins has been reported to inhibit α-amylase and α-glucosidase activity (56). Therefore, even though there were no significant differences in DMI between the control and treatment groups, we can infer that, despite the increased VFA production observed in the *in vitro* experiment, saponin may have decreased pancreatic enzyme activity, which could have reduced the bioavailability of nutrients in the treatment group, ultimately leading to a decrease in growth and carcass performances.

Additionally, according to Riddell et al. (57), probiotics show their maximum effects during stressful conditions. Past studies have reported that probiotic supplementation during stressful periods in ruminant, such as weaning, dietary shifts, and the beginning of lactation, can significantly affect the performance of ruminant (58). Therefore, in the current experiment, the relatively well-housed environment may have helped prevent stressful conditions, limiting the potential effects on animal performance. Another speculated reason for the lack of effects on growth performance could be compensatory digestion that occurs in the post-ruminal digestive tract. The hindgut accounts for approximately 2% of the cow's body weight and has been found to contribute to 8 to 17% of the TVFA absorption proportion from the digestive systems of ruminants (59). The hindgut also accounted for an average of total-tract starch disappearance, averaging 2.9% (range = 0.0 to 6.8%) in steers. Additionally, low digestibility can compensate in the hindgut when ruminal digestibility is low (60). Given these reasons, CA supplementation may not increase the growth performances of Korean-native steers.

Back-fat thickness refers to the external fat thickness of the carcass and is one of the less desirable parts of the carcass, as it is the most sensitive part when calculating the yield grade (KAPE, 2019). As shown, back-fat thickness was significantly decreased in the treatment group. Consistent with these results, Geng et al. (61) reported that feedlot bulls fed with yeast, increased triglycerides and free fatty acid levels in blood. Increased serum triglycerides level could elicit from the lipolysis of pre-deposited fat. Furthermore, Kim et al. (62) demonstrated that *Bacillus* probiotics increased lipid oxidative genes in liver and decreased lipid accumulation in mice subcutaneous tissues. Similarly, a previous study reported that *C. militaris* extract (CE), specifically cordyrrole a treatment against obesity induced by a high-fat diet in mice, decreased body weight gain and food efficiency ratio (63). They observed that the size of adipocytes was decreased by CE treatment, and active constituents of *C. militaris* inhibited adipocyte differentiation. Considering the significant reduction in backfat thickness and the increase in serum non-esterified fatty acid (NEFA) levels in the supplementation group that we could estimate that the results may have been influenced by the reasons mentioned above. Also, regarding carcass characteristics, previous studies have reported that higher carcass weight in cattle is associated with increased back-fat thickness (64). Carcass weight affects meat quality through its effect on fattiness. While there are other factors affecting the quality of meat, it's crucial to consider genetic factors, which can vary depending on the animal species and the level of nutrients (65). Also, it's important to note that the lipid metabolism of ruminants differs from that of monogastric animals, thus further research is needed.

The color of meat is the most important factor for consumers when purchasing meat (66). Meat color is affected by several factors, such as genetics, animal age, nutritional status, and slaughter conditions (67). Fresh meat typically has a bright color and is used as an indicator of its freshness. In this experiment, meat color tended to decrease in the treatment group. The heavier carcass weight of cattle, due to the deposition of energy content from feed, promotes slower glycogen degradation until slaughtering. Heavier cattle also exhibit improved meat color (L*, a*, and b*) values, indicating slower glycogen degradation until slaughtering (68). This is associated with higher glycogen deposition, which accelerates the formation of lactic acid in the muscle. During the pre-slaughter season of cattle, increased physical activity and stress can lead to a higher demand for glycogen and altered pH in the meat, resulting in a change in color (darkening of the meat). Low acidity in the meat leads to a darker color in the muscle. Consequently, we can infer that heavier carcasses deposit more energy, leading to increased production of lactic acid in the muscle. This change in lactic acid levels affects the lower pH in the meat, resulting in a difference in meat color.

Blood hematology parameters are not affected by the supplementation of CA, which means that feeding 0.07% of CA (DM basis) had no negative effect on the immunological responses in Korean-native steers. This could be due to the CA effects vary depending on the animal's condition (fluctuation of blood), and the low sample size may have contributed to these results (69). Collectively, these results suggest that supplementation with 0.07% CA did not improve the performance of Korean-native steers. In general, the compounds of CA exert their maximum effectiveness in stressful environments, while this study was conducted in a normal environment. Therefore, further studies are suggested to investigate the effects of these factors under diverse conditions for ruminants, especially in stressful conditions.

5 Conclusion

Taken together, CA showed no harmful effects on rumen fermentation when supplements were up to 0.48%. However, supplementation of a complex of natural feed additives on Korean native steers significantly decreased average daily gain and back-fat thickness. In addition, there were no significant effects on final body weight, serum metabolites, or the health status of Korean native steers. Therefore, to overcome the limitations of this study, we suggest conducting further research under stressful environments and experimenting with various conditions for animals to determine the efficacy of the feed additives and find ways to enhance their effectiveness.

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 Institute of Animal Care and Use Committee of Konkuk University (approval no. KU22079). The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

M-SJ: Conceptualization, Data curation, Investigation, Methodology, Project administration, Writing –review & editing. Y-HJ: Methodology, Project administration, Writing – original draft. Y-RK: Data curation, Writing – original draft. JG: Writing – review & editing. J-GL: Resources, Writing – original draft. H-GL: Conceptualization, Supervision, Writing – review & editing.

Funding

The author(s) declare financial support was received for the research, authorship, and/or publication of this article. This paper was supported by Konkuk University in 2021.

Conflict of interest

J-GL was employed by DM Bio Co., Ltd.

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

Publisher's note

References

1. Del Hierro JN, Herrera T, Fornari T, Reglero G, Martin D. The gastrointestinal behavior of Saponins and its significance for their bioavailability and bioactivities. *J Funct Foods*. (2018) 40:484–97. doi: 10.1016/j.jff.2017.11.032

2. Peng Q, Cheng L, Kang K, Tian G, Al-Mamun M, Xue B, et al. Effects of yeast and yeast cell wall polysaccharides supplementation on beef cattle growth performance, rumen microbial populations and lipopolysaccharides production. *J Integr Agric*. (2020) 19:810–9. doi: 10.1016/S2095-3119(19)62708-5

3. Allen HK, Levine UY, Looft T, Bandrick M, Casey TA. Treatment, promotion, commotion: antibiotic alternatives in food-producing animals. *Trends Microbiol*. (2013) 21:114–9. doi: 10.1016/j.tim.2012.11.001

4. Doyle N, Mbandlwa P, Kelly WJ, Attwood G, Li Y, Ross RP, et al. Use of lactic acid bacteria to reduce methane production in ruminants, a critical review. *Front Microbiol*. (2019) 10:2207. doi: 10.3389/fmicb.2019.02207

5. Seo JK, Kim S-W, Kim MH, Upadhaya SD, Kam DK, Ha JK. Direct-fed Microbials for ruminant animals. *Asian Australas J Anim Sci*. (2010) 23:1657–67. doi: 10.5713/ajas.2010.r.08

6. Ban Y, Guan LL. Implication and challenges of direct-fed microbial supplementation to improve ruminant production and health. *J Anim Sci Biotechnol*. (2021) 12:109. doi: 10.1186/s40104-021-00630-x

7. Scharek-Tedin L, Pieper R, Vahjen W, Tedin K, Neumann K, Zentek J. *Bacillus Cereus* Var. Toyoi modulates the immune reaction and reduces the occurrence of

diarrhea in piglets challenged with *Salmonella Typhimurium* Dt104. *J Anim Sci*. (2013) 91:5696–704. doi: 10.2527/jas.2013-6382

8. Zerby H, Bard J, Loerch S, Kuber P, Radunz A, Fluharty F. Effects of diet and aspergillus oryzae extract or saccharomyces cervisiae on growth and carcass characteristics of lambs and steers fed to meet requirements of natural markets. *J Anim Sci*. (2011) 89:2257–64. doi: 10.2527/jas.2010-3308

9. Jędrejko KJ, Lazur J, Muszyńska B. Cordyceps Militaris: an overview of its chemical constituents in relation to biological activity. *Foods*. (2021) 10:2634. doi: 10.3390/foods10112634

10. Yue K, Ye M, Zhou Z, Sun W, Lin X. The genus cordyceps: a chemical and pharmacological review. *J Pharm Pharmacol*. (2013) 65:474–93. doi: 10.1111/j.2042-7158.2012.01601.x

11. Tuli HS, Sharma AK, Sandhu SS, Kashyap D. Cordycepin: a bioactive metabolite with therapeutic potential. *Life Sci*. (2013) 93:863–9. doi: 10.1016/j.lfs.2013.09.030

12. Yeo JM, Lee SJ, Lee SM, Shin SH, Lee SH, Ha JK, et al. Effects of cordyceps militaris mycelia on in vitro rumen microbial fermentation. *Asian Australas J Anim Sci*. (2009) 22:201–5. doi: 10.5713/ajas.2009.80579

13. Yeo JM, Lee SJ, Shin SH, Lee SH, Ha JK, Kim WY, et al. Effects of cordyceps militaris mycelia on fibrolytic enzyme activities and microbial populations in vitro. *Asian Australas J Anim Sci*. (2011) 24:364–8. doi: 10.5713/ajas.2011.90085

14. Guo M, Shao S, Wang D, Zhao D, Wang M. Recent progress in polysaccharides from *Panax Ginseng* C. *Food Funct*. (2021) 12:494–518. doi: 10.1039/D0FO01896A

15. Kim JE, Jang SG, Lee CH, Lee JY, Park H, Kim JH, et al. Beneficial effects on skin health using polysaccharides from red ginseng by-product. *J Food Biochem*. (2019) 43:E12961. doi: 10.1111/jfbc.12961

16. Chong-Zhi W, Anderson S, Wei D, Tong-Chuan H, Chun-Su Y. Red ginseng and cancer treatment. *Chin J Nat Med*. (2016) 14:7–16. doi: 10.3724/SP.J.1009.2016.00007

17. Kang H, Park S-B, Kim C. Effect of dietary supplementation of red ginseng by-product on laying performance, blood biochemistry, serum immunoglobulin and microbial population in laying hens. *Asian Australas J Anim Sci*. (2016) 29:1464–9. doi: 10.5713/ajas.15.0664

18. Hamid MMA, Moon J, Yoo D, Kim H, Lee YK, Song J, et al. Rumen fermentation, methane production, and microbial composition following in vitro evaluation of red ginseng byproduct as a protein source. *J Anim Sci Technol*. (2020) 62:801–11. doi: 10.5187/jast.2020.62.6.801

19. Souza VL, Lopes NM, Zacaroni OF, Silveira VA, Pereira RAN, Freitas JA, et al. Lactation performance and diet digestibility of dairy cows in response to the supplementation of *Bacillus Subtilis* spores. *Livest Sci*. (2017) 200:35–9. doi: 10.1016/j.livsci.2017.03.023

20. Chanjula P, Cherdthong A. Effects of spent mushroom cordyceps militaris supplementation on apparent digestibility, rumen fermentation, and blood metabolite parameters of goats. *J Anim Sci*. (2018) 96:1150–8. doi: 10.1093/jas/skx079

21. Wu Q, Cao M, Xie H, Huang Q, Zhou X, Guo J, et al. Effects of probiotic administration on the digestibility characteristics and growth performance of finishing beef cattle fed a total mixed ration containing different levels of corn Stover. *Anim Sci J*. (2022) 93:e13751. doi: 10.1111/asj.13751

22. Van Soest PV, Robertson JB, Lewis BA. Methods for dietary fiber, neutral detergent fiber, and nonstarch polysaccharides in relation to animal nutrition. *J Dairy Sci*. (1991) 74:3583–97. doi: 10.3168/jds.S0022-0302(91)78551-2

23. Kh M. Estimation of the energetic feed value obtained from chemical analysis and in vitro gas production using rumen fluid. *Anim Res Dev*. (1988) 28:7–55.

24. ANKOM Technology. *Ankom gas production system Operator's manual*. Macedon, NY: ANKOM Technology (2018).

25. Park K, Lee H. Effects of nitrogen gas Flushing in comparison with argon on rumen fermentation characteristics in in vitro studies. *J Anim Sci Technol*. (2020) 62:52–7. doi: 10.5187/jast.2020.62.1.52

26. Schofield P, Pitt R, Pell A. Kinetics of Fiber digestion from in vitro gas production. *J Anim Sci*. (1994) 72:2980–91. doi: 10.2527/1994.72112980x

27. Chaney AL, Marbach EP. Modified reagents for determination of urea and Ammonia. *Clin Chem*. (1962) 8:130–2. doi: 10.1093/clinchem/8.2.130

28. Bodas R, Lõpez S, Fernandez M, Garcïa-Gonzãlez R, Rodrïguez A, Wallace R, et al. In vitro screening of the potential of numerous plant species as anti-methanogenic feed additives for ruminants. *Anim Feed Sci Technol*. (2008) 145:245–58. doi: 10.1016/j.anifeedsci.2007.04.015

29. Durmic Z, Hutton P, Revell D, Emms J, Hughes S, Vercoe P. In vitro fermentative traits of Australian Woody perennial plant species that may be considered as potential sources of feed for grazing ruminants. *Anim Feed Sci Technol*. (2010) 160:98–109. doi: 10.1016/j.anifeedsci.2010.07.006

30. Getachew G, Robinson P, Depeters E, Taylor S. Relationships between chemical composition, dry matter degradation and in vitro gas production of several ruminant feeds. *Anim Feed Sci Technol*. (2004) 111:57–71. doi: 10.1016/S0377-8401(03)00217-7

31. Kim W, Hanigan M, Lee S, Lee S, Kim D, Hyun J, et al. Effects of Cordyceps Militaris on the growth of rumen microorganisms and in vitro rumen fermentation with respect to methane emissions. *J Dairy Sci*. (2014) 97:7065–75. doi: 10.3168/jds.2014-8064

32. Mcsweeney C, Mackie R. *Micro-organisms and ruminant digestion: state of knowledge, trends and future prospects*. United States: Food and Agriculture Organization (2012).

33. Koike S, Kobayashi Y. Fibrolytic rumen bacteria: their ecology and functions. *Asian Australas J Anim Sci*. (2009) 22:131–8. doi: 10.5713/ajas.2009.r.01

34. Li R, Tang N, Jia X, Xu Y, Cheng Y. Antidiabetic activity of galactomannan from Chinese Sesbania Cannabina and its correlation of regulating intestinal microbiota. *J Funct Foods*. (2021) 83:104530. doi: 10.1016/j.jff.2021.104530

35. Newbold C, Mcintosh F, Wallace R. Changes in the microbial population of a rumen-simulating fermenter in response to yeast culture. *Can J Anim Sci*. (1998) 78:241–4. doi: 10.4141/A97-086

36. Cappellozza BI, Segura A, Milora N, Galschioet C, Schjelde M, Copani G. Stability of Bacillus and *Enterococcus Faecium* 669 probiotic strains when added to different feed matrices used in dairy production. *Animals*. (2023) 13:2350. doi: 10.3390/ani13142350

37. Esawy MA, Ahmed EF, Helmy WA, Mansour NM, El-Senousy WM, El-Safty MM. Production of Levansucrase from novel honey *Bacillus Subtilis* isolates capable of producing antiviral Levans. *Carbohydr Polym*. (2011) 86:823–30. doi: 10.1016/j.carbpol.2011.05.035

38. Suntara C, Cherdthong A, Wanapat M, Uriyapongson S, Leelavatcharamas V, Sawaengkaew J, et al. Isolation and characterization of yeasts from rumen fluids for potential use as additives in ruminant feeding. *Vet Sci*. (2021) 8:52. doi: 10.3390/vetsci8030052

39. Vallejo-Hernãndez LH, Elghandour MM, Greiner R, Anele UY, Rivas-Cãceres RR, Barros-Rodrïguez M, et al. Environmental impact of yeast and exogenous xylanase on mitigating carbon dioxide and enteric methane production in ruminants. *J Clean Prod*. (2018) 189:40–6. doi: 10.1016/j.jclepro.2018.03.310

40. Flachowsky G. Carbon-footprints for food of animal origin, reduction potentials and research need. *J Appl Anim Res*. (2011) 39:2–14. doi: 10.1080/09712119.2011.570047

41. Jeyanathan J, Martin C, Morgavi D. The use of direct-fed Microbials for mitigation of ruminant methane emissions: a review. *Animal*. (2014) 8:250–61. doi: 10.1017/S1751731113002085

42. Kim Y-J, Jeon J-N, Jang M-G, Oh JY, Kwon W-S, Jung S-K, et al. Ginsenoside profiles and related gene expression during foliation in *Panax Ginseng* Meyer. *J Ginseng Res*. (2014) 38:66–72. doi: 10.1016/j.jgr.2013.11.001

43. Lorent J, Lins L, Domenech OS, Quetin-Leclercq J, Brasseur R, Mingeot-Leclercq M-P. Domain formation and Permeabilization induced by the Saponin A-Hederin and its Aglycone Hederagenin in a cholesterol-containing bilayer. *Langmuir*. (2014) 30:4556–69. doi: 10.1021/la4049902

44. Kholif AE. A review of effect of Saponins on ruminal fermentation, health and performance of ruminants. *Vet Sci*. (2023) 10:450–0. doi: 10.3390/vetsci10070450

45. Newbold CJ, De La Fuente G, Belanche A, Ramos-Morales E, Mcewan NR. The role of ciliate protozoa in the rumen. *Front Microbiol*. (2015) 6:1313. doi: 10.3389/fmicb.2015.01313

46. Allison MJ, Bryant MP. Biosynthesis of branched-chain amino acids from branched-chain fatty acids by rumen Bacteria. *Arch Biochem Biophys*. (1963) 101:269–77. doi: 10.1016/S0003-9861(63)80012-0

47. Mitchell KE, Socha MT, Kleinschmit DH, Moraes LE, Roman-Garcia Y, Firkins JL. Assessing milk response to different combinations of branched-chain volatile fatty acids and valerate in jersey cows. *J Dairy Sci*. (2023) 106:4018–29. doi: 10.3168/jds.2022-22545

48. Sun P, Li J, Bu D, Nan X, Du H. Effects of *Bacillus Subtilis* natto and different components in culture on rumen fermentation and rumen functional Bacteria in vitro. *Curr Microbiol*. (2016) 72:589–95. doi: 10.1007/s00284-016-0986-z

49. Faccenda A, Zambom MA, De Avila AS, Garcias J, Eckstein EI, Fornari JL, et al. Nutrient digestibility and ruminal parameters of cattle fed dried brewers grains and *Saccharomyces Cerevisiae*. *Livest Sci*. (2019) 225:109–15. doi: 10.1016/j.livsci.2019.05.003

50. Zhu W, Wei Z, Xu N, Yang F, Yoon I, Chung Y, et al. Effects of *Saccharomyces Cerevisiae* fermentation products on performance and rumen fermentation and microbiota in dairy cows fed a diet containing low quality forage. *J Anim Sci Biotechnol*. (2017) 8:1–9. doi: 10.1186/s40104-017-0167-3

51. Hassan A, Gado H, Anele UY, Berasain MAM, Salem AZM. Influence of dietary probiotic inclusion on growth performance, nutrient utilization, ruminal fermentation activities and methane production in growing lambs. *Anim Biotechnol*. (2019) 31:365–72. doi: 10.1080/10495398.2019.1604380

52. Sallam SM, Abdelmalek ML, Kholif AE, Zahran SM, Ahmed MH, Zeweil HS, et al. The effect of *Saccharomyces Cerevisiae* live cells and aspergillus Oryzae fermentation extract on the Lactational performance of dairy cows. *Anim Biotechnol*. (2020) 31:491–7. doi: 10.1080/10495398.2019.1625783

53. Guo Y, Li Z, Deng M, Li Y, Liu G, Liu D, et al. Effects of a multi-strain probiotic on growth, health, and fecal bacterial Flora of neonatal dairy calves. *Anim Biosci*. (2022) 35:204–16. doi: 10.5713/ab.21.0084

54. Sharma K, Kaur R, Kumar S, Saini RK, Sharma S, Pawde SV, et al. Saponins: a concise review on food related aspects, applications and health implications. *Food Chem Adv*. (2023) 2:100191. doi: 10.1016/j.focha.2023.100191

55. Yoshikawa M, Sugimoto S, Kato Y, Nakamura S, Wang T, Yamashita C, et al. Acylated Oleanane-type triterpene Saponins with acceleration of gastrointestinal transit and inhibitory effect on pancreatic lipase from flower buds of Chinese tea plant (*Camellia Sinensis*). *Chem Biodivers*. (2009) 6:903–15. doi: 10.1002/cbdv.200800153

56. Dou F, Xi M, Wang J, Tian X, Hong L, Tang H, et al. A-glucosidase and-amylase inhibitory activities of Saponins from traditional Chinese medicines in the treatment of diabetes mellitus. *Pharmazie*. (2013) 68:300–4. doi: 10.1691/ph.2013.2753

57. Riddell J, Gallegos A, Harmon D, Mcleod K. Addition of a bacillus based probiotic to the diet of preruminant calves: influence on growth, health, and blood parameters. *Int J Appl Res Vet Med*. (2010) 8:78–85.

58. Kelsey AJ, Colpoys JD. Effects of dietary probiotics on beef cattle performance and stress. *J Vet Behav*. (2018) 27:8–14. doi: 10.1016/j.jveb.2018.05.010

59. Gressley T, Hall M, Armentano L. Ruminant nutrition symposium: productivity, digestion, and health responses to hindgut acidosis in ruminants. *J Anim Sci*. (2011) 89:1120–30. doi: 10.2527/jas.2010-3460

60. Demeyer D. Quantitative aspects of microbial metabolism in the rumen and hindgut In: JP Jouany, editor. *Rumen microbial metabolism and ruminant digestion*. Paris: INRA (1991). 217–37.

61. Geng CY, Ren LP, Zhou ZM, Chang Y, Meng QX. Comparison of active dry yeast (*Saccharomyces Cerevisiae*) and yeast culture for growth performance, carcass traits, meat quality and blood indexes in finishing bulls. *Anim Sci J*. (2016) 87:982–8. doi: 10.1111/asj.12522

62. Kim B, Kwon J, Kim M-S, Park H, Ji Y, Holzapfel W, et al. Protective effects of Bacillus probiotics against high-fat diet-induced metabolic disorders in mice. *PLoS One*. (2018) 13:E0210120. doi: 10.1371/journal.pone.0210120

63. Kim SB, Ahn B, Kim M, Ji H-J, Shin S-K, Hong IP, et al. Effect of Cordyceps militaris extract and active constituents on metabolic parameters of obesity induced by high-fat diet in C58bl/6j mice. *J Ethnopharmacol*. (2014) 151:478–84. doi: 10.1016/j.jep.2013.10.064

64. Kwon K, Margarette K, Jeon H-E, Han S-J, Woo H-C, Heo S, et al. Market weight, slaughter age, and yield grade to determine economic carcass traits and primal cuts yield of Hanwoo beef. *J Anim Sci Technol*. (2022) 64:143–54. doi: 10.5187/jast.2021.e136

65. Sung K-I, Ghassemi Nejad J, Hong S-M, Ohh S-J, Lee B-H, Peng J-L, et al. Effects of forage level and chromium-methionine chelate supplementation on performance, carcass characteristics and blood metabolites in Korean native (Hanwoo) steers. *J Anim Sci Technol*. (2015) 57:1–7. doi: 10.1186/s40781-015-0043-7

66. Mancini R, Hunt M. Current research in meat color. *Meat Sci*. (2005) 71:100–21. doi: 10.1016/j.meatsci.2005.03.003

67. Sakowski T, Grodkowski G, Gołebiewski M, Slõsarz J, Kostusiak P, Solarczyk P, et al. Genetic and environmental determinants of beef quality—a review. *Front Vet Sci*. (2022) 9:819605. doi: 10.3389/fvets.2022.819605

68. Mahmood S, Dixon W, Bruce H. Cattle production practices and the incidence of dark cutting beef. *Meat Sci*. (2019) 157:107873. doi: 10.1016/j.meatsci.2019.107873

69. Jo J-H, Ghassemi Nejad J, Peng D-Q, Kim H-R, Kim S-H, Lee H-G. Characterization of short-term heat stress in Holstein dairy cows using altered indicators of metabolomics, blood parameters, Milk Microrna-216 and characteristics. *Animals*. (2021) 11:722. doi: 10.3390/ani11030722

10.3389/fvets.2023.1300518

Glossary

ADF	Acid detergent fiber
ADG	Average daily gain
aNDF	a-amylase treated neutral detergent fiber
BCFA	Branched chain fatty acid
BCAA	Branched chain amino acid
BUN	Blood urea nitrogen
CA	Complex natural feed additive
CBC	Complete blood cell count
C. militaris	*Cordyceps militaris*
CMS	Condensed molasses soluble\
DFM	Direct fed microbial
DMI	Dry matter intake
Exp	the exponential function
IBW	Initial body weight
IVDMD	*In-vitro* dry matter digestibility
K	fractional rate of gas production (h^{-1})
L	discrete lag time (h)
NEFA	Non esterified fatty acids
RGP	Red ginseng by-product
TCHO	Total cholesterol
TGP	Total gas production
TVFA	Total volatile fatty acid
V_{max}	The asymptotic gas production (h^{-1})

frontiers | Frontiers in Veterinary Science

TYPE Original Research
PUBLISHED 16 January 2024
DOI 10.3389/fvets.2023.1340964

OPEN ACCESS

EDITED BY
Tao Wang,
Jilin Agricultural University, China

REVIEWED BY
Daniel Hernandez-Patlan,
National Autonomous University of Mexico, Mexico
Yun Ji,
China Agricultural University, China

*CORRESPONDENCE
Marwa I. Abd El-Hamid
✉ mero_micro2006@yahoo.com
Doaa Ibrahim
✉ doibrahim@vet.zu.edu.eg

RECEIVED 19 November 2023
ACCEPTED 26 December 2023
PUBLISHED 16 January 2024

CITATION
Abd El-Hamid MI, El-Azzouny MM, El-Malt RMS, Elkenawy ME, Abdelwarith AA, Younis EM, Youssef W, Dawod RE, Elged DWAH, Habaka MAM, El Oksh ASA, Mekawy S, Davies SJ and Ibrahim D (2024) Future impact of thymoquinone-loaded nanoemulsion in rabbits: prospects for enhancing growth, immunity, antioxidant potential and resistance against *Pasteurella multocida*.
Front. Vet. Sci. 10:1340964.
doi: 10.3389/fvets.2023.1340964

Future impact of thymoquinone-loaded nanoemulsion in rabbits: prospects for enhancing growth, immunity, antioxidant potential and resistance against *Pasteurella multocida*

Marwa I. Abd El-Hamid[1]*, Mona M. El-Azzouny[2], Rania M. S. El-Malt[2], Mona E. Elkenawy[3], Abdelwahab A. Abdelwarith[4], Elsayed M. Younis[4], Wessam Youssef[5], Rehab E. Dawod[6], Dalia W. A. H. Elged[7], Manal A. M. Habaka[8], Amal S. A. El Oksh[9], Soad Mekawy[10], Simon J. Davies[11] and Doaa Ibrahim[12]*

[1]Department of Microbiology, Faculty of Veterinary Medicine, Zagazig University, Zagazig, Egypt, [2]Department of Bacteriology, Animal Health Research Institute (AHRI), Agriculture Research Center (ARC), Zagazig, Egypt, [3]Department of Biochemistry, Animal Health Research Institute (AHRI), Agriculture Research Center (ARC), Mansoura, Egypt, [4]Department of Zoology, College of Science, King Saudi University, Riyadh, Saudi Arabia, [5]Department of Biotechnology, Animal Health Research Institute (AHRI), Agriculture Research Center (ARC), Giza, Egypt, [6]Department of Bacteriology, Animal Health Research Institute (AHRI), Agriculture Research Center (ARC), Damietta, Egypt, [7]Toxicology and Biochemical Department, Animal Health Research Institute (AHRI), Agriculture Research Center (ARC), Zagazig, Egypt, [8]Department of Poultry and Rabbits Diseases, Animal Health Research Institute (AHRI), Agriculture Research Center (ARC), Zagazig, Egypt, [9]Department of Biotechnology, Reference Laboratory for Quality Control of Poultry Production (RLQP), Animal Health Research Institute (AHRI), Agriculture Research Center (ARC), Zagazig, Egypt, [10]Department of Clinical Pathology, Animal Health Research Institute (AHRI), Agriculture Research Center (ARC), Zagazig, Egypt, [11]Aquaculture Nutrition Research Unit (ANRU), Carna Research Station, College of Science and Engineering, Ryan Institute, University of Galway, Galway, Ireland, [12]Department of Nutrition and Clinical Nutrition, Faculty of Veterinary Medicine, Zagazig University, Zagazig, Egypt

Phytochemical nanoemulsions, such as thymoquinone nanoemulsions (TQN), are regarded as innovative alternatives to antimicrobials that significantly improve the performance, digestion, antioxidant potential and immunity of rabbits. Thus, the potential effects of TQN on growth, digestibility, antioxidant potential, immunity and resistance against *Pasteurella multocida* (*P. multocida*) in rabbits were assessed. Herein, 240 rabbits were offered either a basal diet or diets fortified with three TQN-graded concentrations. At 60 days of age, rabbits were challenged with multidrug-resistant (MDR) virulent *P. multocida* strain. Our outcomes described that dietary inclusion of TQN, especially at higher concentrations, significantly enhanced the growth performance of rabbits, which was supported by increasing the levels of jejunal lipase, amylase and trypsin enzymes. Of note, the levels of muscle and jejunal antioxidant enzymes [superoxide dismutase (SOD), glutathione peroxidase (GPX), catalase (CAT) and total antioxidant capacity (T-AOC)], serum immunological markers (IgG, IgG, IgM and total Igs) and blood phagocytic percentage were significantly provoked after TQN fortification; meanwhile, the levels of muscle and jejunal MDA, serum biochemical parameters (total cholesterol, TG and LDL), abdominal fat

percentage, breast and thigh cholesterol were significantly decreased following TQN supplementations. Our findings showed that TQN protected rabbits against *P. multocida* experimental challenge as evidenced by reducing *P. multocida* counts in rabbits' lungs, downregulating the transcription levels of *P. multocida* virulence-related genes (*ptfA, toxA* and *nanB*) at 48 and 96 h post-infection and ameliorating the expression levels of cytokines-related genes (*IL-1β, IL-10, IL-8, IL-6*, *DEFB1*, *TNF-α*, *TLR-4* and *TLR-2*) at 96 h post-infection. Our findings suggest the utilization of TQN in rabbits' diets due to their stimulating effects on digestibility as well as their growth-promoting, anti-inflammatory, antioxidant, antibacterial, anti-virulence and immunostimulant properties, which enhance the rabbits' *P. multocida* resistance.

KEYWORDS

thymoquinone nanoemulsions, growth, immunostimulant, antioxidant, anti-virulence, *Pasteurella multocida*

1 Introduction

Rabbit's production is becoming increasingly significant because of its growing role as a major source of animal protein, especially in low-income developing countries (1). Rabbits are highly susceptible to many bacterial diseases severely influencing the rabbit industry and causing substantial economic losses (2). Due to rabbits close contact with other animal species and the environment, they can become carriers of bacterial pathogens (3). Moreover, they are frequently infected with a bacterium called *Pasteurella multocida* (*P. multocida*), which is the common cause of respiratory illness. *P. multocida* is a significant Gram-negative bacterium linked to a variety of animal illnesses. *P. multocida* strains are classified into 16 lipopolysaccharides (LPS) somatic serogroups (1–16) and 5 capsular serotypes (A, B, D, E or F) (4). Some *P. multocida* serogroups can induce snuffles in rabbits (pasteurellosis), which causes severe epidemics and significant financial losses for rabbit breeding worldwide (5, 6). Rabbit pasteurellosis frequently manifests as chronic mucopurulent respiratory distress leading to high mortality rates in rabbits; however, the illness can also be characterized by other clinical manifestations such as septicemia, otitis and abscesses or can develop without exhibiting any clinical indications (7). The consequence of *P. multocida* infections is affected by the complicated interactions of various bacteria-specific and host characteristics (8). The LPS and polysaccharide capsule are significant virulence determinants implicated in the pathogenicity of *P. multocida* (9). Nevertheless, a large number of additional putative virulence factors are connected to the pathogenesis of *P. multocida* such as a wide range of outer membrane proteins, exotoxins, extracellular enzymes, iron acquisition and regulation proteins, fimbriae and colonization and adhesion factors (10). Of note, antimicrobial agents have been utilized for a long time for the treatment and prevention of bacterial infections such as *P. multocida* resulting in the emergence of multidrug-resistant (MDR) pathogens that are known to have a serious threat to the treatment and control of infectious diseases in both animals and humans (11–15). Additionally, the excessive utilization of antimicrobials has serious negative effects including the development of antimicrobial residues in rabbit meat, disturbing the gut microflora and reducing the host defense system (16). As a result, it is critical to find novel alternative antibiotics such as phytochemicals to control bacterial infections brought on by these MDR strains (17, 18).

One of the natural substitutes for antimicrobials is phytochemicals, which are secondary metabolites produced by plants as a result of their interactions with the surrounding environment (19, 20). Phytochemicals such as essential oils (EOs) can be employed as nutritional supplements in animal diets because of their probable positive impacts on health, bacterial loads, immune defense, meat quality, digestion, utilization of nutrients, antioxidant potential and growth performance (19, 21, 22). These beneficial characteristics are related to their function in enhancing mucosal barriers and gut integrity, which augments host immunity and digestion (18, 21, 23). Thymoquinone (TQ) is regarded as the primary phenolic constituent of black cumin (*Nigella sativa;* NS) EO and it has been used for its therapeutic properties in food and animal industries since ancient times (11, 19). Moreover, TQ can be used as a feed additive in animals' diets owing to its possible beneficial effects on growth performance, utilization of nutrients, immune defense, antioxidant status and microbial load via enhancing digestive and antioxidant enzymes production, gastrointestinal integrity, cytokines-related genes expression and minimizing the harmful bacterial counts (19, 24–26). Nevertheless, the antimicrobial activities of EOs such as TQ might be minimized via volatility, instability issues, low water solubility and oral bioavailability as well as their unpleasant taste (27, 28). To avoid these drawbacks, EOs nanoemulsions such as thymoquinone nanoemulsion (TQN) could be utilized because of their great chemical and physical stability in the aqueous media (28, 29). Due to the EOs' nanoemulsions nanometric size, they can enhance the EOs' bioactivity because the nanocarriers can provide precise control over the active substances released at the target location via improving the deep tissue penetration, enhancing the cellular uptake, shielding them from environmental exposure and lowering their volatility (30, 31).

The use of nanotechnology in rabbit breeding remains in its nascent stage. Despite this interest, to the best of our knowledge, there have been no studies on the application of TQN in rabbit breeding. Thus, the present work was undertaken to investigate, for the first time, the *in vivo* effect of TQN on rabbits' growth performance, digestive and antioxidant enzymes, biochemical and immunological markers in addition to *P. multocida* loads in rabbits' lung and the

expression levels of virulence-and cytokines-related genes post-challenge with virulent *P. multocida* strain.

2 Materials and methods

2.1 Ethical statement

All procedures for experiments were performed following the rules and authorized specifications of the Institutional Animal Care and Use Committee (IACUC), Faculty of Veterinary Medicine, Zagazig University, Egypt under the reference number ZU-IACUC/2/F/195/2022.

2.2 Thymoquinone nanoemulsion preparation and characterization

Thymoquinone (03416-100MG, Mol. Wt. 164.20), polyoxyethylene (20) sorbitan monooleate (Tween 80, food grade) and sodium alginate (medium viscosity, A-2033) were obtained from Sigma-Aldrich (St. Louis, MO, United States). In aqueous phase, sodium alginate was completely dispersed in hot water at a temperature of 70°C with continuous stirring until it was completely dissolved. To prepare the oil phase, a lab digital Ultra-Turrax disperser (IKA, Germany) at 3400 rpm for 2 min was utilized and a primary or coarse emulsion was prepared by combining the thymoquinone EO (1% v/v), sodium alginate solution and Tween 80 (1% v/v) as a surfactant. The primary emulsion was then homogenized for 15 min at 10,000 rpm resulting in the creation of the nanoemulsion solution with controlling the temperature via an ice-water cooling jacket to avoid heat build-up during homogenization. A Sonopuls HD 2200 ultrasonicator (Bandelin, Berlin, Germany) was used to sonicate this combination for 10 min at 700 W (31). The average particle size and morphology of synthesized TQN was evaluated via transmission electron microscopy (Figure 1A) at the National Centre for Radiation Research and Technology, Egyptian Atomic Energy Authority, Cairo, Egypt and Fourier transform infrared spectroscopy (Figure 1B) at Radioactive Isotopes and Generators, Atomic Energy Authority, Egypt. Moreover, Zeta potential measurements (Figure 1C) and initial particle size (Figure 1D) were carried out using the dynamic light scattering (Zetasizer Nano ZS, Malvern, UK). The encapsulation efficiency (*EE%*) of TQN was calculated spectrophotometrically via UV-1800 UV–VIS spectrophotometer (Shimadzu, Kyoto, Japan) at 256 nm according to the method described formerly (32) using the following formula: total [(TQN amountis−free TQN amount)/total TQN amount] × 100.

2.3 Feeding protocol and experimental design of rabbits

For conducting the present experimental trial, 240 New Zealand weaned male rabbits (30-day-old) were purchased from a local commercial rabbit producer. Once the rabbits arrived, they were weighed separately and then randomly placed into four groups with

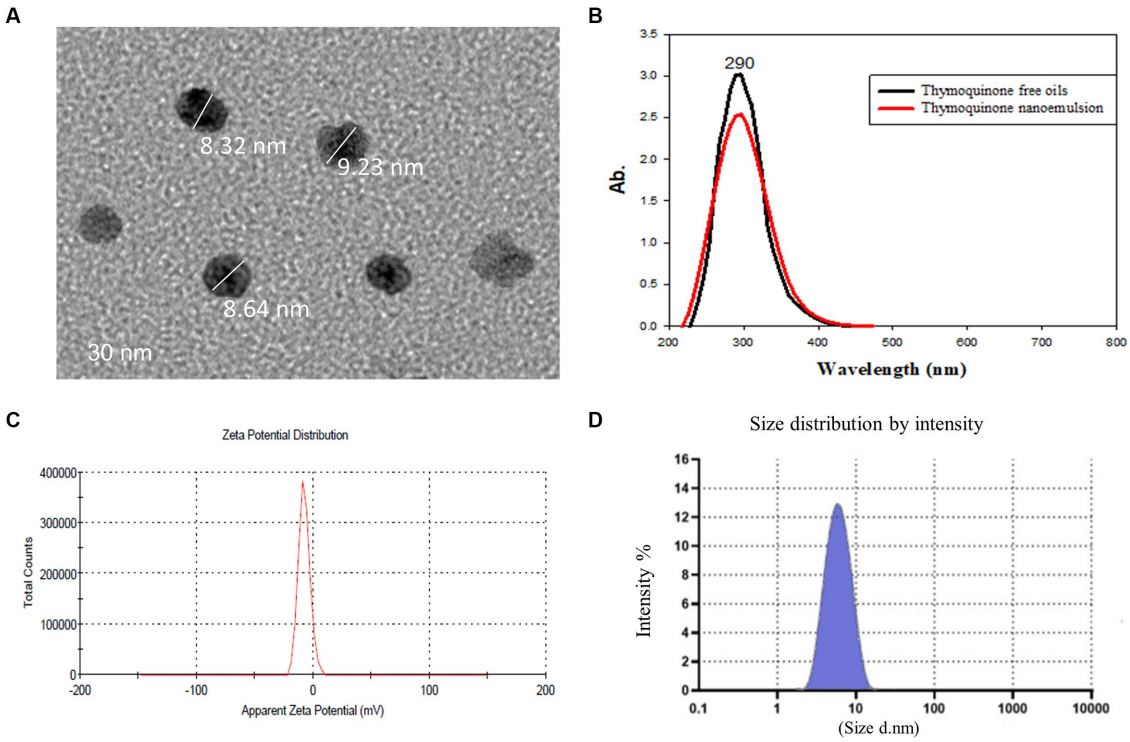


FIGURE 1
Transmission electron microscopy **(A)**, Fourier transform infrared spectroscopy **(B)**, zeta potential **(C)** and particle size distribution **(D)** of thymoquinone nanoemulsions.

60 rabbits and six replicates in each group (10 rabbits/replicate). The four experimental groups consisted of a control group fed the basal diet and three other groups offered meals supplemented with graded levels of TQN including 100, 200 and 300 mg/kg diet, which were spread uniformly over the feed by spraying after the pelleting process during the 30 days feeding trail. The animals were kept in cages under hygienic conditions during the feeding phase and offered pelleted diets with unlimited access to food and water *ad libitum*. The basal diets were created using the nutrient recommendations (33) as displayed in Table 1.

2.4 Growth performance attributes

The initial body weight of rabbits in each group was estimated at the start of the growing period. After that, the rabbits' body weight and feed intake (FI) were recorded weekly to determine the growth performance aspects during the feeding period (60 days old). The feed conversion ratio (FCR), body weight gain (BWG) and FI were calculated as previously described (11, 34–36).

2.5 Collection of samples

After the end of the rearing period (60 days old), blood samples were aseptically collected from ear veins of 24 experimental rabbits (6/group). The blood samples were then separated into two portions; the first one was obtained in sterile centrifuge tubes with an anticoagulant (ethylenediaminetetraacetic acid, Sigma, United States) to be used for determining the phagocytic percentage and hematological parameters and the second portion was obtained in a sterile centrifuge tube without an anticoagulant for separation of sera through centrifugation at 2,000 rpm for 10 min and the obtained sera were kept at −20°C for assessing the immunological and biochemical markers. Additionally, 3 rabbits from each replicate were randomly captured, fasted overnight, weighed and then sacrificed via cervical dislocation according to the guidelines of the World Rabbit Science Association (37) to determine the yields of abdominal fat. After that, the breast muscle and jejunal samples were utilized for determining the activities of digestive and antioxidant enzymes. Moreover, the breast and thigh muscle samples were used to detect the total cholesterol levels in the thigh and breast.

TABLE 1 The control experimental diet's ingredients and nutrition levels.

	%
Ingredient	
Barley grains	16.3
Molasses	3.00
Wheat bran	19
Berseem hay	33.2
Soybean meal, 44%	15.70
Yellow corn	10
Common salt	0.5
Calcium dibasic phosphate	1.5
Anticoccidial	0.2
Antitoxin	0.3
Premix[a]	0.3
Nutrient composition	
Digestible energy (KcaL/Kg)	2555.60
Phosphorus (%)	0.59
Calcium (%)	1.09
Crude fiber (%)	12.56
Ether extract (%)	2.33
Crude protein (%)	16.33

[a]Premix: each 5 kg diet consists of vitamins: B12, 2 mg; B6, 200 mg; B2, 600 mg; B1, 200 mg; E, 3,200 mg; D3, 3,000,000 IU and A, 1800,000 IU; magnesium, 100 g; Zn, 12,000 mg; Se, 20 mg; Co, 20 mg; I, 200 mg; Cu, 3,000 mg; Mn, 10,000 mg; Fe, 10,000 mg; choline, 10,000 mg; nicotinic acid, 4,400 mg and Ca antothenate, 2000 mg.

2.6 Analysis of the digestive and antioxidant enzymes' activities

The activities of amylase, lipase and trypsin enzymes were determined in the jejunal samples utilizing commercial kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) according to the company's directions. Additionally, the activities of antioxidant enzymes including superoxide dismutase (SOD), glutathione peroxidase (GPX) and catalase (CAT) as well as the levels of malondialdehyde (MDA) and total antioxidant capacity (T-AOC) in breast muscle and jejunal tissues were determined using commercial kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) following the producer's manuals. The principle of the used commercial kits is based on the reaction between prepared samples and specified chemicals and the colored products were assayed colorometrically.

2.7 Biochemical and immunological markers

Red blood cells (RBCs) were assessed by a means of a Neubauer hemocytometer (Sigma, Germany) and hemoglobin (Hb) values were estimated via the cyanomethemoglobin colorimeteric technique. The levels of serum alanine and aspartate aminotransferase (ALT and AST), low-density lipoprotein (LDL), total cholesterol (TC) and total triglycerides (TG) were detected utilizing analytical kits (Spinreact Co., Santa Coloma, Spain) following the procedures' manuals. The yields of abdominal fat were represented as a proportion of the body weight. Moreover, the breast and thigh muscle samples were used to enzymatically detect the total cholesterol in the thigh and breast via gas chromatography as in agreement with Association of Official Agricultural Chemists (AOAC) International 2002-AOAC 994.10 standard (38). The examined samples were processed chemically via saponification with 5% KOH in methanol, pH = 2, extraction in ether, concentration and suspension in chloroform and they were subsequently analyzed using gas chromatography.

The serum concentrations of immune-related variables including immunoglobulin M (IgM), IgG and IgA were evaluated utilizing the enzyme-linked immunosorbent assay kits (Sigma Aldrich, MO, United States) as per the manufacturer's regulations. Furthermore,

serum total immunoglobulins (Igs) and blood phagocytic percentage were determined as stated previously (39, 40).

2.8 *Pasteurella multocida* challenge trial

The MDR virulent *P. multocida* strain used in the current challenge study was isolated from rabbits with respiratory signs of snuffles and phenotypically identified according to conventional microbiological techniques. Briefly, the strain was grown into blood agar medium and the developed colonies were stained with Gram's stain and then examined microscopically. Definitive identification was further conducted using various biochemical tests comprising oxidase, catalase, methyl red, Voges-Proskauer, indole, citrate, H_2S production and urease consistent with standard techniques (6, 41). The utilized strain was molecularly identified using PCR examination of a species specific gene fragment; *kmt1* gene as in compliance with the previously pronounced procedure (42). Additionally, the strain was confirmed to be MDR after examining its antimicrobial susceptibility pattern according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) standard (43). Moreover, it was affirmed to be virulent via PCR testing for the presence of genes encoding dermonecrotoxin (*toxA*), sialidases (*nanB*) and colonization and adhesion-related protein (*ptfA*) utilizing primers and PCR cycling protocols formerly described (6, 44, 45). The infecting strain was enriched in brain heart infusion broth (Oxoid, UK), passed twice in healthy rabbits for improving its pathogenicity and then it was re-isolated from sacrificed rabbits and used for the challenge trial. All rabbits were checked to make sure they were *P. multocida*-free before starting the challenge trial via bacteriological examination of conjunctival, nasal and rectal swabs for isolation and phenotypic and molecular identification of *P. multocida* as previously detailed. At 60 days of age (the end of rearing period), 18 animals representing 6 replicates from each group were challenged intranasally with 0.1 mL of pure *P. multocida* inocula containing 2×10^5 CFU/mL in sterile phosphate buffer saline. The experimentally infected rabbits were monitored for 96 h to look for any emergence of clinical signs. Additionally, clinically diseased, moribund and freshly dead rabbits were aseptically submitted for re-isolation and identification of the infecting *P. multocida* strain.

2.8.1 Quantification of *Pasteurella multocida*

At 48 and 96 h post-infection with *P. multocida*, 6 rabbits per each experimental group were euthanized and colony-forming units (CFUs) of *P. multocida* strains were detected in lung, spleen and liver tissues after culturing onto blood agar medium supplemented with 5% fresh sheep blood and clindamycin.

2.8.2 Gene expression analysis of virulence- and cytokines-related genes utilizing reverse transcription-quantitative PCR technique

Lung and splenic tissues were collected from 6 rabbits per each experimental group and used to extract total RNA using the QIAamp RNeasy Mini kit (Qiagen, Hilden, Germany) according to the producer's manuals. A Nano Drop 2000 spectrophotometer (Thermo Scientific Inc., Waltham, MA, United States) was used for evaluating the RNA concentration and purity. The gene expression profiling was performed utilizing the 2x QuantiTect SYBR Green RT-PCR Kit (Qiagen, Hilden, Germany) via one-step reverse transcription-quantitative polymerase chain reaction (RT-qPCR) tests. The mRNA transcript levels of virulence-related genes (*ptfA, toxA* and *nanB*) were determined at 48 and 96 h post-infection and the expression levels of cytokines-related genes including interleukin-1β (*IL-1β*), *IL-10, IL-8, IL-6*, beta-defensin 1 (*DEFB1*), tumor necrosis factor-alpha (*TNF-α*), toll-like receptor 4 (*TLR-4*) and *TLR-2* were determined at 96 h post-infection. A Stratagene Mx3005P real-time thermal cycler (Agilent Technologies, Inc., Santa Clara, CA, United States) was utilized to perform each RT-qPCR reaction in triplicate. The transcript levels of the housekeeping genes, glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and *kmt1* genes were served as endogenous controls for the expression levels of the examined genes. Table 2 describes all primer sequences of the studied genes. Post-PCR melting curve analyses were used to confirm the specificity of the PCR amplifications and the purity of the qPCR products after completing the RT-qPCR procedures. The $2^{-\Delta\Delta Ct}$ technique was used to analyze the relative modifications in gene transcription levels (46).

2.9 Statistical analysis

Our data were analyzed using the general linear model of SPSS Inc. program version 20 (IBM Corp., Armonk, NY, United States) and the statistical significance difference between the experimental groups was evaluated using Tukey's posthoc test. Levene's and Shapiro–Wilk's tests were used to determine the homogeneity and normality among the treatment groups, respectively. The results were expressed as the standard error of means (SEM) and the significance was identified at $p < 0.05$. The GraphPad Prism program version 8 (San Diego, CA, United States) was used to create the graphs in the current study.

3 Results

3.1 Thymoquinone nanoemulsion characterization

The average particle size and zeta potential of synthesized TQN are illustrated in Figure 1, where TQN exhibited a negative average zeta potential of -23 ± 0.89 mV and initial particle size of 8.73 ± 1.20 nm with polydispersity index of 0.022 and EE% of $87.65 \pm 0.42\%$.

3.2 Growth performance and activities of digestive enzymes

Table 3 displays the findings of rabbits' growth performance criteria following dietary inclusion of different levels of thymoquinone nanoemulsions. After ending the rearing period (60 days old), rabbits offered diets with TQN supplementations at the levels of 200 and 300 mg/kg showed prominently significant ($p < 0.001$) rises in BWG (1,613 and 1,463 g, respectively). On the other hand, the BWG of rabbits supplemented with 100 mg/kg of TQN (1,392 g) and those offered a control basal diet (1,378 g) showed no significant variations.

TABLE 2 Primers' sequences of the investigated genes used in reverse transcription-quantitative PCR techniques.

Specificity/target gene	Primer sequence (5′-3′)	Accession No./reference
House keeping		
GAPDH	F-GGTGGTGCTAAGCGTGTTA	NM205518
	R-CCCTCCACAATGCCAA	
kmt1	F-ATCCGCTATTTACCCAGTGG	(42)
	R-GCTGTAAACGAACTCGCCAC	
Virulence attributes		
ptfA	F-TGTGGAATTCAGCATTTTAGTGTGTC	(45)
	R-TCATGAATTCTTATGCGCAAAATCCTGCTGG	
toxA	F-CTTAGATGAGCGACAAGG	
	R-GAATGCCACACCTCTATAG	
nanB	F-CATTGCACCTAACACCTCT	
	R-GGACACTGATTGCCCTGAA	
Cytokines		
IL-1β	F-TTCCGGATGTATCTCGAGCA	NC_013670
	R-GTGGATCGTGGTCGTCTTCA	
IL-10	F-AAAAGCTAAAAGCCCCAGGA	NM001082045.1
	R-CGGGAGCTGAGGTATCAGAG	
IL-8	F-CTCTCTTGGCAACCTTCCTG	KT216053.1
	R-TTGCACAGTGAGGTCCACTC	
IL-6	F-GCCAACCCTACAACAAGA	NC_013678
	R-AGAGCCACAACGACTGAC	
DEFB1	F-AGCCTGTCTGCCTGGAGTAG	XM017337690.1
	R-GATGAGGAGAGGCTTCATGG	
TNF-α	F-CTGCACTTCAGGGTGATCG	XM_008262537.2
	R-CTACGTGGGCTAGAGGCTTG	
TLR-4	F-AGATGAAGTTGTTCCCTCCG	NM_001082732.2
	R-GTGGGCTTAGAACAACTGGAAC	
TLR-2	F-TGCCTCCTTGTTACCTATGC	NM_00108271
	R-AGATGAAGTTGTTCCCTCCG	

GAPDH, glyceraldehyde 3-phosphate dehydrogenase; *ptfA*, *Pasteurella multocida* type 4 fimbrial subunit gene; *toxA*, dermonecrotoxin gene; *nanB*, gene encoding *P. multocida* sialidase protein; *IL*, interleukin; *DEFB1*, beta-defensin 1; *TNF-α*, tumor necrosis factor-alpha and *TLR*, toll-like receptor.

TABLE 3 Impact of different levels of thymoquinone nanoemulsions on rabbits' growth performance traits and jejunal digestive enzymes' activities over the whole rearing period (60 days old).

Parameter	Experimental groups					
	Control	TQNI	TQNII	TQNIII	*p* value	SEM
Growth performance						
Body weight, g	2190^{c}	2205^{c}	2269^{b}	2419^{a}	< 0.001	14.56
Body weight gain, g	1378^{c}	1392^{c}	1463^{b}	1613^{a}	< 0.001	11.10
Feed intake, g	4661^{a}	4559^{a}	3755^{b}	4028^{c}	0.02	13.30
Feed conversion ratio	3.38^{a}	3.28^{b}	2.57^{c}	2.50^{c}	0.03	1.34
Digestive enzymes						
Amylase (U/g prot)	254.34^{c}	255.88^{c}	265.16^{b}	299.90^{a}	<0.001	1.03
Lipase (U/g prot)	437.05^{d}	459.18^{c}	479.56^{b}	490.02^{a}	<0.001	1.47
Trypsin (U/g prot)	280.86^{b}	295.79^{a}	300.2^{a}	302.04^{a}	<0.001	1.07

Control, rabbits fed basal control diets; TQNI, TQNII and TQNIII, rabbits fed basal diets with thymoquinone nanoemulsions (TQN) supplementations at graded levels consisting of 100, 200 and 300 mg/kg diets, respectively; SEM, standard error of the mean. $^{a-d}$ Means in a row with different superscript letters represent statistical variations ($p < 0.05$).

Additionally, the best ($p = 0.03$) FCR was seen in rabbits offered diets with TQN supplementation at doses of 200 and 300 mg/kg (2.57 and 2.50, respectively) in comparison with the control group (3.38).

The effect of supplementing graded levels of TQN on the activities of digestive enzymes in the rabbits' jejunal tissues is shown in Table 3. The results showed that increasing TQN levels significantly ($p < 0.001$) raised the activities of jejunal amylase and lipase enzymes in a dose-dependent manner at 60 days old. Additionally, the most notable ($p < 0.001$) increases in the activities of jejunal amylase, lipase and trypsin enzymes (299.90, 490.02 and 302.04 U/g prot, respectively) were noticed in rabbits administered TQN at a concentration of 300 mg/kg.

3.3 Analysis of antioxidant-related parameters and malondialdehyde content

Table 4 describes the impact of dietary inclusion of different levels of TQN on the activities of antioxidant parameters in addition to MDA contents in breast muscle and jejunal tissues of rabbits. Surprisingly, significant improvements ($p < 0.001$) were seen in the activities of T-AOC, SOD, GPX and CAT in the breast muscle and jejunal tissues of rabbits supplemented with higher levels of TQN unlike the control group. Moreover, the most significant ($p < 0.001$) increase in the levels of T-AOC (14.9 U/mg prot), SOD (35.35 µ/mL), GPx (322.32 µmoL/mg) and CAT (5 U/L) were detected in the breast muscles of rabbits fortified with TQN at concentrations of 300 mg/kg in comparison with the control group (8.26 U/mg prot, 31.03 µ/mL, 295.78 µmoL/mg and 3.2 U/L, respectively). Furthermore, the most significant ($p < 0.001$) elevation in the activities of T-AOC (25.8 U/mg prot), SOD (20.16 µ/mL), GPx (302.8 µmoL/mg) and CAT (4.02 U/L) was seen in the jejunal tissues of rabbits fortified with TQN at concentrations of 300 mg/kg in comparison with the control group (17.92 U/mg prot, 17.9 µ/mL, 253.18 µmoL/mg and 2.64 U/L, respectively). On the other hand, muscle and jejunal MDA levels were significantly decreased ($p < 0.001$) across all TQN-supplemented groups as the level of TQN fortification increased. Additionally, the most significant ($p < 0.001$) decreases in the activities of muscle and jejunal MDA (0.2 and 1.28 nmoL/mL, respectively) were determined in rabbits offered TQN at the level of 300 mg/kg concerning the control group (0.38 and 2.06 nmoL/mL, respectively).

3.4 Analysis of serum biochemical parameters, abdominal fat and muscle cholesterol

The impact of dietary graded levels of TQN on the rabbits' serum biochemical parameters, abdominal fat and muscle cholesterol are summarized in Table 5. Notably, there were no significant differences ($p > 0.05$) in the RBCs counts, Hb concentrations and serum levels of AST and ALT among all experimental groups. Moreover, the lipid profile revealed that total cholesterol, TG and LDL levels were decreased in the sera of rabbits with increasing dietary levels of TQN. The most significant ($p < 0.05$) reductions in the serum levels of TC, TG and LDL (108.36, 75.82 and 94.44 mg/ dL, respectively) were detected among rabbits fortified with TQN at a level of 300 mg/Kg when compared with the control group (112.44, 78.9 and 111.5 mg/ dL, respectively).

Remarkably, supplementing rabbits with TQN had a decreasing impact on their abdominal fat percentages. Moreover, the most significant ($p = 0.003$) decline in the abdominal fat was seen in rabbits offered TQN dietary supplementation at a concentration of 300 mg/ Kg (1.91%) concerning the control group (2.11%). Compared to the thigh, rabbits' breast muscle had less cholesterol levels and raising the intake of dietary TQN decreased the cholesterol levels in both tissues concerning the control group. Furthermore, the most significant ($p < 0.01$) minimizations in the concentrations of thigh and breast

TABLE 4 Effect of various levels of thymoquinone nanoemulsions on the activities of antioxidant markers in the breast muscle and jejunal tissues of rabbits.

Antioxidant markers	Experimental groups					
	Control	TQNI	TQNII	TQNIII	*p* value	SEM
Breast muscle						
T-AOC (U/mg prot)	8.26^{c}	10.04^{b}	11.00^{b}	14.90^{a}	< 0.001	0.25
SOD (µ/mL)	31.03^{c}	32.09^{b}	34.92^{a}	35.35^{a}	< 0.001	0.08
GPX (µmoL/mg)	295.78^{c}	295.88^{c}	319.7^{b}	322.32^{a}	< 0.001	0.36
CAT (U/L)	3.20^{c}	3.48^{c}	4.14^{b}	5.00^{a}	< 0.001	0.08
MDA (nmoL/mL)	0.38^{a}	0.29^{a}	0.24^{b}	0.20^{c}	< 0.001	0.01
Jejunal tissues						
T-AOC (U/mg prot)	17.92^{c}	18.64^{c}	21.62^{b}	25.80^{a}	< 0.001	0.15
SOD (µ/mL)	17.90^{c}	18.12^{bc}	18.92^{b}	20.16^{a}	< 0.001	0.12
GPX (µmoL/mg)	253.18^{c}	256.12^{c}	276.58^{b}	302.8^{a}	< 0.001	1.5
CAT (U/L)	2.64^{c}	3.27^{b}	3.72^{a}	4.02^{a}	< 0.001	0.05
MDA (nmoL/mL)	2.06^{a}	1.70 ^{ab}	1.48^{b}	1.28^{c}	< 0.001	0.04

T-AOC, total antioxidant capacity; SOD, superoxide dismutase; GPX, glutathione peroxidase; CAT, catalase; MDA, malondialdehyde; control, rabbits fed basal control diets; TQNI, TQNII and TQNIII, rabbits fed basal diets with thymoquinone nanoemulsions (TQN) supplementations at graded levels consisting of 100, 200 and 300 mg/kg diets, respectively; SEM, standard error of the mean. $^{a-c}$Mans in a row with different superscript letters represent statistical variations ($p < 0.05$).

TABLE 5 Impact of graded levels of thymoquinone nanoemulsions on the levels of hematology, serum biochemical parameters, abdominal fat and muscle cholesterol levels in rabbits.

Parameter	Experimental groups					
	Control	TQNI	TQNII	TQNIII	*p* value	SEM
RBCs ($\times 10^6$/μL)	2.62	2.67	2.69	2.72	0.07	0.10
Hb (g/dL)	11.29	11.27	11.30	11.33	0.80	0.12
WBC, 10^3/mL	12.55	11.99	12.03	12.13	0.60	0.15
Platelets, 10^3/mL	125.66	126.87	125.99	126.12	0.73	0.46
ALT (U/L)	21.18	21.36	20.88	21.09	0.575	0.12
AST (U/L)	51.40	51.36	50.54	51.66	0.094	0.152
TG (mg/dL)	78.9^a	78.58^a	76.62^b	75.82^b	< 0.001	0.188
TC (mg/dL)	121.44^a	118.84^b	113.24^c	108.36^d	< 0.001	0.322
LDL (mg/dL)	111.50^a	99.58^b	98.44^b	94.44^c	< 0.001	0.378
Abdominal fat (%)	2.11^a	2.06^b	2.05^b	1.91^b	0.003	0.017
Breast cholesterol (mg/100 mg)	59.36^a	59.27^a	57.43^b	56.52^b	< 0.001	0.209
Thigh cholesterol (mg/100 mg)	62.24^a	61.73^{ab}	61.73^{ab}	60.52^b	0.028	0.185

RBCs, red blood cells; Hb, hemoglobin; ALT, alanine aminotransferase; AST, aspartate aminotransferase; TG, total triglycerides; TC, total cholesterol; LDL, low-density lipoprotein; control, rabbits fed basal control diets; TQNI, TQNII and TQNIII, rabbits fed basal diets with thymoquinone nanoemulsions (TQN) supplementations at graded levels consisting of 100, 200 and 300 mg/kg diets, respectively; SEM, standard error of the mean. $^{a-d}$Means in a row with different superscript letters represent statistical variations ($p < 0.05$).

cholesterol (60.52 and 56.52 mg/100 mg, respectively) were pronounced in rabbits fortified with TQN at the level of 300 mg/Kg regarding the control group (62.24 and 59.36 mg/100 mg, respectively).

3.5 Analysis of serum immunological parameters and blood phagocytic percentage

Data relating to the serum immunological parameters and blood phagocytic percentage post supplemnting rabbits with graded TQN levels are displayed in Table 6. Concerning the concentrations of serum IgG, IgM, IgA, total Igs and blood phagocytic percentage, they were significantly ($p < 0.001$) upraised in rabbits offered TQN dietary inclusion in a dose dependant manner. Notably, rabbits offered TQN at a concentration of 300 mg/Kg showed the highest significant ($p < 0.001$) immune response as evinced by increased serum levels of IgG, IgM, IgA, total Igs and blood phagocytic percentage (13.3, 0.69, 0.81 and 16.52 mg/ dL and 49.12%, respectively) when compared with the control group (10.84. 0.48, 0.62 and 12.7 mg/ dL and 25.4%, respectively).

3.6 Effect of thymoquinone nanoemulsions dietary inclusion on *Pasteurella multocida* counts

At 48 and 96 h post-infection with MDR virulent *P. multocida* strain, TQN-supplemented rabbits showed no observable clinical symptoms of snuffles or subcutaneous hemorrhages in contrast to control rabbits, which showed respiratory clinical signs of snuffles, subcutaneous hemorrhages and decline in their activities. The quantification outcomes of *P. multocida* in the lung, spleen and liver tissues of experimentally infected rabbits are demonstrated in Figure 2. At 48 and 96 h post-infection, *P. multocida* was numerically and significantly ($p < 0.05$) reduced in the lung and spleen tissues of rabbits offered TQN supplementations in a dose-dependent manner. Notably, our data showed that *P. multocida* populations were at their lowest levels in lung, spleen and liver tissues of rabbits receiving TQN supplementations at a level of 300 mg/Kg at both time intervals post-infection with *P. multocida* strain (up to 1.89, 0.11 and 0.03 $\log_{10}$ CFU/g, respectively).

3.7 Gene expression analysis of virulence-related genes post-infection with *Pasteurella multocida*

The relative transcription levels of *P. multocida* virulence-related genes via RT-qPCR at 48 and 96 h post-infection with MDR virulent *P. multocida* are shown in Figure 3. The outcomes demonstrated that TQN supplementations, especially at higher levels significantly ($p < 0.05$) downregulated the transcription levels of *ptfA, toxA* and *nanB* virulence genes compared to the control non-supplemented group. Of note, TQN dietary supplementation at a concentration of 300 mg/Kg had a noticeable decreasing impact on the expression of the examined virulence genes at 48 and 96 h post-infection with *P. multocida* strain, with a special reference to *nanB* gene (0.41 and 0.33–fold change, respectively).

3.8 Gene expression analysis of cytokines-related genes post-infection with *Pasteurella multocida*

The results of mRNA expression levels of cytokines-related genes via RT-qPCR at 96 h post-infection with MDR virulent *P. multocida* strain are illustrated in Figure 4. Our findings showed that supplementing the rabbits with TQN significantly ($p < 0.05$) upregulated the transcription levels of *IL-10* (Figure 4D), *TLR-4*

TABLE 6 Impact of different concentrations of thymoquinone nanoemulsions on the levels of serum immunological parameters and blood phagocytic percentage in rabbits.

Parameter	Experimental groups					
	Control	TQNI	TQNII	TQNIII	*p* value	SEM
IgG (mg/dL)	10.84^{c}	11.64^{b,c}	11.96^{b}	13.30^{a}	< 0.001	0.142
IgM (mg/dL)	0.48^{c}	0.58^{b}	0.64^{a,b}	0.69^{a}	< 0.001	0.11
IgA (mg/dL)	0.62^{c}	0.66^{c}	0.74^{b}	0.81^{a}	< 0.001	0.008
Total Igs (mg/dL)	12.70^{d}	14.68^{c}	15.52^{b}	16.52^{a}	< 0.001	0.094
Phagocytic percentage (%)	25.40^{d}	38.92^{c}	42.50^{b}	49.12^{a}	< 0.001	0.305

Ig, immunoglobulin; Igs, immunoglobulins; control, rabbits fed basal control diets; TQNI, TQNII and TQNIII, rabbits fed basal diets with thymoquinone nanoemulsions (TQN) supplementations at graded levels consisting of 100, 200 and 300 mg/kg diets, respectively; SEM, standard error of the mean. $^{a\text{-}d}$Means in a row with different superscript letters represent statistical variations ($p < 0.05$).

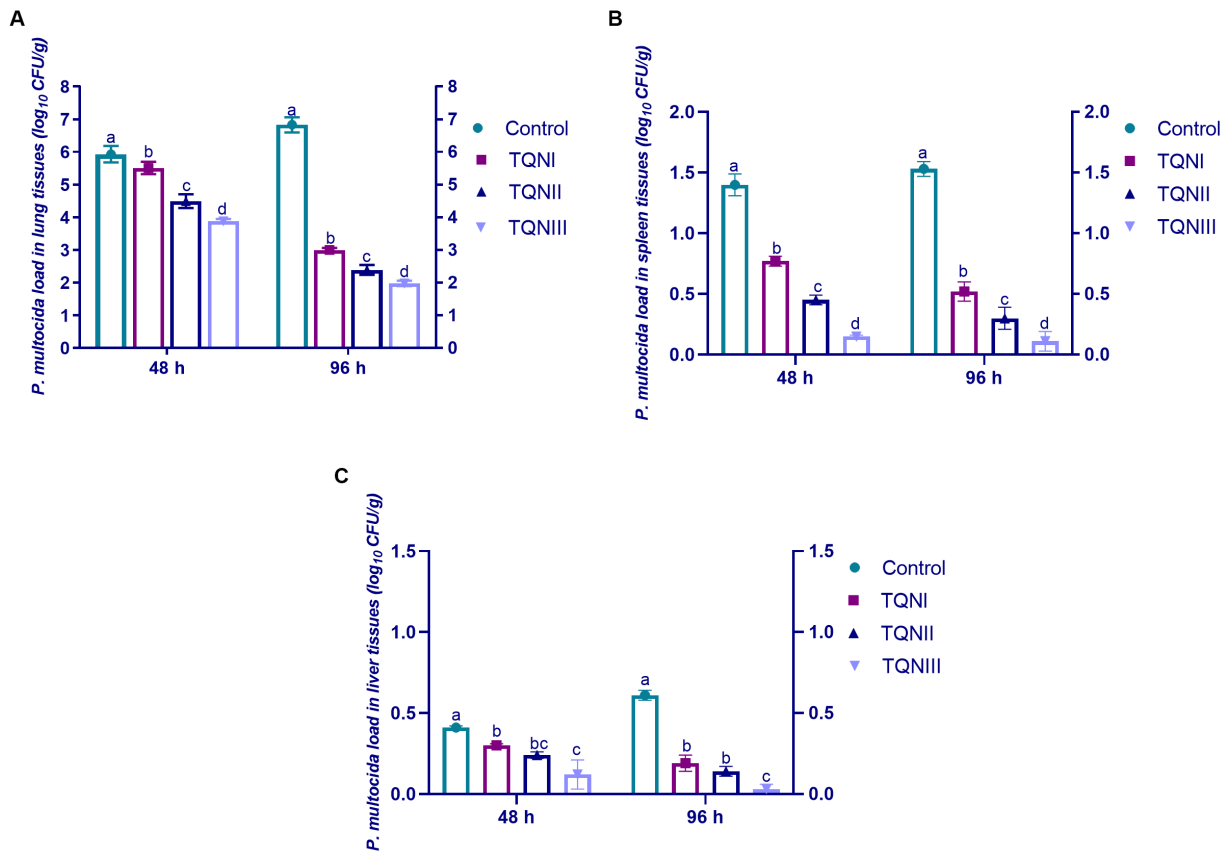


FIGURE 2
Quantification of *P. multocida* loads in the lung **(A)**, spleen **(B)** and liver **(C)** tissues of rabbits in response to thymoquinone nanoemulsions (TQN) supplementations at 48 and 96 h post-infection with MDR virulent *Pasteurella multocida* strain. Values are means ± standard error of the mean (SEM) in bars. Control, rabbits fed basal control diets; TQNI, TQNII and TQNIII, rabbits received basal diets with TQN supplementations at graded levels consisting of 100, 200 and 300 mg/kg diets, respectively. $^{a\text{-}d}$Means with various superscript letters denote statistical variations ($p < 0.05$).

(Figure 4E), *DEFB1* (Figure 4G) and *TLR-2* (Figure 4H) genes comparing with the control non-supplemented rabbits at 96 h post-infection with *P. multocida* strain. Moreover, the results displayed that increasing TQN levels significantly ($p < 0.05$) decreased the expression levels of *IL-8* (Figure 4A), *TNF-α* (Figure 4B), *IL-6* (Figure 4C) and *IL-1β* (Figure 4F) genes un like the control group at 96 h post-infection with *P. multocida*. Of note, rabbits offered 300 mg/kg of TQN had the highest significant ($p < 0.05$) increase in the expression levels of *IL-10*, *TLR-4*, *DEFB1* and *TLR-2* genes (up to 1.59– fold change) and the most significant ($p < 0.05$) downregulation in the transcription levels of *IL-8*, *TNF-α*, *IL-6* and *IL-1β* genes (up to 0.69– fold change) concerning the control group.

4 Discussion

Recent developments in global rabbit industry have encouraged the utilization of a variety of practical and cost-effective strategies to

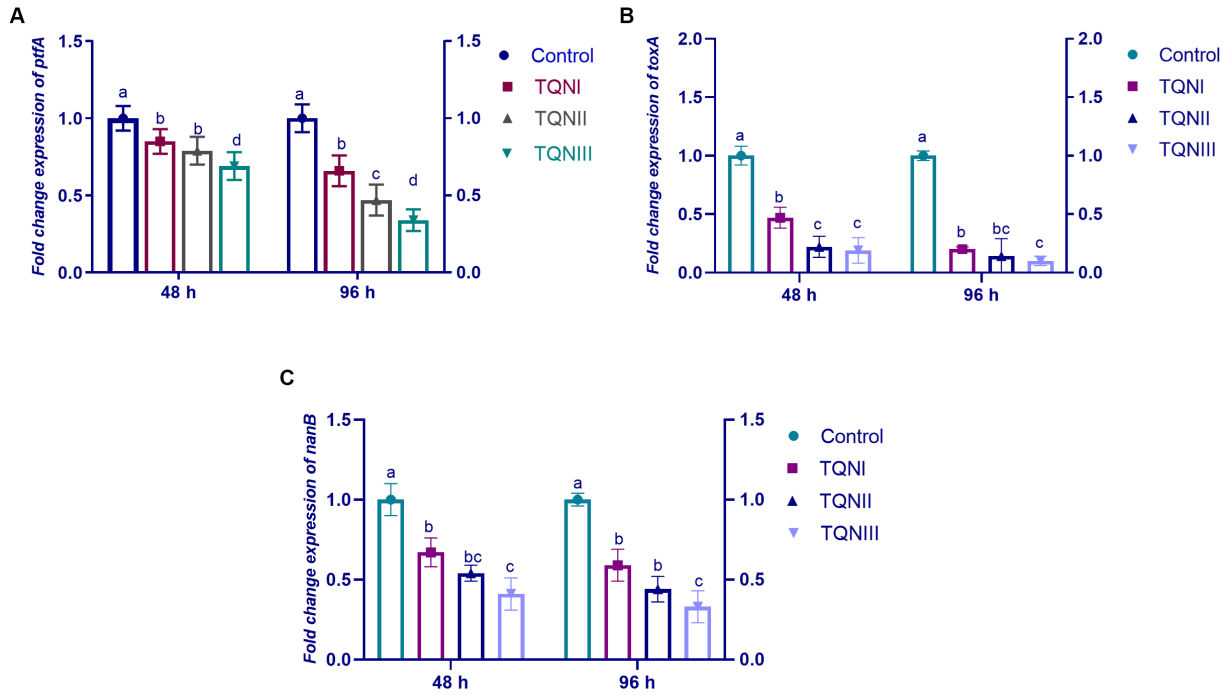


FIGURE 3
Impact of dietary fortification with graded levels of thymoquinone nanoemulsions (TQN) at 48 and 96 h post-infection with MDR virulent *Pasteurella multocida* strain on relative transcription levels of *P. multocida* virulence genes; *ptfA* **(A)**, *toxA* **(B)** and *nanB* **(C)**. Values are means ± standard error of the mean (SEM) in bars. Control, rabbits fed basal control diets; TQNI, TQNII and TQNIII, rabbits received basal diets with TQN supplementations at graded levels consisting of 100, 200 and 300 mg/kg diets, respectively. $^{a-d}$Means with various superscript letters denote statistical variations ($p < 0.05$).

recommend the sustainability of rabbit production. Additionally, the excessive usage of antimicrobials led to the development of MDR strains, which limit their use; thus, several researches have focused on finding alternative substances from natural herbs (11, 20). Phytochemicals are thought to have a key role in rabbits' diets. Among them, TQ was employed as a potential antimicrobial, anti-carcinogenic, antioxidant, anti-inflammatory, immunostimulant and growth-promoting agent (11, 19, 24, 25). Of note, the use of nanotechnology in rabbit breeding remains in its nascent stage and unfortunately, to the best of our knowledge, there are no data on the application of TQN in the rabbit industry. Thus, our study investigated, for the first time, the efficacy of TQN on rabbits' growth performance, digestive and antioxidant enzymes' activities, phagocytic percentage, immunological and biochemical markers in addition to *P. multocida* resistance in challenged rabbits.

In the present work, dietary inclusion of TQN had enhancing impacts on rabbits' growth attributes unlike the control group. Additionally, rabbits receiving TQN at a level of 300 mg/Kg exhibited the maximum BWG and superior FCR throughout the rearing period. In agreement with our outcomes, *Nigella sativa* (NS) seed had growth-enhancing impacts by provoking the BWG and FCR in rabbits (24, 47, 48) and broilers (28). Furthermore, rabbits offered dietary NS extract showed ameliorated FCR and enhanced BWG (11). In broilers, a previous research demonstrated that NS seed had enhancing impact on the digestive system, which led to better growth performance (49). In accordance, previous reports noted that dietary inclusion of TQ had provoked the BWG and FCR in broilers (50) and fish (19). Moreover, a previous report displayed that nano-encapsulated cumin EO had ameliorated the BWG and FCR in broilers (51). However, the effect of TQN on growth performance in rabbits was not studied until now. The high growth performance of rabbits offered TQN supplementation could be attributed to their role in preserving the structure and enhancing the functions of the digestive system as previously recorded (19, 36, 52). The production of digestive enzymes could be altered through hormones, genes and dietary supplements (53). In parallel with the enhanced growth performance in TQN-supplemented groups, the activities of jejunal digestive enzymes (amylase, lipase and trypsin) were likewise raised indicating their improving impact on digestibility and nutrient utilization. In agreement with our outcomes, a recent study reported that NS extract had improving impacts on rabbits' growth, feed utilization, digestive enzymes and gut microbial flora (11). A recent report revealed also that TQ enhanced digestion (54) and this may be linked to its function as a potent stimulator of digestive enzymes and its ability to increase nutrient retention with a favorable impact on nutrient utilization and growth performance (55). Previous studies stated the enhancing effects on broilers' digestive enzymes following dietary fortification with eugenol nanoemulsions (56), garlic nano-hydrogel (57), thymol nanoemulsion (31) and EOs mixture (18). However, the effect of TQN on the activities of digestive enzymes in rabbits has not yet been investigated. Of note, EOs may affect growth rate by increasing nutrient digestibility through the regulation of the intestinal microbiota and upregulation of endogenous enzymes (58). Additionally, it was formerly proven that phytochemicals can modify the digestive transcriptional patterns (59). The differences in the impacts of EOs between various researches may be resulted from the variability in supplementation doses, components, extraction techniques and sources (19). The enhancing effect of thymoquinone nanoemulsion on rabbits' performance might

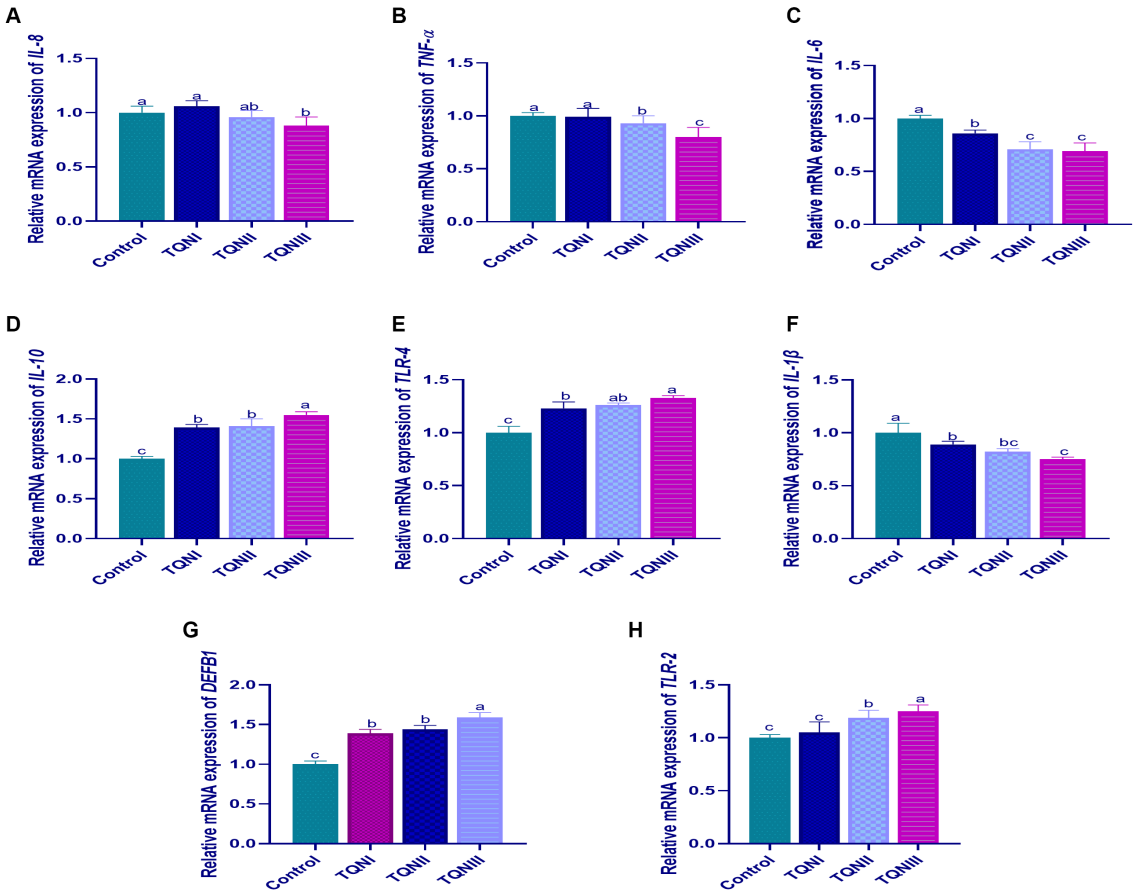


FIGURE 4
Transcriptional levels of cytokines-related genes; IL-8 [interleukin-8; **(A)**], TNF-α [tumor necrosis factor-alpha; **(B)**], IL-6 [interleukin-6; **(C)**], IL-10 [interleukin-10; **(D)**], TLR-4 [toll-like receptor 4; **(E)**], IL-1β [interleukin-1β; **(F)**], DEFB1 [beta-defensin 1; **(G)**] and TLR-2 [toll-like receptor 2; **(H)**] detectable via RT-qPCR in the splenic tissues of rabbits offered graded levels of thymoquinone nanoemulsions (TQN) supplementations at 96 h post-infection with MDR virulent *Pasteurella multocida* strain. Values are means ± standard error of the mean (SEM) in bars. Control, rabbits fed basal control diets; TQNI, TQNII and TQNIII, rabbits received basal diets with TQN supplementations at graded levels consisting of 100, 200 and 300 mg/kg diets, respectively. $^{a-c}$Means with various superscript letters denote statistical variations ($p < 0.05$).

be explained by improving the bioactivity and bioavailability of TQ because TQN permit easier uptake by cells in the gut and deeper penetration to tissues resulting in an effective rise in the transcription levels of genes encoding digestive enzymes (31).

The immune system and overall health of animals are closely related to the antioxidant defense system. Subjecting animals to stressful situations could increase the formation of reactive oxygen species (ROS), which cause oxidative stress and significant cell damage. The antioxidant defense system helps the animals to keep endogenous ROS at shallow concentrations and reduce oxidative damage brought on by the elevated reactive nature of ROS (60). Due to the ongoing formation and clarity of free radicals by the animal's antioxidant system, their levels are maintained under a dynamic balance in normal physiological conditions (61). On the other hand, increasing ROS generation might promote the lipid peroxidation of cell membranes and have a detrimental effect on animal health and performance (62). Antioxidant enzymes, including GPX, SOD and CAT, are thought to be the primary lines of defense against the production of harmful ROS, resulting in direct detoxification (63, 64). The T-AOC is regarded as an index to reflect the body's antioxidant levels (65). Contrarily, larger levels of free radicals lead to an excess of MDA, which is one of the byproducts of cells' lipid peroxidation; thus, the MDA concentration is typically used as a sign of oxidative stress (66). Animal diets supplemented with antioxidants can eliminate free radicals, which can coordinate the animal's antioxidant system. In this situation, TQN is a natural antioxidant, but researches on the processes by which it affects the rabbits' antioxidant system and whether its application would offer an extra advantage for enhancing this function are currently lacking and require more investigation. Our results showed a significant increase in the concentrations of T-AOC and GPX, CAT and SOD enzymes and a decrease in the level of MDA in the breast muscle and jejunal tissues of rabbits offered dietary TQN in comparison with the control group suggesting its role in activating the antioxidant enzymatic processes. Similarly, TQ dietary supplementation increased the activities of CAT, SOD and GPX antioxidant enzymes and lowered the concentrations of MDA in rabbits (67). In accordance, dietary fortifications with liposomal encapsulated TQ elevated the levels of T-AOC, CAT antioxidant enzyme and decreased the level of MDA in rabbits (68). Furthermore, dietary inclusion of microalgae mixture increased the activities of T-AOC and GPX, CAT and SOD antioxidant enzymes and decreased the level of MDA in fish, which enhanced the cells' resistance to

oxidative stress (69). Numerous studies have stated that TQ has antioxidant properties, which are responsible for eliminating free radicals and provide a significant improvement to oxidative stress responses (19, 70, 71). Herein, dietary inclusion of TQN boosted the antioxidant potential of rabbits, which might be attributed to the effectiveness of nanoemulsions in enhancing the bioactivity and bioavailability of TQ, which allow easier uptake by cells and deeper penetration to tissues leading to enhancement of antioxidant defense system.

The liver enzymes aminotransferases (AST and ALT) reflect the health condition of the liver (39). Herein, TQN supplementations did not change the levels of AST and ALT, which suggested that TQN has a protective impact on the rabbits' liver tissues. In agreement with our outcomes, dietary inclusion with liposomal encapsulated curcumin and nano-curcumin did not alter the activities of AST and ALT (39, 72). Similarly, previous studies stated that dietary inclusion of EOs did not alter the levels of AST and ALT in fish (19) and broilers (40). Furthermore, dietary EOs (73) and coconut oil (74) fortifications reduced the serum level of ALT and AST in rabbits, respectively. Our findings displayed a significant decrease in the activities of total cholesterol, TG and LDL in rabbits receiving dietary TQN, which suggests that TQN might have a modulatory effect in regulating the level of enzymes involved in lipid metabolism. Moreover, lowered serum cholesterol concentrations were also reflected in reduced cholesterol levels in thigh and breast muscles after TQN dietary supplementations. Our findings might be attributed to boosting the cholesterol total fecal excretion through the bile and inhibiting its absorption in the gut (40, 75). Additionally, phytochemical nanoemulsions may alter hepatic gene expressions and prevent the biosynthesis of cholesterol by decreasing important lipogenic factors that promote the production of bile acids and boost cholesterol clearance (76). Moreover, TQ dietary supplementation minimized the serum levels of TC and TG in fish (19). In accordance, EOs supplementations decreased the serum activities of TC, TG and LDL in broilers (40). Accordingly, similar outcomes were observed when abdominal fat, cholesterol levels in the thigh and breast muscles and serum levels of TC, TG and LDL were reduced in broilers receiving dietary NS supplementations (77).

The health of animals is primarily maintained by the immune system. Essential oils have a favorable impact on the animals' immune defense as they improve the activities of lymphocyte and immunoglobulin synthesis (78, 79). The rabbits' immunological response and antioxidant defense systems are positively correlated providing defense against invasive harmful microorganisms. Strengthening the immune defense of rabbits through dietary natural antioxidants could counteract the issue of stressful situations during the weaning phase. Total Igs have significant functions in the immunological activities including phagocytosis and neutralization of harmful microorganisms making them important components of the humoral immune defense (80). Additionally, IgM, IgG and IgA are the three main immunoglobulin isotypes that react to both systemic and local microorganisms (81). Our outcomes showed that rabbits offered dietary TQN, particularly at a level of 300 mg/kg showed increases in the levels of IgG, IgM, IgA and total Igs and phagocytic percentage when compared with the control group. These findings were attributed to provoking the rabbits' immune system, which accelerated the synthesis of cytokines that are essential for controlling the immune response (76). Similarly, dietary coconut oil supplementation enhanced the phagocytic activity and immune response in rabbits (74) and dietary inclusion with NS seed enhanced the serum levels of IgG and IgM in rabbits (24). Moreover, TQ dietary supplementation enhanced the serum level of IgM in fish (19). Furthermore, EOs fortifications provoked the serum levels of IgG and IgM and blood phagocytic percentage in rabbits (74). In accordance, a recent study displayed that phytochemical curcumin encapsulation increased the levels of total Igs and IgM in fish (39). Accordingly, previous reports displayed that EOs supplementation enhanced the activities of IgG, IgM and IgA and phagocytic percentage in broilers (40) and piglets (82).

Pasteurella multocida can colonize the respiratory tract of rabbits causing snuffles (pasteurellosis) and significant financial losses for rabbit breeding (5, 6). For many years, antimicrobials have been considered a key tool in the treatment of bacterial infections; but as a result of their excessive usage, MDR strains have emerged recently (21). From this perspective, the EOs' possible benefits have been considered as a possible approach to combat bacterial infections (18, 19, 22). There is a lack of information regarding EOs' ability to prevent or treat *P. multocida* infections in farmed rabbits. Herein, quantitative analysis of *P. multocida* counts in challenged rabbits displayed that dietary inclusion with TQN significantly minimized *P. multocida* loads at 48 and 96 h post-infection with MDR virulent *P. multocida* strain concerning the control group. Similarly, a recent study reported that NS extract significantly minimized methicillin-resistant *Staphylococcus aureus* counts in challenged rabbits suggesting its antibacterial activities (11); but, the impact of TQN on *P. multocida* populations in the lung tissues of challenged rabbits was not studied till now. Regarding *P. multocida* resistance in challenged rabbits following TQN fortifications, previous studies stated that NS extract can alter the innate immune response function by minimizing bacterial survival, raising nitric oxide generation and improving the phagocytic ability of macrophages (11, 83).

Nutritional immunology is a unique approach to disease prevention in the rabbit industry via the use of dietary supplements to get around the limitations of vaccination programs (11, 84). Moreover, improving rabbits' nutrition and veterinary care for preventing infections will increase the economic and productive efficiency of rabbits' husbandry. Notably, numerous treatment strategies target recently microbial pathogenicity rather than microbial survival (85). Thus, the TQN anti-virulence activities were investigated by determining the transcription levels of *ptfA, toxA* and *nanB* virulence genes in response to its supplementation at 48 h and 96 h post-infection with *P. multocida* strain. Herein, the transcription levels of *ptfA, toxA* and *nanB* virulence genes were significantly downregulated in challenged rabbits fortified with TQN supplementation, especially at high levels, unlike the control group at 48 h and 96 h post-infection with MDR virulent *P. multocida* strain. Similarly, a previous report displayed the *in vivo* anti-virulence characteristics of dietary TQ supplementations *against Aeromonas sobria* in challenged fish (19). Furthermore, a recent study stated the *in vivo* anti-virulence properties of thymol nanoemulsion against *Salmonella Enteritidis* in challenged broilers (30). Notably, a recent study displayed that marjoram extract downregulated the expressions of *P. multocida* virulence-related genes *in vitro* (6); however, the *in vivo* anti-virulence effect of TQN in rabbits experimentally infected with *P. multocida* has not yet been studied. The anti-virulence characteristics of TQN might be resulted from the inhibition of quorum sensing (QS), which is the microbial gene

regulation system that controls the transcription of various virulence markers (19). A recent report stated that the EO suppresses QS at sub-inhibitory concentrations and reduces a range of QS markers in a dose-dependent effect (86). The direct impact of EO on the production of QS signaling molecules and the deactivation of cognate receptors may be responsible for its anti-QS effects. As a result, it inhibited the transcription of virulence genes responsible for cooperative behaviors (30, 85).

The persistence of gut integrity and barrier functions can be influenced by the interactive effects of phytochemicals on the superior immune response via altering the transcription of various cytokines, mucin and pattern recognition receptors (87). It is well-recognized that cytokines have a significant regulatory role in controlling the gut inflammatory reactions (84). Notably, the tight junctions serve as both physical and functional barriers against the entry of harmful pathogens and other chemicals making them essential elements of the gut barrier functions (88). Numerous markers such as mucin, tight junction molecules and defensins reflect different elements of the gut barrier. Moreover, TLR signaling improves the integrity of tight junctions by improving the transcription of important genes related to tight junctions (11). Of note, TQ has immunostimulant activities in a variety of animals' immunologic and inflammatory illnesses (89) and it exerts an ameliorating impact on the anti-inflammatory cytokines such as *IL-10* and the proinflammatory cytokines including *TNF-α*, *IL-8*, *IL-6* and *IL-1β*, which inhibits the development of intestinal inflammation and maintains intestinal hemostasis (90). Contrarily, when bacteria invade the gut epithelial cells, gut immune cells begin cytokines synthesis, which in turn promotes immune defense against bacteria (91). In this context, *P. multocida* may increase the expression levels of *TNF-α*, *IL-8* and *IL-6* genes, which in turn raises the gastrointestinal epithelium's permeability (92). *IL-1β* is one of the most important pro-inflammatory cytokines, which promotes its own expression as well as the transcription of other chemokines and pro-inflammatory cytokines leading to recruiting the inflammatory reactions and initiating the formation of antimicrobial cells (93). Additionally, *IL-10* has a primarily antagonistic effect on inflammation in addition to its critical function in suppressing immunological and inflammatory reactions (94). Our findings showed that, in parallel with improving the serum immunological parameters and phagocytic indices, dietary TQN fortifications significantly downregulated the expression levels of *TNF-α*, *IL-8*, *IL-6* and *IL-1β* genes and upregulated the transcription levels of *IL-10*, *TLR-4*, *DEFB1* and *TLR-2* genes in rabbits at 96 h post-infection with *P. multocida* in comparison with the control group. Our findings indicate that TQN fortifications successfully counteracted the strong inflammatory reactions in *P. multocida* experimentally-infected groups suggesting its potent anti-inflammatory and immunostimulant activities. In agreement with our findings, TQ can control the movement of inflammatory cells by altering the transcription of cytokines and/or chemokines, which has the effect of reducing the immune system's response to inflammation (89). In a comparable study, NS extract reduced the expression levels of *TNF-α*, *IL-8*, *IL-6* and *IL-1β* genes and increased the transcription levels of *IL-10*, *TLR-4*, *DEFB1* and *TLR-2* genes in rabbits (11) and dietary supplementations of NS powder improved the immunological responses in broilers (95). These findings may provide an explanation for TQ anti-inflammatory properties (90). Thymoquinone has already been shown to have improving effects on the immune defense via enhancing the response of antibodies and restoring the immunological and inflammatory changes (96). From our point of view, TQN have antimicrobial, antioxidant, immunostimulant and anti-inflammatory properties that enhance serum cellular and humoral immunity, which in turn reduces the proliferation of harmful microorganisms and inflammation in rabbits.

5 Conclusion

Overall, the outcomes of our work indicated the improving effects of TQN dietary supplementation during the entire experimental period on rabbits' growth, digestion, immunity and antioxidant potential as realized by increasing the levels of digestive and antioxidant enzymes as well as biochemical and immunological markers. Moreover, dietary inclusion of TQN for rabbits challenged with *P. multocida* reduced the severity of clinical manifestations and bacterial translocation or localization by reducing the counts of *P. multocida* in rabbits' lungs, downregulating the expression levels of *P. multocida* virulence-related genes and ameliorating the transcription levels of cytokines-related genes. Therefore, our findings suggest using TQN as a novel dietary supplement, which is advertised to play a fundamental role in controlling *P. multocida* infection in rabbits.

Data availability statement

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

Ethics statement

The animal study was approved by all procedures for experiments were performed following the rules and authorized specifications of the Institutional Animal Care and Use Committee (IACUC), Faculty of Veterinary Medicine, Zagazig University, Egypt under the reference number (ZU-IACUC/2/F/195/2022). The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

MA: Data curation, Investigation, Methodology, Resources, Writing – original draft, Writing – review & editing. ME-A: Investigation, Writing – original draft. RE-M: Methodology, Software, Validation, Writing – review & editing. ME: Formal analysis, Funding acquisition, Resources, Software, Visualization, Investigation, Writing – original draft. AA: Supervision, Writing – original draft, Writing – review & editing. EY: Conceptualization, Investigation, Visualization, Writing – original draft. WY: Data curation, Investigation, Writing – original draft, Writing – review & editing. RD: Conceptualization, Data curation, Formal analysis, Funding acquisition, Writing – original draft. DE: Investigation, Methodology, Project administration, Writing – review & editing. MH: Software, Supervision, Validation,

Visualization, Writing – review & editing. AO: Data curation, Funding acquisition, Resources, Visualization, Writing – review & editing. SM: Methodology, Resources, Visualization, Writing – original draft. SD: Writing – original draft. DI: Conceptualization, Formal analysis, Methodology, Supervision, Writing – original draft, Writing – review & editing.

Funding

The author(s) declare financial support was received for the research, authorship, and/or publication of this article. This work was supported by the researches supporting project number (RSP2024R36), King Saud University, Riyadh, Saudi Arabia.

Conflict of interest

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

Publisher's note

All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

References

1. Cullere M, Dalle Zotte A. Rabbit meat production and consumption: state of knowledge and future perspectives. *Meat Sci*. (2018) 143:137–46. doi: 10.1016/J.MEATSCI.2018.04.029

2. Soriano-Vargas E, Vega-Sánchez V, Zamora-Espinosa JL, Acosta-Dibarrat J, Aguilar-Romero F, Negrete-Abascal E. Identification of *Pasteurella multocida* capsular types isolated from rabbits and other domestic animals in Mexico with respiratory diseases. *Trop Anim Health Prod*. (2012) 44:935–7. doi: 10.1007/S11250-011-9995-X/FIGURES/1

3. Ferreira TSP, Moreno LZ, Felizardo MR, de Gobbi DDS, Filsner PHDLN, De Moura Gomes VT, et al. Pheno- and genotypic characterization of *Pasteurella multocida* isolated from cats, dogs and rabbits from Brazil. *Comp Immunol Microbiol Infect Dis*. (2016) 45:48–52. doi: 10.1016/J.CIMID.2016.02.004

4. Arumugam ND, Ajam N, Blackall PJ, Asiah NM, Ramlan M, Maria J, et al. Capsular serotyping of *pasteurella multocida* from various animal hosts – a comparison of phenotypic and genotypic methods. *Trop Biomed*. (2011) 28:55–63. https://pubmed.ncbi.nlm.nih.gov/21602769/

5. Boyce J, Harper M, Wilkie I, Adler B. *Pasteurella: pathogenesis of bacterial infections in animals*. Oxford, UK: Wiley-Blackwell (2010).

6. Abd El-Hamid MI, El-Sayed ME, Ali AR, Abdallah HM, Arnaout MI, El-mowalid GA. Marjoram extract down-regulates the expression of *Pasteurella multocida* adhesion, colonization and toxin genes: a potential mechanism for its antimicrobial activity. *Comp Immunol Microbiol Infect Dis*. (2019) 62:101–8. doi: 10.1016/j.cimid.2018.11.007

7. Rodríguez J, Martín MJ, Ruiz MA, Clares B. Current encapsulation strategies for bioactive oils: from alimentary to pharmaceutical perspectives. *Food Res Int*. (2016) 83:41–59. doi: 10.1016/J.FOODRES.2016.01.032

8. Verma S, Sharma M, Katoch S, Verma L, Kumar S, Dogra V, et al. Profiling of virulence associated genes of *Pasteurella multocida* isolated from cattle. *Vet Res Commun*. (2013) 37:83–9. doi: 10.1007/S11259-012-9539-5/TABLES/3

9. Katsuda K, Hoshinoo K, Ueno Y, Kohmoto M, Mikami O. Virulence genes and antimicrobial susceptibility in *Pasteurella multocida* isolates from calves. *Vet Microbiol*. (2013) 167:737–41. doi: 10.1016/J.VETMIC.2013.09.029

10. Katoch S, Sharma M, Patil RD, Kumar S, Verma S. In vitro and in vivo pathogenicity studies of *Pasteurella multocida* strains harbouring different ompA. *Vet Res Commun*. (2014) 38:183–91. doi: 10.1007/S11259-014-9601-6

11. Elmowalid GAE, Ahmad AAM, El-Hamid MIA, Ibrahim D, Wahdan A, El Oksh ASA, et al. *Nigella sativa* extract potentially inhibited methicillin resistant *Staphylococcus aureus* induced infection in rabbits: potential immunomodulatory and growth promoting properties. *Animals*. (2022) 12:2635. doi: 10.3390/ANI12192635/S1

12. Ammar AM, El-Hamid MIA, Mohamed YH, Mohamed HM, Al-khalifah DHM, Hozzein WN, et al. Prevalence and antimicrobial susceptibility of bovine Mycoplasma species in Egypt. *Biology (Basel)*. (2022) 11:1083. doi: 10.3390/BIOLOGY11071083

13. Ammar AM, Abd El-Hamid MI, El-Malt RMS, Azab DS, Albogami S, Al-Sanea MM, et al. Molecular detection of fluoroquinolone resistance among multidrug-, extensively drug-, and Pan-drug-resistant Campylobacter species in Egypt. *Antibiotics*. (2021) 10:1342. doi: 10.3390/ANTIBIOTICS10111342

14. Ahmed HA, Tahoun ABMB, Abou Elez RMM, Abd El-Hamid MI, Abd Ellatif SS. Prevalence of *Yersinia enterocolitica* in milk and dairy products and the effects of storage temperatures on survival and virulence gene expression. *Int Dairy J*. (2019) 94:16–21. doi: 10.1016/J.IDAIRYJ.2019.02.010

15. Abd El-Hamid MI, Awad NFS, Hashem YM, Abdel-Rahman MA, Abdelaziz AM, Mohammed IAA, et al. In vitro evaluation of various antimicrobials against field mycoplasma gallisepticum and *mycoplasma synoviae* isolates in Egypt. *Poult Sci*. (2019) 98:6281–8. doi: 10.3382/PS/PEZ576

16. Mesev EV, LeDesma RA, Ploss A. Decoding type I and III interferon signalling during viral infection. *Nat Microbiol*. (2019) 4:914–24. doi: 10.1038/s41564-019-0421-x

17. Abdel-Raheem SM, Abd El-Hamid MI, Ibrahim D, El-Malt RMS, El-Ghareeb WR, Ismail HA, et al. Future scope of plant-derived bioactive compounds in the management of methicillin-resistant *Staphylococcus aureus*: in vitro antimicrobial and antivirulence prospects to combat MRSA. *Microb Pathog*. (2023) 183:106301. doi: 10.1016/J.MICPATH.2023.106301

18. Hashem YM, Abd El-Hamid MI, Awad NFS, Ibrahim D, Elshater NS, El-Malt RMS, et al. Insights into growth-promoting, anti-inflammatory, immunostimulant, and antibacterial activities of Toldin CRD as a novel phytobiotic in broiler chickens experimentally infected with *Mycoplasma gallisepticum*. *Poult Sci*. (2022) 101:102154. doi: 10.1016/j.psj.2022.102154

19. Ibrahim D, Shahin SE, Alqahtani LS, Hassan Z, Althobaiti F, Albogami S, et al. Exploring the interactive effects of thymol and thymoquinone: moving towards an enhanced performance, gross margin, immunity and *Aeromonas sobria* resistance of Nile Tilapia (*Oreochromis niloticus*). *Animals*. (2022) 12:3034. doi: 10.3390/ani12213034

20. Ammar AM, El-Naenaeey E-SY, El-Hamid MIA, El-Gedawy AA, El-Malt RMS. Campylobacter as a major foodborne pathogen: a review of its characteristics, pathogenesis, antimicrobial resistance and control. *J Microbiol Biotechnol Food Sci*. (2021) 10:609–19. doi: 10.15414/JMBFS.2021.10.4.609-619

21. Aljazzar A, Abd El-Hamid MI, El-Malt RMS, Rizk El-Gharreb W, Abdel-Raheem SM, Ibrahim AM, et al. Prevalence and antimicrobial susceptibility of Campylobacter species with particular focus on the growth promoting, immunostimulant and anti-*Campylobacter jejuni* activities of eugenol and trans-cinnamaldehyde mixture in broiler chickens. *Animals*. (2022) 12:905. doi: 10.3390/ANI12070905

22. Ammar AM, El-Naenaeey E-SY, El-Malt RMS, El-Gedawy AA, Khalifa E, Elnahriry SS, et al. Prevalence, antimicrobial susceptibility, virulence and genotyping of *Campylobacter jejuni* with a special reference to the anti-virulence potential of eugenol and beta-resorcylic acid on some multi-drug resistant isolates in Egypt. *Animals*. (2021) 11:3. doi: 10.3390/ani11010003

23. Abd El-Hamid MI, Ibrahim SM, Eldemery F, El-Mandrawy SAM, Metwally AS, Khalifa E, et al. Dietary cinnamaldehyde nanoemulsion boosts growth and transcriptomes of antioxidant and immune related genes to fight *Streptococcus agalactiae* infection in Nile tilapia (*Oreochromis niloticus*). *Fish Shellfish Immunol*. (2021) 113:96–105. doi: 10.1016/J.FSI.2021.03.021

24. El-Gindy Y, Zeweil H, Zahran S, El-Rahman MA, Eisa F. Hematologic, lipid profile, immunity, and antioxidant status of growing rabbits fed black seed as natural antioxidants. *Trop Anim Health Prod*. (2020) 52:999–1004. doi: 10.1007/S11250-019-02091-X/FIGURES/1

25. Ates MB, Ortatatli M, Oguz H, Ozdemir O, Terzi F, Ciftci MK, et al. The ameliorative effects of *Nigella sativa*, thymoquinone, and bentonite against aflatoxicosis in broilers via AFAR and Nrf2 signalling pathways, and down-regulation of caspase-3. *Br Poult Sci*. (2021) 63:332–9. doi: 10.1080/00071668.2021.1998366

26. Zohree A, Abdelmoteleb AM, Hamed O. Role of *Nigella sativa* oil and alpha lipoic acid on amikacin induced hepatorenal toxicity in rabbits. *Egypt J Anim Heal*. (2022) 2:24–31. doi: 10.21608/EJAH.2022.276336

27. Kaur R, Kaur L. Encapsulated natural antimicrobials: a promising way to reduce microbial growth in different food systems. *Food Control*. (2021) 123:107678. doi: 10.1016/J.FOODCONT.2020.107678

28. Kalam MA, Raish M, Ahmed A, Alkharfy KM, Mohsin K, Alshamsan A, et al. Oral bioavailability enhancement and hepatoprotective effects of thymoquinone by self-nanoemulsifying drug delivery system. *Mater Sci Eng C*. (2017) 76:319–29. doi: 10.1016/J.MSEC.2017.03.088

29. Ibrahim D, Kishawy ATY, Khater SI, Khalifa E, Ismail TA, Mohammed HA, et al. Interactive effects of dietary quercetin nanoparticles on growth, flesh antioxidant capacity and transcription of cytokines and *Aeromonas hydrophila* quorum sensing orchestrating genes in Nile tilapia (*Oreochromis niloticus*). *Fish Shellfish Immunol*. (2021) 119:478–89. doi: 10.1016/J.FSI.2021.10.034

30. Bendary MM, Ibrahim D, Mosbah RA, Mosallam F, Hegazy WAH, Awad NFS, et al. Thymol nanoemulsion: a new therapeutic option for extensively drug resistant foodborne pathogens. *Antibiotics*. (2021) 10:25. doi: 10.3390/ANTIBIOTICS10010025

31. Ibrahim D, Abdelfattah-Hassan A, Badawi M, Ismail TA, Bendary MM, Abdelaziz AM, et al. Thymol nanoemulsion promoted broiler chicken's growth, gastrointestinal barrier and bacterial community and conferred protection against *Salmonella Typhimurium*. *Sci Rep*. (2021) 11:7742. doi: 10.1038/S41598-021-86990-W

32. Shah S, Pal A, Kaushik VK, Devi S. Preparation and characterization of venlafaxine hydrochloride-loaded chitosan nanoparticles and in vitro release of drug. *J Appl Polym Sci*. (2009) 112:2876–87. doi: 10.1002/APP.29807

33. De Blas C, Wiseman J. *The nutrition of the rabbit*. Wallingford, UK: CAB International (1998).

34. Ibrahim D, Abdelfattah-Hassan A, Arisha AH, El-Aziz RMA, Sherief WRIA, Adil SH, et al. Impact of feeding anaerobically fermented feed supplemented with acidifiers on its quality and growth performance, intestinal villi and enteric pathogens of mulard ducks. *Livest Sci*. (2020) 242:104299. doi: 10.1016/J.LIVSCI.2020.104299

35. Ibrahim D, Sewid AH, Arisha AH, Abd El-Fattah AH, Abdelaziz AM, Al-Jabr OA, et al. Influence of *Glycyrrhiza glabra* extract on growth, gene expression of gut integrity, and *Campylobacter jejuni* colonization in broiler chickens. *Front Vet Sci*. (2020) 7:1080. doi: 10.3389/FVETS.2020.612063/BIBTEX

36. Farahat M, Ibrahim D, Kishawy ATY, Abdallah HM, Hernandez-Santana A, Attia G. Effect of cereal type and plant extract addition on the growth performance, intestinal morphology, caecal microflora, and gut barriers gene expression of broiler chickens. *Animal*. (2021) 15:100056. doi: 10.1016/J.ANIMAL.2020.100056

37. Blasco A, Ouhayoun J. Harmonization of criteria and terminology in rabbit meat research. *World Rabbit Sci*. (1996) 1:93–6. doi: 10.4995/wrs.1993.189

38. AOAC International. Spices and other condiments In: W Horwitz, editor. *Official methods of analysis of AOAC international. Vol. II food composition, additives, natural contaminants*. Gaithersburg, MD: AOAC International (2002).

39. Alhawas B, Abd El-Hamid MI, Hassan Z, Ibrahim GA, Neamat-Allah ANF, Rizk El-Ghareeb W, et al. Curcumin loaded liposome formulation: enhanced efficacy on performance, flesh quality, immune response with defense against *Streptococcus agalactiae* in Nile tilapia (Orechromis niloticus). *Fish Shellfish Immunol*. (2023) 138:108776. doi: 10.1016/J.FSI.2023.108776

40. Kishawy ATY, Al-khalaifah HS, Nada HS, Roushdy EM, Zaglool AW, Ismail TA, et al. Black pepper or radish seed oils in a new combination of essential oils modulated broiler chickens' performance and expression of digestive enzymes, lipogenesis, immunity, and autophagy-related genes. *Vet Sci*. (2022) 9:43. doi: 10.3390/vetsci9020043

41. Zangenah S, Güleryüz G, Boräng S, Ullberg M, Bergman P, Özenci V. Identification of clinical Pasteurella isolates by MALDI-TOF—a comparison with VITEK 2 and conventional microbiological methods. *Diagn Microbiol Infect Dis*. (2013) 77:96–8. doi: 10.1016/J.DIAGMICROBIO.2013.06.024

42. Townsend KM, Frost AJ, Lee CW, Papadimitriou JM, Dawkins HJS. Development of PCR assays for species- and type-specific identification of *Pasteurella multocida* isolates. *J Clin Microbiol*. (1998) 36:1096–100. doi: 10.1128/JCM.36.4.1096-1100.1998

43. EUCAST, T.E.C. on A.S.T. Breakpoint tables for interpretation of MICs and zone diameters version 13.0. Available at: https://www.eucast.org/clinical_breakpoints (Accessed November 9, 2023).

44. Sarangi LN, Priyadarshini A, Kumar S, Thomas P, Gupta SK, Nagaleekar VK, et al. Virulence genotyping of *pasteurella multocida* isolated from multiple hosts from India. *Sci World J*. (2014) 2014:1–10. doi: 10.1155/2014/814109

45. Tang X, Zhao Z, Hu J, Wu B, Cai X, He Q, et al. Isolation, antimicrobial resistance, and virulence genes of *Pasteurella multocida* strains from swine in China. *J Clin Microbiol*. (2009) 47:951–8. doi: 10.1128/JCM.02029-08

46. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. *Methods*. (2001) 25:402–8. doi: 10.1006/METH.2001.1262

47. Hammad AM, Aly SS, Hassan HA, Abbas NH, Eltahan A, Khalifa E, et al. Occurrence, phenotypic and molecular characteristics of vancomycin-resistant enterococci isolated from retail raw milk in Egypt. *Foodborne Pathog Dis*. (2022) 19:192–8. doi: 10.1089/FPD.2021.0054

48. Mehrez AZ, El-Harairy MA, Salama MMM. Effect of using blck seed on growth performance and economical efficiency of rabbits. *J Anim Poult Prod*. (2011) 2:13–21. doi: 10.21608/JAPPMU.2011.83332

49. Jamróz D, Kamel C. Plant extracts enhance broiler performance. In non-ruminant nutrition: antimicrobial agents and plant extracts on immunity, health and performance. *J Anim Sci*. (2002) 80:41–6.

50. Rastad A, Sadeghi AA, Chamani M, Shawrang P. Effects of thymoquinone on performance and carcass characteristics of broiler chickens under oxidative stress. *Biol Forum*. (2015) 7:979–85.

51. Amiri N, Afsharmanesh M, Salarmoini M, Meimandipour A, Hosseini SA, Ebrahimnejad H. Effects of nanoencapsulated cumin essential oil as an alternative to the antibiotic growth promoter in broiler diets. *J Appl Poult Res*. (2020) 29:875–85. doi: 10.1016/J.JAPR.2020.08.004

52. El-naby ASA, Al-sagheer AA, Negm SS, Naiel MAE. Dietary combination of chitosan nanoparticle and thymol affects feed utilization, digestive enzymes, antioxidant status, and intestinal morphology of *Oreochromis niloticus*. *Aquaculture*. (2020) 515:734577. doi: 10.1016/j.aquaculture.2019.734577

53. Peterson BC, Bosworth BG, Li MH, Beltran R, Santos GA. Assessment of a phytogenic feed additive (Digestarom P.E.P. MGE) on growth performance, processing yield, fillet composition, and survival of channel catfish. *J World Aquac Soc*. (2014) 45:206–12. doi: 10.1111/JWAS.12103

54. Hannan MA, Rahman MA, Sohag AAM, Uddin MJ, Dash R, Sikder MH, et al. Black cumin (*Nigella sativa* L.): a comprehensive review on phytochemistry, health benefits, molecular pharmacology, and safety. *Nutrients*. (2021) 13:1784. doi: 10.3390/NU13061784

55. Sureshkumar S, Balasubramanian B, Asghar MU, Cano S, Wilk M, Korczynski M, et al. Effect of dietary supplementation of black cumin seeds (*Nigella sativa*) on performance, carcass traits, and meat quality of Japanese quails (*Coturnix coturnix* japonica). *Animals*. (2022) 12:1298. doi: 10.3390/ANI12101298

56. Ibrahim D, Eldemery F, Metwally AS, Abd-Allah EM, Mohamed DT, Ismail TA, et al. Dietary eugenol nanoemulsion potentiated performance of broiler chickens: orchestration of digestive enzymes, intestinal barrier functions and cytokines related gene expression with a consequence of attenuating the severity of *E. coli* O78 infection. *Front Vet Sci*. (2022) 9:847580. doi: 10.3389/FVETS.2022.847580

57. Ibrahim D, Ismail TA, Khalifa E, Abd El-Kader SA, Mohamed DTI, Mohamed DTI, et al. Supplementing garlic nanohydrogel optimized growth, gastrointestinal integrity and economics and ameliorated necrotic enteritis in broiler chickens using a *Clostridium perfringens* challenge model. *Animals*. (2021) 11:2027. doi: 10.3390/ANI11072027

58. Lee KW, Everts H, Kappert HJ, Frehner M, Losa R, Beynen AC. Effects of dietary essential oil components on growth performance, digestive enzymes and lipid metabolism in female broiler chickens. *Br Poult Sci*. (2003) 44:450–7. doi: 10.1080/00071660310000855O8

59. Liu Y, Song M, Che TM, Bravo D, Maddox CW, Pettigrew JE. Effects of capsicum oleoresin, garlic botanical, and turmeric oleoresin on gene expression profile of ileal mucosa in weaned pigs. *J Anim Sci*. (2014) 92:3426–40. doi: 10.2527/JAS.2013-6496

60. Wilhelm Filho D, Tribess T, Gáspari C, Claudio FD, Torres MA, Magalhães ARM. Seasonal changes in antioxidant defenses of the digestive gland of the brown mussel (*Perna perna*). *Aquaculture*. (2001) 203:149–58. doi: 10.1016/S0044-8486(01)00599-3

61. Winston GW, Di Giulio RT. Prooxidant and antioxidant mechanisms in aquatic organisms. *Aquat Toxicol*. (1991) 19:137–61. doi: 10.1016/0166-445X(91)90033-6

62. Peng M, Xu W, Tan P, Du J, Mai K, Zhou H, et al. Effect of dietary fatty acid composition on growth, fatty acids composition and hepatic lipid metabolism in juvenile turbot (*Scophthalmus maximus* L.) fed diets with required n3 LC-PUFAs. *Aquaculture*. (2017) 479:591–600. doi: 10.1016/j.aquaculture.2017.06.032

63. Vickers N. Animal communication: when I'm calling you, will you answer too? *Curr Biol*. (2017) 27:R713–5. doi: 10.1093/cercor/bhx111

64. Martínez-Álvarez RM, Morales AE, Sanz A. Antioxidant defenses in fish: biotic and abiotic factors. *Rev Fish Biol Fish*. (2005) 15:75–88. doi: 10.1007/S11160-005-7846-4

65. Mahfouz R, Sharma R, Sharma D, Sabanegh E, Agarwal A. Diagnostic value of the total antioxidant capacity (TAC) in human seminal plasma. *Fertil Steril*. (2009, 2009) 91:805–11. doi: 10.1016/j.fertnstert.2008.01.022

66. Gaweł S, Wardas M, Niedworok E, Wardas P. Malondialdehyde (MDA) as a lipid peroxidation marker. *Wiad Lek Wars Pol*. (2004) 57:587–95. https://pubmed.ncbi.nlm.nih.gov/15765761/

67. Attia A, Ragheb A, Sylwestrowicz T, Shoker A. Attenuation of high cholesterol-induced oxidative stress in rabbit liver by thymoquinone. *Eur J Gastroenterol Hepatol*. (2010) 22:826–34. doi: 10.1097/MEG.0B013E328336000D

68. Fahmy HM, Saad EAEMS, Sabra NM, El-Gohary AA, Mohamed FF, Gaber MH. Treatment merits of latanoprost/thymoquinone – encapsulated liposome for glaucomatus rabbits. *Int J Pharm*. (2018) 548:597–608. doi: 10.1016/J.IJPHARM.2018.07.012

69. Ibrahim D, Abd El-Hamid MI, Al-Zaban MI, Elhady M, El-Azzouny MM, Elfeky TM, et al. Impacts of fortifying Nile Tilapia (*Oreochromis niloticus*) diet with different strains of microalgae on its performance, fillet quality and disease resistance to *Aeromonas hydrophila* considering the interplay between antioxidant and inflammatory response. *Antioxidants*. (2022) 11:2181. doi: 10.3390/ANTIOX11112181

70. Harphoush S, Wu G, Qiuli G, Zaitoun M, Ghanem M, Shi Y, et al. Thymoquinone ameliorates obesity-induced metabolic dysfunction, improves reproductive efficiency exhibiting a dose-organ relationship. *Syst Biol Reprod Med*. (2019) 65:367–82. doi: 10.1080/19396368.2019.1626933

71. Rahmani AH, Almatroudi A, Babiker AY, Ali Khan A, Alsahli MA. Thymoquinone, an active constituent of black seed attenuates CCl4 induced liver injury in mice via modulation of antioxidant enzymes, PTEN, P53 and VEGF protein. *Open Access Maced J Med Sci*. (2019) 7:311. doi: 10.3889/OAMJMS.2019.050

72. Abdelkhalek N, El-Adl M, El-Ashram A, Othman M, Gadallah H, El-Diasty M, et al. Immunological and antioxidant role of curcumin in ameliorating fipronil toxicity

in Nile tilapia (*Oreochromis niloticus*). *Aquac Res*. (2021) 52:2791–801. doi: 10.1111/ARE.15131

73. Alam RTM, Fawzi EM, Alkhalf MI, Alansari WS, Aleya L, Abdel-Daim MM. Anti-inflammatory, immunomodulatory, and antioxidant activities of allicin, norfloxacin, or their combination against *Pasteurella multocida* infection in male New Zealand rabbits. *Oxidative Med Cell Longev*. (2018) 2018:1–10. doi: 10.1155/2018/1780956

74. El-Abasy M, Abdelhady D, Kamel T, Shukry M. Ameliorative effect of coconut oil on hematological, immunological and serum biochemical parameters in experimentally infected rabbits. *Alexandria J Vet Sci*. (2016) 50:36–48. doi: 10.5455/ajvs.229021

75. Ramasamy K, Abdullah N, Wong MCVL, Karuthan C, Ho YW. Bile salt deconjugation and cholesterol removal from media by Lactobacillus strains used as probiotics in chickens. *J Sci Food Agric*. (2010) 90:65–9. doi: 10.1002/JSFA.3780

76. Shin SK, Ha TY, McGregor RA, Choi MS. Long-term curcumin administration protects against atherosclerosis via hepatic regulation of lipoprotein cholesterol metabolism. *Mol Nutr Food Res*. (2011) 55:1829–40. doi: 10.1002/MNFR.201100440

77. Singh PK, Kumar A. Effect of dietary black cumin (*Nigella sativa*) on the growth performance, nutrient utilization, blood biochemical profile and carcass traits in broiler chickens. *Anim Nutr Feed Technol*. (2018) 18:409–19. doi: 10.5958/0974-181X.2018.00038.0

78. Faramarzi S, Bozorgmehrifard MH, Khaki A, Moomivand H, Ezati MS, Rasoulinezhad S, et al. Study on the effect of *Thymus vulgaris* essential oil on humoral immunity and performance of broiler chickens after La Sota vaccination. *Ann Biol Res*. (2013) 4:290–4.

79. Krishan G, Narang A. Use of essential oils in poultry nutrition: a new approach. *J Adv Vet Anim Res*. (2014, 2014) 1:156–62. doi: 10.5455/javar.2014.a36

80. Magnadottir B. Immunological control of fish diseases. *Mar Biotechnol*. (2010) 12:361–79. doi: 10.1007/S10126-010-9279-X/METRICS

81. Salinas I, Zhang YA, Sunyer JO. Mucosal immunoglobulins and B cells of teleost fish. *Dev Comp Immunol*. (2011) 35:1346–65. doi: 10.1016/J.DCI.2011.11.009

82. Li SY, Ru YJ, Liu M, Xu B, Péron A, Shi XG. The effect of essential oils on performance, immunity and gut microbial population in weaner pigs. *Livest Sci*. (2012) 145:119–23. doi: 10.1016/J.LIVSCI.2012.01.005

83. Elmowalid G, Amar AM, Ahmad AAM. *Nigella sativa* seed extract: 1. Enhancement of sheep macrophage immune functions in vitro. *Res Vet Sci*. (2013) 95:437–43. doi: 10.1016/J.RVSC.2013.02.015

84. Abd El-Hamid MI, Ibrahim D, Hamed RI, Nossieur HH, Elbanna MH, Baz H, et al. Modulatory impacts of multi-strain probiotics on rabbits' growth, nutrient transporters, tight junctions and immune system to fight against *Listeria monocytogenes* infection. *Animals*. (2022) 12:2082. doi: 10.3390/ANI12162082

85. Zhang D, Gan RY, Zhang JR, Farha AK, Li H, Zhu F, et al. Antivirulence properties and related mechanisms of spice essential oils: a comprehensive review. *Compr Rev Food Sci Food Saf*. (2020) 19:1018–55. doi: 10.1111/1541-4337.12549

86. Miller LC, O'Loughlin CT, Zhang Z, Siryaporn A, Silpe JE, Bassler BL, et al. Development of potent inhibitors of pyocyanin production in *pseudomonas aeruginosa*. *J Med Chem*. (2015) 58:1298–306. doi: 10.1021/JM5015082/SUPPL_FILE/JM5015082_SI_001.PDF

87. Patra AK, Amasheh S, Aschenbach JR. Modulation of gastrointestinal barrier and nutrient transport function in farm animals by natural plant bioactive compounds – a comprehensive review. *Crit Rev Food Sci Nutr*. (2018) 59:3237–66. doi: 10.1080/10408398.2018.1486284

88. Tabler TW, Greene ES, Orlowski SK, Hiltz JZ, Anthony NB, Dridi S. Intestinal barrier integrity in heat-stressed modern broilers and their ancestor wild jungle fowl. *Front Vet Sci*. (2020) 7:538427. doi: 10.3389/FVETS.2020.00249/BIBTEX

89. Salem ML. Immunomodulatory and therapeutic properties of the *Nigella sativa* L. seed. *Int Immunopharmacol*. (2005) 5:1749–70. doi: 10.1016/J.INTIMP.2005.06.008

90. Kapan M, Tekin R, Onder A, Firat U, Evliyaoglu O, Taskesen F, et al. Thymoquinone ameliorates bacterial translocation and inflammatory response in rats with intestinal obstruction. *Int J Surg*. (2012) 10:484–8. doi: 10.1016/J.IJSU.2012.06.006

91. Kayamuro H, Yoshioka Y, Abe Y, Arita S, Katayama K, Nomura T, et al. Interleukin-1 family cytokines as mucosal vaccine adjuvants for induction of protective immunity against influenza virus. *J Virol*. (2010) 84:12703–12. doi: 10.1128/JVI.01182-10

92. Drolia R, Tenguria S, Durkes AC, Turner JR, Bhunia AK. Listeria adhesion protein induces intestinal epithelial barrier dysfunction for bacterial translocation. *Cell Host Microbe*. (2018) 23:470–484.e7. doi: 10.1016/J.CHOM.2018.03.004

93. Wang J, Zeng Y, Wang S, Liu H, Zhang D, Zhang W, et al. Swine-derived probiotic *Lactobacillus plantarum* inhibits growth and adhesion of enterotoxigenic Escherichia coli and mediates host defense. *Front Microbiol*. (2018) 9:354038. doi: 10.3389/FMICB.2018.01364/BIBTEX

94. Wei X, Zhang Y, Zhou H, Tian F, Ni Y. Antimicrobial activities and in vitro properties of cold-adapted Lactobacillus strains isolated from the intestinal tract of cold water fishes of high latitude water areas in Xinjiang, China. *BMC Microbiol*. (2019) 19:247. doi: 10.1186/S12866-019-1623-3

95. Al-Mufarrej SI. Immune-responsiveness and performance of broiler chickens fed black cumin (*Nigella Sativa* L.) powder. *J Saudi Soc Agric Sci*. (2014) 13:75–80. doi: 10.1016/J.JSSAS.2013.01.006

96. Amin B, Hosseinzadeh H. Black cumin (*Nigella sativa*) and its active constituent, thymoquinone: an overview on the analgesic and anti-inflammatory effects. *Planta Med*. (2016) 82:8–16. doi: 10.1055/S-0035-1557838/ID/RY1283-1/BIB

frontiers | Frontiers in Veterinary Science

TYPE Original Research
PUBLISHED 19 January 2024
DOI 10.3389/fvets.2023.1330841

OPEN ACCESS

EDITED BY
Kai Wang,
Chinese Academy of Agricultural Sciences (CAAS), China

REVIEWED BY
Miaomiao Wu,
Hunan Agricultural University, China
Jun Zhang,
Northwest A&F University, China

*CORRESPONDENCE
Zhe Sun
✉ sunzhe198615@163.com
Guixin Qin
✉ qgx@jlau.edu.cn

RECEIVED 31 October 2023
ACCEPTED 14 December 2023
PUBLISHED 19 January 2024

CITATION
Aschalew ND, Zhang L, Wang Z, Xia Y, Yin G, Dong J, Zhen Y, Zhang X, Wang T, Sun Z and Qin G (2024) Effects of yeast culture and oxalic acid supplementation on *in vitro* nutrient disappearance, rumen fermentation, and bacterial community composition.
Front. Vet. Sci. 10:1330841.
doi: 10.3389/fvets.2023.1330841

Effects of yeast culture and oxalic acid supplementation on *in vitro* nutrient disappearance, rumen fermentation, and bacterial community composition

Natnael D. Aschalew[1,2], Longyu Zhang[1], Ziyuan Wang[1], Yuanhong Xia[1], Guopei Yin[1], Jianan Dong[1], Yuguo Zhen[1,3], Xuefeng Zhang[1,3], Tao Wang[1,3], Zhe Sun[1,3,4]* and Guixin Qin[1,3]*

[1]Key Laboratory of Animal Nutrition and Feed Science of Jilin Province, Key Laboratory of Animal Production Product Quality and Security Ministry of Education, JLAU-Borui Dairy Science and Technology R&D Center, College of Animal Science and Technology, Jilin Agricultural University, Changchun, China, [2]College of Agriculture and Environmental Science, Dilla University, Dilla, Ethiopia, [3]Postdoctoral Scientific Research Workstation, Feed Engineering Technology Research Center of Jilin Province, Changchun Borui Science and Technology Co., Ltd., Changchun, China, [4]College of Life Sciences, Engineering Research Center of Bioreactor and Pharmaceutical Development, Ministry of Education, Jilin Agricultural University, Changchun, China

Hemicellulose is an important polysaccharide in ruminant nutrition, but it has not been studied as thoroughly as cellulose. Further research is needed to explore supplements that can improve its digestibility and ruminal buffering effects. Our previous research demonstrated the efficacy of oxalic acid (OA) as an essential nutrient in yeast culture (YC) for improving rumen fermentation performance. Consequently, we conducted *in vitro* rumen digestion experiments to examine the effects of YC and OA on rumen fermentation and bacterial composition. Two diets containing different levels of hemicellulose were formulated: diet 1 with 10.3% and diet 2 with 17% hemicellulose. Three levels of YC (0.00, 0.625, and 1.25 g/kg) and three doses of OA (0.0, 0.4, and 0.8 g/kg, DM) were added into each diet with a 3 × 3 factorial design. A comprehensive assessment was conducted on a total of 18 experimental treatments at fermentation periods of 0, 6, 12, 24, and 48 h. In the first experiment (diet 1), the supplementation of YC, OA, and their interaction significantly increased *in vitro* DM disappearance (IVDMD) and NDF disappearance (IVNDFD; $p < 0.001$). In the second experiment (diet 2), the supplementation of OA and the interaction between YC and OA ($p < 0.001$) increased IVDMD and IVCPD, but had no significant effects on IVNDFD. The interactions of YC and OA significantly increased ammonia nitrogen ($p < 0.001$). The production of acetic acid, propionic acid, and total volatile fatty acids (TVFA), and pH levels were significantly higher in treatments supplemented with YC and OA ($p < 0.001$). YC and OA in both diets significantly altered the rumen bacterial community leading to increased Shannon and Simpson diversity indices ($p < 0.001$). In both diets, OA supplementation significantly increased the relative abundance of the phylum *Bacteroidetes* and *Prevotella* genus. The result also showed a positive correlation between the *Prevotella* and *Selenomonas* genera with IVDMD, IVNDFD, propionic acid, and TVFA production, suggesting that these dominant bacteria enhanced nutrient disappearance in the rumen. In conclusion, adding YC and OA resulted in modifications to the bacterial community's composition and diversity, and improved nutrient disappearance. These changes indicate improved rumen fermentation efficiency, which is promising for future *in vivo* studies.

KEYWORDS

rumen fermentation, bacterial community composition, *in vitro* disappearance, oxalic acid, volatile fatty acids, yeast culture

1 Introduction

The increasing demand for intensive sheep production has led to the strategic use of expensive feed ingredients to boost productivity. As a result, producers are providing grains, grain processing byproducts, and concentrates to their sheep in order to achieve higher returns (1). This primarily impacts the animals' health by reducing the pH levels in their rumen, resulting in subacute ruminal acidosis and other metabolic disorders. In response, feed manufacturing companies have recently begun incorporating corn bran into the total mixed ration (TMR) due to its high hemicellulose content (2), as it is the richest source of hemicellulose.

Hemicellulose is a polysaccharide comprising all cell wall constituents except cellulose and pectin (3), that plays a significant role in ruminant nutrition as an important component of fiber, but it has not been as extensively researched as cellulose. Anaerobic microorganisms, including hemicellulolytic and nonhemicellulolytic microbes, as well as extracellular enzymes can break down and ferment hemicellulose in the rumen to produce volatile fatty acids (VFA) and stabilize the rumen ecosystem of the host (4). Currently, there is a lack of research on the effectiveness of supplements in enhancing hemicellulose digestibility and ruminal buffering effects. Consequently, in order to enhance hemicellulose digestion, it is necessary to design and implement other alternatives.

Saccharomyces cerevisiae is a unicellular fungus commonly added to animal feed as yeast culture (YC) or live yeast (LY) supplement that facilitates carbohydrate fermentation (5). Adding YC to the ruminant's diet can stimulate fiber digestion (6) and change fermentation by improving the rumen microflora composition (7, 8), but it is not a nutrient source (9). Plata and Bárcena-Gama (10) conducted a study showing that adding YC to the diet could facilitate the breakdown of hemicellulose in the rumen. On the other hand, studies have shown that adding YC to the diet can reduce ammonia nitrogen concentration (11, 12) and rumen microbiome (13).

Oxalic acid (OA) is a low molecular weight organic acid that is synthesized by several organisms, including fungi, bacteria, plants, and animals (14, 15) and is most abundantly found in the environment (14). Roughage feeds supplemented with organic acids improved rumen fermentation efficiency and fiber degradation (16, 17). Other researchers found that OA decreased DM digestibility, ammonia nitrogen concentration, and butyrate production and had a negative impact on rumen microbial activities (18). However, it is still being produced sufficiently in the environment (14, 15, 19). Therefore, finding options to effectively use this potentially available organic acid especially as a feed supplement is important for livestock producers, feed processing companies and reagents manufacturing industries. Furthermore, we previously reported that oxalic acid (OA) plays a crucial role in enhancing rumen fermentation and nutrient digestion in YC. Consequently, the supplementation levels of YC and OA were determined based on the recommendations derived from these findings (20). Hence, this research hypothesized that combining YC and OA may enhance rumen fermentation efficiency, nutrient digestion, and alter bacterial community composition. Therefore, two 3 × 3 factorial *in vitro* experiments were designed to examine the effects of YC and OA on nutrient disappearance, rumen fermentation parameters, and bacterial community composition.

2 Materials and methods

2.1 Animals

Four short-tailed, male, Han sheep (average live weight, 36 ± 3.52 kg, *mean* ± *SD*) were used as donors of rumen fluid. The sheep were fitted with a round-shaped and flexible rumen cannula. They were fed pelleted concentrate and oat hay in a 1:1 concentrate to forage ratio twice daily at 07:30 AM and 05:30 PM and had free access to fresh, clean water.

2.2 Experimental treatment formulation and design

Based on the fiber requirements of sheep (21) specifically neutral detergent fiber (NDF) and acid detergent fiber (ADF) levels, two hemicellulosic diets were formulated. These diets were named diet 1 and diet 2, their hemicellulose levels were 10.3% and 17%, respectively. The two diets were supplemented with 3 levels of YC (0.000, 0.625, and 1.250 g/kg) and 3 doses of OA (0.0, 0.4, and 0.8 g/kg of feed, DM basis) in a 3 × 3 factorial design. A total of 18 treatments (9 in each diet) were designed and evaluated. The YC and OA levels are expressed as grams per kilogram of feed on a dry matter basis. Table 1 presents detailed information about the experimental treatments in both diets. The ingredients and nutritional chemical composition of the two diets are shown in Table 2. The YC was manufactured under the controlled microenvironment of the JLAU-Borui Dairy Science and Technology R&D Center, and the oxalic acid was purchased from the RHAWN reagents supplying company (Shanghai, China).

2.3 Nutritional chemical composition analyses

The dried feed samples were ground to pass through a 1 mm screen using a Wiley mill (Thomas Scientific, Swedesboro, NJ) for all nutritional composition analyses. The chemical composition was determined using AOAC (22) methods: method 934.01 for DM, method 942.05 for ash, and method 984.13 for N determination. The acid detergent fiber (ADF) and neutral detergent fiber (NDF) contents were evaluated using the ANKOM^{200} Fiber Analyzer (ANKOM

10.3389/fvets.2023.1330841

TABLE 1 The experimental treatments with levels of supplements used in the study.

Diets	Treatments	Yeast culture levels (g/kg)	Oxalic acid levels (g/kg)
Diet 1 (10.3% hemicellulose)	C	-	-
	y	0.625	-
	Y	1.25	-
	o	-	0.40
	O	-	0.80
	yo	0.625	0.40
	yO	0.625	0.80
	Yo	1.25	0.40
	YO	1.25	0.80
Diet 2 (17% hemicellulose)	ctrl	-	-
	L	0.625	-
	H	1.25	-
	l	-	0.40
	h	-	0.80
	Ll	0.625	0.40
	Lh	0.625	0.80
	Hl	1.25	0.40
	Hh	1.25	0.80

Where: C = Diet 1 (10.3% hemicellulose); y = diet 1 with low yeast culture; Y = Diet 1 with high yeast culture; o = diet 1 with low oxalic acid; O = diet 1 with high oxalic acid; yo = diet 1 with low yeast culture and low oxalic acid; yO = diet 1 with low yeast culture and high oxalic acid; Yo = diet 1 with high yeast culture and low oxalic acid; YO = diet 1 with high yeast culture and high oxalic acid; ctrl = Diet 2 (17% hemicellulose); L = diet 2 with low yeast culture; H = diet 2 with high yeast culture; l = diet 2 with low oxalic acid; h = diet 2 with high oxalic acid; Ll = diet 2 with low yeast culture and low oxalic acid; Lh = diet 2 with low yeast culture and high oxalic acid; Hl = diet 2 with high yeast culture and low oxalic acid; and Hh = diet 2 with high yeast culture and high oxalic acid. The hemicellulose, yeast culture, and oxalic acid levels are expressed on a dry matter basis.

Technology, Macedon, NY, United States). Reagents such as sodium sulfite and α-amylase (ANKOM Technology) were used in the NDF analysis, while sulfuric acid was used for ADF extraction. The hemicellulose level was computed using the difference between the NDF and ADF levels.

2.4 *In vitro* nutrient disappearance

To measure the nutrient disappearance, an ANKOM DAISY^{II} incubator (ANKOM Technology, Macedon, NY, United States) was used. Before the morning feeding, the rumen fluid was collected from four rumen-cannulated sheep and filtered through four layers of cheesecloth. Subsequently, a total volume of 1,200 mL of buffer solution (23) and 400 mL of rumen fluid were added to each digestion jar. One gram of experimental treatment was measured and added to the F-57 ANKOM filter bag. An empty F-57 filter bag was included in each phase for the correction factor. The filter bags were heat-sealed and placed directly into the digestion jars. The jars were purged with CO_2 for 30 s to create an anaerobic environment, then tightly covered and incubated for 0, 6, 12, 24, and 48 h in an ANKOM DAISY^{II} incubator. Each treatment was replicated 15 times (5 replications for each parameter) at each fermentation time to measure *in vitro* dry matter (IVDMD), neutral detergent fiber (IVNDFD), and crude protein (IVCPD) disappearance.

A total of 1,350 ANKOM F-57 filter bags were used for measuring nutrient disappearance. After fermentation, each bag was rinsed with cold tap water until the water ran clear and was agitated to remove any remaining water. Then, each bag was air-dried and oven dried at 55°C for 72 h with bags being turned upside down every 12 h to ensure uniform drying. The weight of each dried bag was then measured. At each fermentation time, some bags were used for further NDF and CP analyses using the methods mentioned in section 2.3. Finally, the following mathematical formulas were used to calculate *in vitro* nutrient disappearance (24, 25).

$$\mathrm{IVDMD}\left(\%\ \mathrm{of\ DM}\right)=\left(\frac{\begin{array}{l}\text{Initial Sample DM weigh}\left(\mathrm{g}\right)\\ -\text{Sample weight residue}\left(\mathrm{g}\right)\end{array}}{\text{Initial Sample DM weight}\left(\mathrm{g}\right)}\right)\times 100$$

$$\mathrm{IVNDFD}\left(\%\ \mathrm{of\ NDF}\right)=\left(\frac{\begin{array}{l}\text{NDF before fermentation}\left(\mathrm{g}\right)\\ -\text{NDF residue}\left(\mathrm{g}\right)\end{array}}{\text{NDF before fermentation}}\right)\times 100$$

$$\mathrm{IVCPD}\left(\%\ \mathrm{of\ CP}\right)=\left(\frac{\begin{array}{l}\text{CP before fermentation}\left(\mathrm{g}\right)\\ -\text{CP residue}\left(\mathrm{g}\right)\end{array}}{\text{CP before fermentation}\left(\mathrm{g}\right)}\right)\times 100$$

10.3389/fvets.2023.1330841

TABLE 2 Ingredients and nutritional composition of the experimental diets (%, DM).

Item	Diet 1 (10.3% hemicellulose)	Diet 2 (17% hemicellulose)
Ingredients		
Corn	38.12	35.71
Soybean meal	16.18	13.27
Corn germ meal (spray)	6.18	6.12
Corn bran	10.30	31.63
Peanut shell	15.46	0.00
Corn oil	0.36	0.00
Bentonite	4.12	4.08
Sugar	4.12	4.08
Premix[a]	5.15	5.10
Total	100	100
Nutritional compositions (% DM)		
Dry matter	90.5	90.3
Digestible energy[b] MJ/kg	11.6	12.2
Crude protein	13.8	13.9
Crude fat	2.7	2.8
Crude fiber	13.1	6.7
Ash	14.6	12.4
Starch	30.1	30.3
Neutral detergent fiber	26.0	25.8
Acid detergent fiber	15.7	8.8
Hemicellulose[c]	10.3	17
Calcium	0.6	0.6
Phosphorus	0.4	0.4

Where: [a]Premix composition (per kilogram): $FeSO_4$, 8,453 mg; $CuSO_4.5H_2O$, 1,480 mg; $MnSO_4$, 13,241 mg; $ZnSO_4.5H_2O$, 8,294 mg; $CoCl_2$, 16 mg; KI, 30 mg; Na_2SeO_3 (1% Se), 377 mg; vitamin A, 755 IU; vitamin D, 113 IU; vitamin E, 887 IU. [b]Calculated based on Ruminants (21). [c]Calculated by the difference between NDF and ADF.

2.5 Rumen fermentation parameters

All samples used to measure *in vitro* ruminal efficiency parameters such as volatile fatty acids (VFA), ammonia nitrogen (NH_3-N), and ruminal microbiome composition were processed using the ANKOM^{RF} gas production system. Two grams of the experimental treatment were added to 250 mL ANKOM bottles. The mixed buffer solution and rumen fluid were purged with CO_2 at each step, added to the sample-containing bottles, and placed inside an incubator shaker with an internal temperature of 39°C and a speed of 80 rpm (Tianjin Honor Instrument Shaker Co. Ltd., Tianjin, China). After each incubation time (0, 6, 12, 24, 48 h) was completed, the pH values were immediately measured. Then, a 2 mL aliquot of fluid was collected and preserved at −20°C until VFA and NH_3-N tests were performed. The samples for microbial composition analysis were stored at −80°C. Specific procedures for each rumen fermentation parameter are detailed below.

2.5.1 Ruminal fluid pH

The ruminal fluid pH was measured with a Sanxin MP523-04 digital pH meter (Shanghai Sanxin Instrumentation, Inc., Shanghai, China) according to the manufacturer's guidelines.

2.5.2 Volatile fatty acids analysis

The analysis of volatile fatty acids (VFA) was carried out using an Agilent 7,890 A gas chromatograph (Agilent Technologies, Santa Clara, California, United States) and a 50 m (internal diameter 0.32 mm) CP-Wax Chrompack silica-fused capillary column (Varian, Palo Alto, California, United States). The initial and final oven temperatures were set at 65 and 195°C, respectively, with helium used as the carrier gas. The detector and injector temperatures were carefully calibrated to 250°C, and the injector volume was precisely configured to 1 µL. The samples were centrifuged at 10,000 × *g* at 4°C for 10 min. The centrifuged sample was then transferred to another 2 mL EP tube, and 0.2 mL of metaphosphoric acid was added. The mixture was refrigerated for approximately 3 h, then removed and centrifuged again at 10,000 × *g* at 4°C for 10 min to obtain the supernatant. A 1 mL of the supernatant sample was placed into the GC beaker and processed by gas chromatography.

2.5.3 Ammonia nitrogen concentration

The NH_3-N concentration was measured using the Chaney and Marbach (26) method, with a Cary Series UV–Vis Spectrophotometer connected to a computer running Cary Series analysis software (Agilent Technologies, California, United States). In detail, the samples were centrifuged at 4,000 × *g* for 10 min at 4°C, and 0.2 mL of

supernatant was combined with 1.8 mL of distilled water and 8 mL of 0.2 M hydrochloric acid in 15-mL centrifuge tubes. From each 15-mL tube, 0.4 mL was transferred to a 10-mL tube, and 2 mL of each solution A and B were added. Lastly, analyzed by a Cary Series UV–Vis Spectrophotometer. The measured extinction values were substituted into the regression formula, and the results were multiplied by a dilution factor to determine the final NH_3-N concentration in the sample.

2.5.4 Cumulative gas production

The ANKOM^{RF} gas production system is designed to measure the gas pressure in psi. The gas production for each bottle was measured and recorded over time. The data was then converted into moles of gas using the 'ideal' gas law and further converted into milliliters (mL) of gas using Avogadro's law.

The ideal gas law:

$$n = p\left(\frac{V}{RT}\right)$$

where: n—is the quantity of gas generated, measured in moles (mol).

p—pressure in kilo pascal (kPa).

V—the glass bottle's head-space volume, expressed in liters (L).

T—the internal temperature in Kelvin (K).

R—gas constant (8.314472 L·kPa·K^{-1}·mol^{-1}).

Avogadro's law states that, under standard conditions, 1 mole will fill 22.4 L at 273.15°K and 101.325 kPa (1 psi = 6.894757293 kilopascal). Therefore, the following formula can be used to convert a gas mole measurement to milliliters (ANKOM manual):

$$Gas\ produced\ (mL) = n \times 22.4 \times 1000$$

2.6 Rumen microbiota composition analysis

2.6.1 DNA sequencing

Sequencing, library preparations, and taxonomic analysis were performed by Shanghai Personal Biotechnology Co. Ltd. (Shanghai, China). A total of 216 ruminal fluid samples produced 30,196,306 original sequences, with 19,625,098 (90,857 sequences per sample) identified as high-quality bacterial sequences. The 16S ribosomal RNA (rRNA) gene was amplified using the polymerase chain reaction (PCR) method. Barcoded tags and the widely used bacterial primers 341F (5′-CCTAYGGGRBGCASCAG-3′) and 806R (5′-GGACTACNN GGGTATCTAAT-3′) were used in the amplification process (27). The V4 region of the gene was amplified from genomic DNA and the resulting amplicons were prepared for sequencing using Illumina's MiSeq technology (28).

2.6.2 Taxonomic composition analysis

Based on the distribution of OTU in different samples, the levels of alpha diversity in each sample were assessed. An association network was constructed for each sample, and the topological index was calculated to identify the key species. The composition and distribution of each sample at phylum, class, order, family, genus, and species levels were analyzed visually and statistically using QIIME2 in the R programming language. This analysis involved removing singleton data points and statistically summarizing all the information in a table. To fully evaluate the richness of the microbial community alpha diversity, the Chao1 (29) index, as well as the Shannon (30) and Simpson (31) indices, were used and analyzed. Additionally, Principal Coordinates Analysis (PCoA) of beta diversity was conducted to compare the distance between the two samples.

2.7 Statistical data analysis

The MIXED procedure of SAS (version 9.4; SAS Institute Inc., Cary, NC) was used to analyze all variables. For statistical analyses, treatments in the two hemicellulosic diets were analyzed and computed separately. A two-way ANOVA was used, and the sources of variation between treatments were taken as fixed effect variables and incubation time as a random effect. The significance level was declared at $p \leq 0.05$ for Duncan's New Multiple Range Test (MRT), which was used to examine the significant variables for multiple comparisons of the mean values. Microbiome community composition data were analyzed using several tools inside R programming language software (32), and the model for both experiments was as follows:

$$Y_{ijk} = \mu + Y_i + O_j + (YO)_{ij} + e_{ijk}$$

Where: Y_{ijk} = the dependent variables; μ = overall mean; Y_i = yeast culture effect ($_i$ = 1–3); O_j = oxalic acid effect ($_j$ = 1–3); $(YO)_{ij}$ = effect due to YC and OA interaction; and e_{ijk} = random residual error.

3 Results

3.1 *In vitro* nutrient disappearance

Figure 1 illustrates the changes that occurred in the amounts of *in vitro* dry matter (IVDMD), neutral detergent fiber (IVNDFD), and crude protein (IVCPD). In diet 1, supplementation with YC, OA, and their interaction increased IVDMD at 24 and 48 h ($p < 0.001$; Figure 1A). At 48 h, YC ($p = 0.05$), OA ($p < 0.01$), and their combinations ($p < 0.01$) also increased IVNDFD (Figure 1B). Additionally, at 48 h, OA ($p < 0.05$) and the YC and OA interactions ($p < 0.01$) significantly increased IVCPD (Figure 1C). In diet 2, YC, OA, and their interaction significantly increased IVDMD ($p < 0.001$; Figure 1D). YC and OA supplementation reduced IVNDFD at 12 h ($p < 0.001$; Figure 1E). Furthermore, all supplemented treatments showed higher IVCPD than the control ($p < 0.001$; Figure 1F). These results indicated that YC, OA, and their interactions may enhance *in vitro* nutrient disappearance.

3.2 Rumen fermentation parameters

In diet 1, YC, OA, and their combination significantly increased the ruminal fluid pH (Figure 2A). The pH value of the control was lower than that of the supplemented treatments at 24 and 48 h

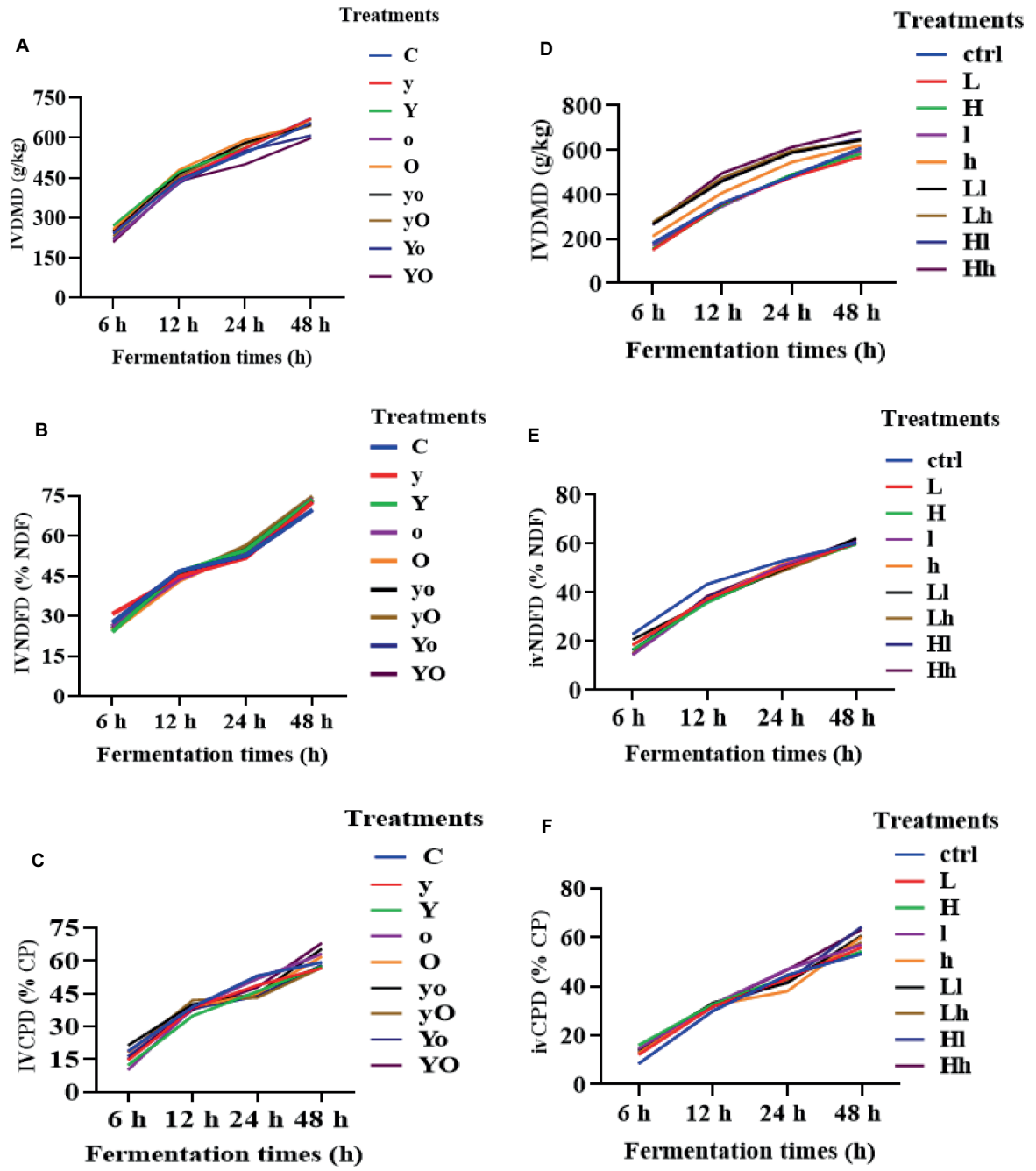


FIGURE 1
The *in vitro* nutrient disappearance of diet 1 treatments DM **(A)**, NDF **(B)**, CP **(C)**, and diet 2 **(D–F)** respectively. Where: C = Diet 1 (10.3% hemicellulose); y = diet 1 with low yeast culture; Y = diet 1 with high yeast culture; o = diet 1 with low oxalic acid; O = diet 1 with high oxalic acid; yo = diet 1 with low yeast culture and low oxalic acid; yO = diet 1 with low yeast culture and high oxalic acid; Yo = diet 1 with high yeast culture and low oxalic acid; YO = diet 1 with high yeast culture and high oxalic acid; ctrl = Diet 2 (17% hemicellulose); L = diet 2 with low yeast culture; H = diet 2 with high yeast culture; l = diet 2 with low oxalic acid; h = diet 2 with high oxalic acid; Ll = diet 2 with low yeast culture and low oxalic acid; Lh = diet 2 with low yeast culture and high oxalic acid; Hl = diet 2 with high yeast culture and low oxalic acid; Hh = diet 2 with high yeast culture and high oxalic acid. IVDMD = *in vitro* dry matter disappearance; IVNDFD = *in vitro* neutral detergent fiber disappearance; IVCPD = *in vitro* crude protein disappearance.

($p < 0.05$). The YC and OA interactions also significantly increased NH_3-N ($p < 0.001$), while individual supplementation with either YC or OA reduced the NH_3-N concentration (Figure 2B). Additionally, the YC and OA interactions reduced the cumulative gas production at 24 h ($p = 0.05$; Figure 2C). In diet 2, individual supplementation of OA significantly increased the ruminal fluid pH ($p < 0.001$; Figure 2D). The YC and OA interactions also increased the NH_3-N concentration at 24 h ($p < 0.001$; Figure 2E).

In diet 1, the supplemented groups showed significantly higher levels of acetic acid ($p < 0.05$, 12 h), TVFA ($p < 0.001$, 24

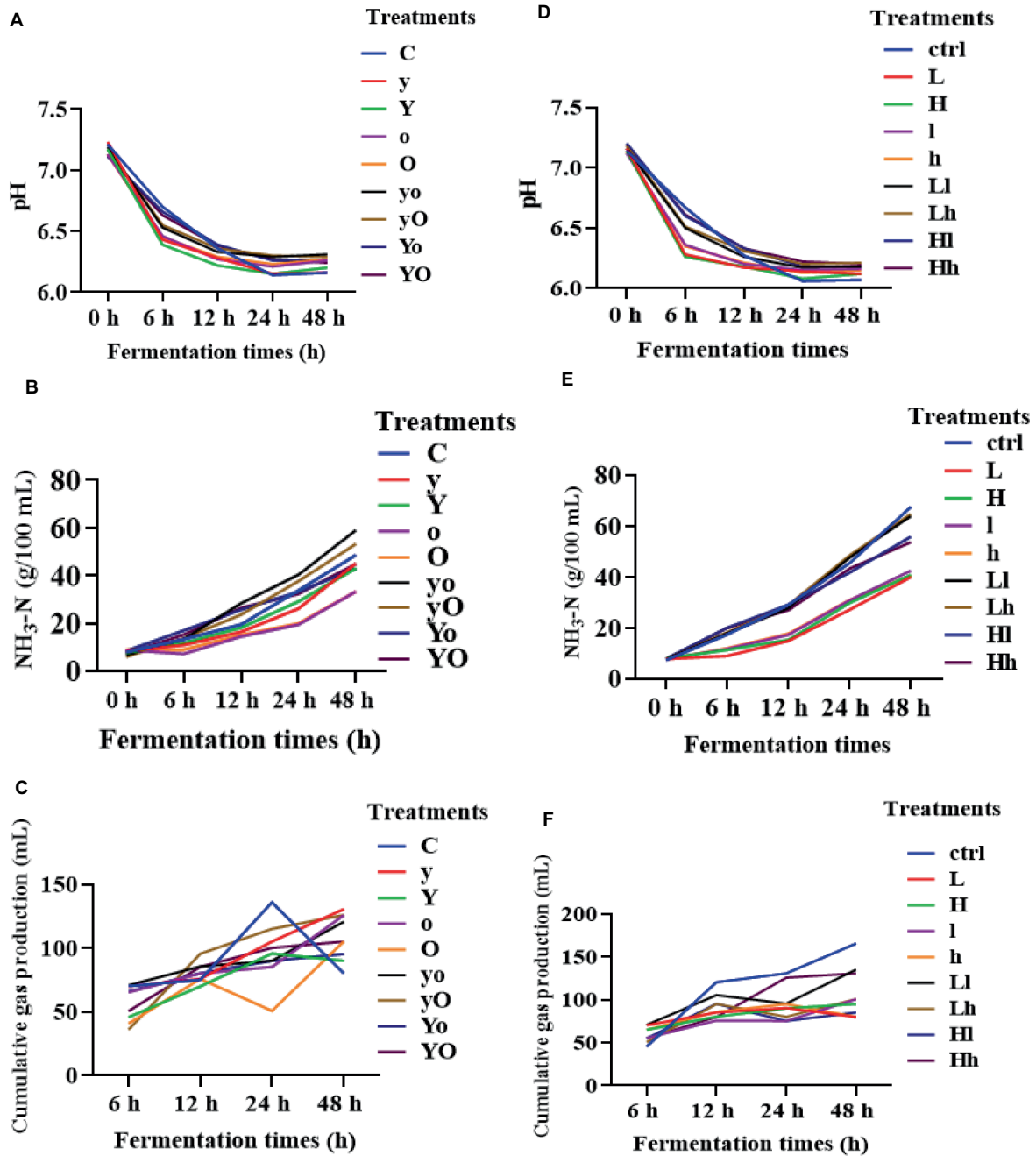


FIGURE 2
Rumen fermentation parameters of diet 1 pH **(A)**, NH_3-N **(B)**, cumulative gas production **(C)**, and diet 2 **(D–F)** respectively. Where: C = Diet 1 (10.3% hemicellulose); y = diet 1 with low yeast culture; Y = diet 1 with high yeast culture; o = diet 1 with low oxalic acid; O = diet 1 with high oxalic acid; yo = diet 1 with low yeast culture and low oxalic acid; yO = diet 1 with low yeast culture and high oxalic acid; Yo = diet 1 with high yeast culture and low oxalic acid; YO = diet 1 with high yeast culture and high oxalic acid; ctrl = Diet 2 (17% hemicellulose); L = diet 2 with low yeast culture; H = diet 2 with high yeast culture; l = diet 2 with low oxalic acid; h = diet 2 with high oxalic acid; Ll = diet 2 with low yeast culture and low oxalic acid; Lh = diet 2 with low yeast culture and high oxalic acid; Hl = diet 2 with high yeast culture and low oxalic acid; Hh = diet 2 with high yeast culture and high oxalic acid; NH_3-N = ammonia nitrogen.

and 48 h), and propionic acid ($p < 0.001$; Table 3). In diet 2, treatments supplemented with OA produced higher levels of acetic acid compared to YC alone and their interaction ($p < 0.001$). Table 4 indicates that treatments supplemented with lower levels of OA, and both lower and higher levels of YC increased TVFA production ($p < 0.05$). additionally, supplementation with YC and OA in this diet significantly reduced the cumulative gas production during fermentation (Figure 2F).

All supplemented treatments in both diets produced higher levels of propionic and total volatile fatty acids compared to the control ($p < 0.001$). The acetic to propionic acid ratio was higher in the control treatments ($p < 0.001$). These results suggest that supplementation

10.3389/fvets.2023.1330841

TABLE 3 Effects of YC and OA supplementation in diet 1 on major VFA production (mmol/L).

Item	Time	Diet 1	Yeast culture (YC)		Oxalic acid (OA)		Interactions				SEM	*p* value		
		C	y	Y	o	O	yo	yO	Yo	YO		YC	OA	YC × OA
Acetic acid	0 h	15.80	15.22	14.45	14.77	15.40	15.89	17.20	16.43	16.75	0.31	0.593	0.268	0.494
	6 h	33.14^{ab}	32.21^{ab}	31.83^{a}	41.20^{d}	38.66^{cd}	33.32^{ab}	31.57^{a}	33.39^{ab}	35.94^{bc}	0.66	<0.001	<0.001	<0.001
	12 h	44.01^{ab}	39.97^{ab}	38.24^{a}	43.00^{ab}	46.46^{b}	43.31^{ab}	40.93^{ab}	42.17^{ab}	43.24^{ab}	0.58	0.012	0.049	0.157
	24 h	58.53^{e}	44.97^{ab}	44.26^{a}	51.30^{cd}	53.43^{de}	51.21^{cd}	47.83^{abcd}	50.19^{bcd}	47.11^{abc}	0.89	<0.001	0.207	<0.001
	48 h	70.95^{b}	49.95^{a}	56.22^{a}	56.37^{a}	57.26^{a}	57.70^{a}	53.02^{a}	55.63^{a}	54.54^{a}	1.16	<0.001	0.018	<0.001
Propionic acid	0 h	7.62	7.47	7.13	7.79	8.05	7.85	8.58	8.59	8.77	0.14	0.513	0.007	0.338
	6 h	14.13^{a}	29.89^{d}	29.35^{d}	34.73^{e}	32.72^{de}	23.45^{c}	22.09^{c}	18.02^{b}	20.62^{b}	1.31	<0.001	<0.001	<0.001
	12 h	18.14^{a}	36.98^{d}	35.15^{d}	37.67^{d}	39.93^{d}	29.48^{c}	27.86^{bc}	23.23^{ab}	24.38^{bc}	1.43	<0.001	0.726	<0.001
	24 h	22.97^{a}	41.78^{e}	40.79^{e}	43.74^{ef}	46.19^{f}	33.88^{d}	30.45^{c}	27.50^{bc}	26.78^{b}	1.57	<0.001	<0.001	<0.001
	48 h	27.47^{a}	44.67^{d}	50.03^{e}	46.59^{de}	49.07^{de}	36.61^{c}	33.02^{bc}	30.71^{ab}	30.63^{ab}	1.66	<0.001	0.003	<0.001
Butyric acid	0 h	3.80	3.75	3.57	3.51	3.75	3.85	4.35	4.04	4.29	0.09	0.331	0.154	0.543
	6 h	9.25^{cd}	11.61^{e}	11.69^{e}	6.94^{ab}	6.52^{a}	10.04^{d}	9.53^{d}	7.40^{ab}	7.93^{bc}	0.36	<0.001	<0.001	0.001
	12 h	13.31^{c}	15.12^{c}	14.26^{c}	9.28^{a}	10.05^{a}	14.0^{5c}	13.28^{bc}	10.34^{a}	10.69^{ab}	0.43	<0.001	<0.001	0.074
	24 h	19.59^{d}	17.89 cd	16.99^{cd}	12.92^{a}	13.24^{ab}	17.58^{cd}	15.86^{bc}	13.91^{ab}	12.04^{a}	0.51	<0.001	<0.001	<0.001
	48 h	22.81^{f}	18.28^{cde}	20.84^{ef}	14.54^{a}	15.59^{abc}	19.60^{de}	17.53^{abc}	15.23^{ab}	15.57^{abc}	0.55	0.052	<0.001	<0.001
TVFA	0 h	28.75	27.93	26.58	27.35	28.58	29.06	31.75	30.50	31.29	0.56	0.541	0.138	0.467
	6 h	59.68^{a}	79.21^{cd}	78.53^{cd}	87.31^{d}	82.08^{d}	71.49^{bc}	67.47^{ab}	62.55^{ab}	68.45^{ab}	1.80	0.002	0.649	<0.001
	12 h	79.83^{a}	99.49^{c}	94.79^{abc}	95.50^{bc}	102.34^{c}	93.39^{abc}	88.46^{abc}	80.56^{ab}	83.49^{ab}	1.73	0.016	<0.001	0.049
	24 h	108.58^{bcd}	114.46^{cde}	111.56^{cde}	116.04^{de}	121.11^{e}	111.95^{cde}	102.82^{abc}	98.77^{ab}	92.25^{a}	1.81	<0.001	0.018	<0.001
	48 h	131.89^{c}	125.13^{abc}	143.50^{c}	130.49^{bc}	133.28^{c}	126.03^{abc}	114.32^{ab}	110.73^{a}	110.07^{a}	2.19	0.002	<0.001	<0.001
A:P	0 h	2.02	1.95	2.01	1.98	1.91	1.97	1.98	2.03	1.94	0.02	0.88	0.62	0.87
	6 h	2.35^{f}	1.08^{a}	1.09^{a}	1.19^{b}	1.18^{b}	1.42^{c}	1.43^{c}	1.85^{e}	1.74^{d}	0.08	<0.001	<0.001	<0.001
	12 h	2.43^{e}	1.08^{a}	1.09^{ab}	1.14^{ab}	1.16^{b}	1.47^{c}	1.47^{c}	1.82^{d}	1.78^{d}	0.08	<0.001	<0.001	<0.001
	24 h	2.55^{d}	1.08^{a}	1.09^{a}	1.17^{a}	1.16^{a}	1.51^{b}	1.57^{b}	1.83^{c}	1.76^{c}	0.09	<0.001	<0.001	<0.001
	48 h	2.59^{d}	1.12^{a}	1.12^{a}	1.21^{a}	1.17^{a}	1.58^{b}	1.61^{b}	1.81^{c}	1.78^{c}	0.09	<0.001	<0.001	<0.001

^{a,b,c,d,e,f}Different superscripts in the same row imply that their mean values are significantly different ($p \leq 0.05$). Where: C = Diet 1 (10.3% hemicellulose); y = diet 1 with low yeast culture; Y = diet 1 with high yeast culture; o = diet 1 with low oxalic acid; O = diet 1 with high oxalic acid; yo = diet 1 with low yeast culture and low oxalic acid; yO = diet 1 with low yeast culture and high oxalic acid; Yo = diet 1 with high yeast culture and low oxalic acid; YO = diet 1 with high yeast culture and high oxalic acid; TVFA = total volatile fatty acid; A:P = acetic to propionic acid ratio.

with YC and OA may have a buffering effect on the rumen, leading to improved NH_3-N concentration and volatile fatty acids production.

3.3 Rumen microbiome composition

3.3.1 Ruminal bacterial alpha diversity indices

For rumen bacteria community composition analyses, a total of 216 samples of ruminal fluid were used, with four replications per fermentation time. In diet 1, supplementation with YC, OA, and their interactions significantly increased the Chao1 value, an indicator of bacterial richness (Figure 3A). Specifically, the OA ($p < 0.001$, at 12 h) and YC and OA interaction ($p < 0.05$, at 48 h) increased the Chao1 values. YC, OA, and their interactions also increased the Shannon (Figure 3B) and Simpson (Figure 3C) diversity indices ($p < 0.001$) at 12 h. In diet 2, the Chao1 index results showed that YC and OA supplementation increased bacterial richness and abundance (Figure 3D). The YC and OA interactions ($p < 0.001$) showed a higher Chao1 index value. However, the Chao1 index value of the YC alone supplemented treatments was significantly lower than that of the control. Supplementation with YC and OA interaction also altered the Shannon (Figure 3E) and Simpson (Figure 3F) diversity indices in this diet. Lower YC and lower OA supplementation significantly increased the Shannon and Simpson indices at 12 h. Additional results on bacterial alpha diversity indices are also shown in Supplementary Figures 1, 2 for diets 1 and 2, respectively. The β-diversity principal coordinate analysis (PCoA) also showed that in each type of supplement, the bacterial community composition was closer to each other at 12 and 48 h but scattered at 0 h in both diets (Figure 4). This PCoA result showed that there is a community composition variation between the control and the YC, OA, and their interaction-supplemented treatments in both diets. However, there is no significant community variation between the lower and higher levels of each supplement. The YC, OA, and their interaction-supplemented treatments bacterial community composition were closer to each other without a significant difference caused by the level of YC and OA.

TABLE 4 Effects of YC and OA supplementation in diet 2 on major VFA production (mmol/L).

Item	Time	Diet 2	Yeast culture (YC)		Oxalic acid (OA)		Interactions				SEM	*p*-value		
		ctrl	L	H	l	h	Ll	Lh	Hl	Hh		YC	OA	YC × OA
Acetic acid	0 h	16.22	15.70	16.80	16.06	16.81	16.35	15.77	16.37	19.12	0.33	0.185	0.383	0.544
	6 h	35.94^{bcd}	30.36^{a}	38.23^{d}	37.52^{cd}	45.88^{e}	31.84^{a}	31.67^{a}	33.05^{ab}	34.13^{abc}	0.91	<0.001	<0.001	<0.001
	12 h	54.68^{d}	37.63^{a}	41.60^{abc}	45.87^{c}	56.74^{d}	38.73^{ab}	41.00^{abc}	44.11^{bc}	41.58^{abc}	1.29	<0.001	0.004	<0.001
	24 h	58.10^{bc}	49.32^{a}	49.54^{ab}	53.64^{ab}	62.92^{c}	48.64^{a}	50.32^{ab}	50.02^{ab}	49.63^{ab}	1.03	<0.001	0.072	0.119
	48 h	61.79^{bc}	57.76^{abc}	54.52^{a}	62.71^{c}	72.10^{d}	56.68^{abc}	58.18^{abc}	55.17^{ab}	54.69^{a}	1.11	<0.001	0.008	0.006
Propionic acid	0 h	8.71	8.18	8.89	8.55	8.98	8.65	8.32	8.26	9.59	0.155	0.393	0.446	0.538
	6 h	15.49^{a}	28.92^{d}	35.50^{f}	32.56^{e}	40.39^{g}	22.72^{c}	22.54^{c}	19.37^{b}	9.48^{b}	1.57	<0.001	<0.001	<0.001
	12 h	22.51^{a}	35.32^{d}	38.56^{de}	39.86^{e}	51.88^{f}	26.98^{bc}	28.09^{c}	25.82^{abc}	24.31^{ab}	1.78	<0.001	<0.001	<0.001
	24 h	24.15^{a}	44.47^{c}	44.66^{c}	45.68^{c}	56.27^{d}	32.67^{b}	33.38^{b}	29.94^{b}	29.18^{b}	1.94	<0.001	0.002	<0.001
	48 h	26.54^{a}	51.24^{de}	48.12^{d}	53.56^{e}	60.69^{f}	37.00^{c}	38.45^{c}	31.97^{b}	31.89^{b}	2.17	<0.001	0.001	<0.001
Butyric acid	0 h	4.07	3.94	4.45	4.14	4.35	4.26	4.06	4.23	5.20	0.13	0.207	0.429	0.652
	6 h	9.61^{bc}	11.68^{d}	13.84^{e}	6.92^{a}	8.75^{b}	9.82^{c}	9.55^{bc}	7.15^{a}	7.57^{a}	0.42	<0.001	<0.001	<0.001
	12 h	15.60^{d}	14.35^{cd}	15.36^{d}	10.18^{a}	13.44^{bc}	12.4^{b}	13.09^{bc}	10.66^{a}	9.93^{a}	0.41	<0.001	<0.001	<0.001
	24 h	18.90^{d}	18.02^{d}	18.19^{d}	12.81^{a}	16.48^{bcd}	16.32^{bcd}	17.14^{cd}	13.68^{abc}	13.53^{ab}	0.46	0.008	<0.001	0.013
	48 h	20.31^{c}	19.45^{c}	18.71^{bc}	16.39^{c}	20.94^{c}	8.61^{bc}	19.38^{c}	15.61^{a}	15.20^{a}	0.37	<0.001	<0.001	0.007
TVFA	0 h	30.42	29.28	31.64	30.20	31.63	30.72	29.55	30.38	35.50	0.63	0.246	0.430	0.598
	6 h	64.04^{a}	76.38^{bc}	93.75^{d}	81.33^{c}	100.52^{d}	68.85^{ab}	68.21^{a}	63.27^{a}	65.07^{a}	2.56	<0.001	<0.001	<0.001
	12 h	97.91^{cd}	94.29^{bcd}	102.91^{d}	101.77^{d}	129.97^{e}	84.12^{ab}	88.57^{abc}	85.94^{ab}	80.8^{a}	2.81	<0.001	<0.001	<0.001
	24 h	108.4^{ab}	121.79^{b}	122.41^{b}	120.26^{b}	145.76^{c}	105.88^{ab}	109.77^{ab}	100.84^{a}	99.62^{a}	2.85	<0.001	0.008	<0.001
	48 h	118.61^{ab}	141.34^{de}	133.77^{cde}	144.77^{e}	166.87^{f}	123.86^{abc}	127.87^{bcd}	112.32^{a}	111.22^{a}	3.38	<0.001	0.009	<0.001
A:P	0 h	1.88	1.89	1.87	1.91	1.91	1.96	1.92	1.93	1.92	0.01	0.74	0.20	0.99
	6 h	2.32^{e}	1.05^{a}	1.08^{a}	1.15^{b}	1.14^{b}	1.40^{c}	1.41^{c}	1.71^{d}	1.75^{d}	0.08	<0.001	<0.001	<0.001
	12 h	2.43^{e}	1.07^{a}	1.08^{a}	1.15^{b}	1.09^{a}	1.44^{c}	1.46^{c}	1.71^{d}	1.71^{d}	0.08	<0.001	<0.001	<0.001
	24 h	2.41^{e}	1.11^{a}	1.11^{a}	1.18^{a}	1.12^{a}	1.49^{b}	1.51^{b}	1.67^{c}	1.70^{c}	0.08	<0.001	<0.001	<0.001
	48 h	2.33^{e}	1.13^{a}	1.13^{a}	1.17^{a}	1.19^{a}	1.53^{b}	1.51^{b}	1.73^{c}	1.71^{c}	0.07	<0.001	<0.001	<0.001

^{a,b,c,d,e}Different superscripts in the same row imply that their mean values are significantly different ($p \leq 0.05$). Where: ctrl = Diet 2 (17% hemicellulose); L = diet 2 with low yeast culture; H = diet 2 with high yeast culture; l = diet 2 with low oxalic acid; h = diet 2 with high oxalic acid; Ll = diet 2 with low yeast culture and low oxalic acid; Lh = diet 2 with low yeast culture and high oxalic acid; Hl = diet 2 with high yeast culture and low oxalic acid; Hh = diet 2 with high yeast culture and high oxalic acid; VFA = volatile fatty acid; TVFA = total volatile fatty acid; A:P = acetic acid to propionic acid ratio.

3.3.2 Ruminal bacterial phylum composition

Bacteroidetes, *Firmicutes*, and *Actinobacteria* were the most dominant phyla in both diets. As shown in Table 5 and Figure 5A, in diet 1, supplementation with OA affected the composition and abundance of rumen bacterial phyla. The abundance of *Bacteroidetes* was significantly higher in the control treatment at 12 h. However, at 48 h, OA supplementation significantly increased the abundance of *Bacteroidetes* ($p < 0.001$). Neither YC supplementation alone nor YC and OA interaction had an impact on the abundance of *Firmicutes*. However, their abundance was reduced in treatments supplemented with OA ($p < 0.001$). The YC and OA interactions increased *Actinobacteria* phylum abundance *($p < 0.001$).* The addition of lower levels of YC and OA significantly increased the abundance of the *Spirochaetes* and *Verrucomicrobia* phyla.

Adding YC and OA to diet 2 also increased the number of *Bacteroidetes*, *Firmicutes*, *Actinobacteria*, and *Proteobacteria*, which are the most dominant phyla ($p < 0.001$; Table 6; Figure 5B). Specifically, treatments supplemented with OA alone showed a higher relative abundance of the *Bacteroidetes* phylum ($p < 0.001$). The lower levels of YC and OA supplementation significantly increased the abundance of the *Bacteroidetes* phylum ($p < 0.001$). The relative abundance of *Firmicutes* was also significantly increased with the addition of YC at both higher and lower levels ($p < 0.001$).

3.3.3 Rumen bacterial genus composition

Prevotella, Selenomonas, Butyrivibrio, Succiniclasticum, and *Ruminococcaceae_Ruminococcus* were the dominant genera identified in both diets. The results of the bacterial genus composition in diet 1 are shown in Table 7; Figure 5C, and Supplementary Table 1. Hence, YC, OA, and their interaction increased the *Prevotella* genus abundance at 48 h ($p < 0.001$). The supplemented treatments also showed a significantly higher abundance of the *Selenomonas* genus at 48 h. However, the *Butyrivibiro* genus was higher in the control treatment. In diet 2, the *Prevotella* genus was found to have a higher abundance in the supplemented experimental treatments than in the control ($p < 0.001$; Table 8; Figure 5D; Supplementary Table 2).

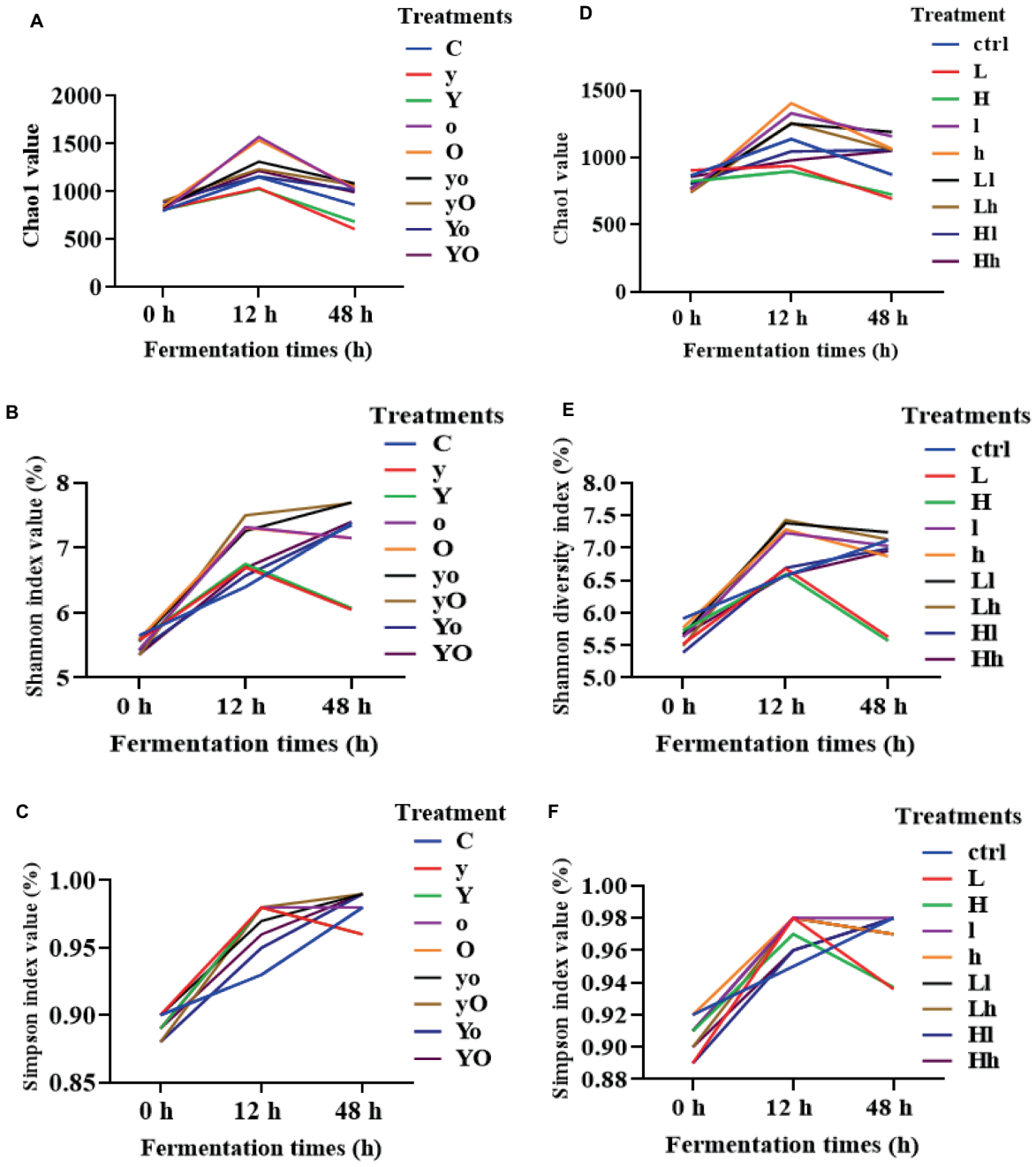


FIGURE 3
Alpha diversity indices of bacteria community composition of diet 1 [Chao1 **(A)**, Shannon **(B)**, Simpson **(C)**, and diet 2 **(D–F)**] respectively. Where: C = Diet 1 (10.3% hemicellulose); y = diet 1 with low yeast culture; Y = diet 1 with high yeast culture; o = diet 1 with low oxalic acid; O = diet 1 with high oxalic acid; yo = diet 1 with low yeast culture and low oxalic acid; yO = diet 1 with low yeast culture and high oxalic acid; Yo = diet 1 with high yeast culture and low oxalic acid; YO = diet 1 with high yeast culture and high oxalic acid; ctrl = Diet 2 (17% hemicellulose); L = diet 2 with low yeast culture; H = diet 2 with high yeast culture; l = diet 2 with low oxalic acid; h = diet 2 with high oxalic acid; Ll = diet 2 with low yeast culture and low oxalic acid; Lh = diet 2 with low yeast culture and high oxalic acid; Hl = diet 2 with high yeast culture and low oxalic acid; Hh = diet 2 with high yeast culture and high oxalic acid.

Selenomonas and *Succiniclasticum* were also the dominant genera, which were highly increased in the supplemented treatments ($p < 0.001$). The *Butyrivibiro* genus was higher in the control treatment. However, this genus was significantly increased in higher YC supplemented treatment at 12 h. As shown in Figure 5, the dominant bacterial genera were highly distributed in the supplemented treatments.

3.4 The relationship between rumen fermentation parameters and bacterial genus composition

A canonical correlation analysis (CCA) was used to analyze Pearson's correlation coefficient for multiple sets of variables as shown in Table 9. In diet 1, *Prevotella* and *Selenomonas* showed significant

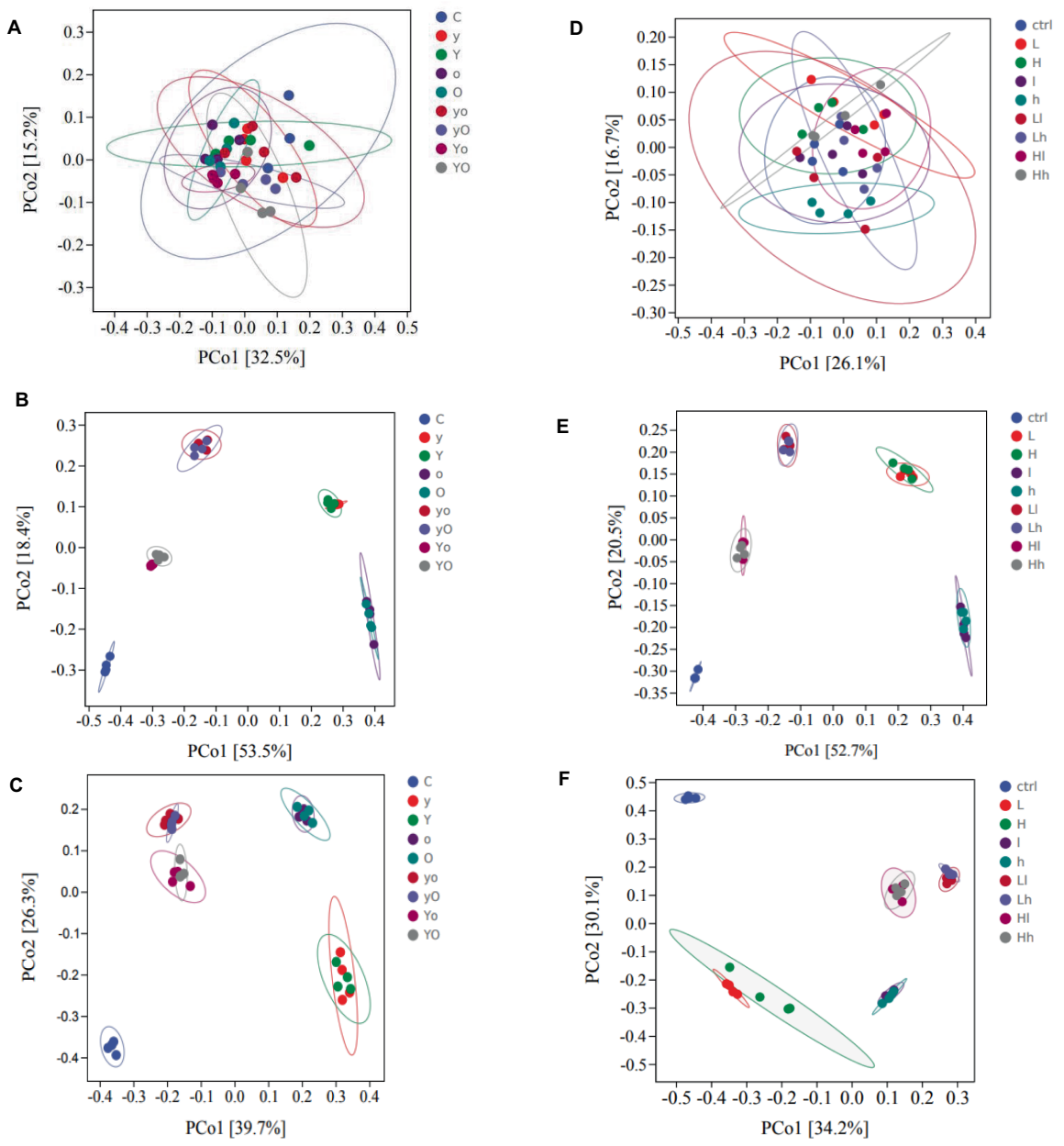


FIGURE 4
Principal coordinate analysis (PCoA) showing the distribution of the bacterial community composition at the genus level of diet 1 (**A**, 0 h), (**B**, 12 h), and (**C**, 48 h); diet 2 (**D**, 0 h), (**E**, 12 h), and (**F**, 48 h) respectively. Where: C = Diet 1 (10.3% hemicellulose); y = diet 1 with low yeast culture; Y = diet 1 with high yeast culture; o = diet 1 with low oxalic acid; O = diet 1 with high oxalic acid; yo = diet 1 with low yeast culture and low oxalic acid; yO = diet 1 with low yeast culture and high oxalic acid; Yo = diet 1 with high yeast culture and low oxalic acid; YO = diet 1 with high yeast culture and high oxalic acid; ctrl = Diet 2 (17% hemicellulose); L = diet 2 with low yeast culture; H = diet 2 with high yeast culture; l = diet 2 with low oxalic acid; h = diet 2 with high oxalic acid; Ll = diet 2 with low yeast culture and low oxalic acid; Lh = diet 2 with low yeast culture and high oxalic acid; Hl = diet 2 with high yeast culture and low oxalic acid; and Hh = diet 2 with high yeast culture and high oxalic acid. The number of samples included were 108 in each diet. Each point in the plot represents a sample, and different colored dots indicate different treatments. The percentages in square brackets of the axis represent the proportion of sample variance data (distance matrix) that can be interpreted by the corresponding axis. The closer the projection distance of the two points on the axis, the more similar the community composition of the two samples in the corresponding dimension.

and positive correlations with IVDMD, propionic acid, and TVFA. The *Sicciniclasticum* genus showed significant and positive correlations with IVCPD, pH, acetic acid, butyric acid, and NH_3-N. However, *Prevotella* showed a significant and negative correlation with NH_3-N. In diet 2, the *Prevotella* genus showed a positive correlation with IVCPD, pH, acetic acid, propionic acid, and TVFA, but had a negative correlation with IVDMD, IVNDFD, NH_3-N, butyric acid, and the acetic to propionic acid ratio. The *Butyrivibrio* genus had a positive association with NH_3-N, acetic acid, butyric acid, and the acetic to propionic acid ratio but showed a negative correlation with other parameters. The *Selenomonas* genus exhibits a significant positive association with pH, propionic acid, butyric acid, TVFA, and

TABLE 5 Effects of supplementation with YC and OA in diet 1 on the dominant bacterial phylum composition (%).

Phylum	Time	Diet 1	Yeast culture (YC)		Oxalic acid (OA)		Interactions				SEM	*p*-value		
		C	y	Y	o	O	yo	yO	Yo	YO		YC	OA	YC × OA
Bacteroidetes	0 h	54.37	58.15	56.19	56.83	58.68	52.08	55.93	61.67	57.51	0.81	0.29	0.84	0.13
	12 h	72.82^{e}	47.71^{b}	47.19^{b}	61.39^{d}	59.88^{cd}	36.20^{a}	38.10^{a}	57.85^{cd}	55.41^{c}	1.91	<0.001	<0.001	<0.001
	48 h	20.43^{a}	23.49^{a}	23.69^{a}	38.17^{b}	40.13^{b}	19.88^{a}	18.10^{a}	20.86^{a}	23.91^{a}	1.35	<0.001	0.001	<0.001
Firmicutes	0 h	29.91	27.12	29.04	32.36	29.69	30.04	26.78	26.27	24.61	0.62	0.02	0.19	0.44
	12 h	18.99^{a}	32.94^{c}	33.66^{cd}	23.05^{ab}	25.39^{b}	39.55^{de}	43.46^{e}	23.79^{ab}	24.21^{ab}	1.38	<0.001	0.048	<0.001
	48 h	61.85^{b}	61.00^{b}	60.24^{b}	40.57^{a}	39.83^{a}	55.62^{b}	56.67^{b}	58.07^{b}	54.51^{b}	1.41	<0.001	<0.001	<0.001
Actinobacteria	0 h	13.18	12.44	12.45	9.03	9.39	15.71	15.76	10.38	16.27	0.75	0.07	0.47	0.18
	12 h	3.92^{a}	19.07^{cd}	18.66^{cd}	11.67^{b}	11.08^{b}	21.41^{d}	14.71^{bc}	14.88^{bc}	16.85^{bcd}	0.93	<0.001	0.13	<0.001
	48 h	3.73^{a}	13.59^{cd}	14.51^{d}	9.33^{b}	8.84^{b}	14.478^{d}	15.07^{d}	11.16^{bc}	10.98^{bc}	0.61	<0.001	0.10	<0.001
Proteobacteria	0 h	0.11	0.09	0.10	0.11	0.13	0.06	0.07	0.06	0.07	0.01	0.02	0.16	0.79
	12 h	0.98^{b}	0.06^{a}	0.10^{a}	1.82^{c}	1.87^{c}	0.20^{a}	0.23^{a}	0.10^{a}	0.09^{a}	0.12	<0.001	<0.001	<0.001
	48 h	8.43^{c}	1.43^{a}	1.17^{a}	8.45^{c}	7.97^{c}	2.91^{ab}	2.84^{ab}	4.40^{b}	4.67^{b}	0.50	<0.001	0.003	0.021
Spirochaetes	0 h	0.95^{b}	0.80^{ab}	0.67^{ab}	0.44^{a}	0.55^{ab}	0.70^{ab}	0.54^{ab}	0.46^{a}	0.52^{a}	0.04	0.19	<0.001	0.19
	12 h	1.39^{cd}	0.03^{a}	0.07^{a}	0.80^{b}	0.74^{b}	1.00^{bc}	1.36^{cd}	1.57^{d}	1.77^{d}	0.11	0.04	<0.001	<0.001
	48 h	0.49^{bc}	0.05^{a}	0.05^{a}	0.80^{cde}	0.72^{bcd}	1.12^{e}	1.04^{de}	0.40^{ab}	0.68^{bcd}	0.07	<0.001	<0.001	<0.001
Verrucomicrobia	0 h	0.73^{bc}	0.63^{abc}	0.79^{c}	0.49^{abc}	0.76^{c}	0.67^{abc}	0.36^{a}	0.54^{abc}	0.39^{ab}	0.03	0.17	0.003	0.003
	12 h	1.22^{d}	0.00^{a}	0.00^{a}	0.00^{a}	0.00^{a}	0.56^{bc}	0.72^{c}	0.47^{bc}	0.43^{b}	0.07	0.02	0.34	<0.001
	48 h	2.55^{bc}	0.004^{a}	0.01^{a}	0.02^{a}	0.03^{a}	2.84^{c}	2.88^{c}	1.82^{b}	1.79^{b}	0.21	<0.001	<0.001	<0.001
Elusimicrobia	0 h	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.45	0.45	0.122
	12 h	0.00^{a}	0.00^{a}	0.01^{a}	0.06^{a}	0.06^{a}	0.02^{a}	0.02^{a}	0.61^{c}	0.49^{b}	0.04	<0.001	<0.001	<0.001
	48 h	0.00^{a}	0.02^{a}	0.02^{a}	0.16^{a}	0.14^{a}	0.11^{a}	0.11^{a}	2.44^{b}	2.26^{b}	0.09	<0.001	<0.001	<0.001

[a,b,c,d,e]Different superscripts in the same row imply that their mean values are significantly different ($p \leq 0.05$). Where: C = Diet 1 (10.3% hemicellulose); y = diet 1 with low yeast culture; Y = diet 1 with high yeast culture; o = diet 1 with low oxalic acid; O = diet 1 with high oxalic acid; yo = diet 1 with low yeast culture and low oxalic acid; yO = diet 1 with low yeast culture and high oxalic acid; Yo = diet 1 with high yeast culture and low oxalic acid; and YO = diet 1 with high yeast culture and high oxalic acid.

the ratio of acetic acid to propionic acid. Conversely, it demonstrated a negative correlation with *in vitro* disappearance measures, NH_3-N, acetic acid, and propionic acid.

4 Discussion

4.1 *In vitro* nutrient disappearance (IVDMD, IVNDFD, IVCPD)

One of the rumen fermentation efficiency measuring parameters is nutrient digestibility or disappearance. However, the researchers have found inconsistent results regarding the effects of yeast culture and oxalic acid on nutrient digestibility. In this study, YC supplementation in the first experiment (diet 1) increased IVDMD and IVNDFD. Similarly, another report showed that supplementing heifers' diets with YC increased DM and NDF digestibility (12) by promoting the growth of fiber-digesting bacteria (33). Supplementing the diet with a lower level of OA also increased the IVDMD and IVNDFD. This result partially contradicts the report by Benbati et al. (18), which stated that DM digestibility was reduced due to OA administration in sheep, but returned to normal after 14 days, possibly due to the responsible microbes adapting to the supplement. The interaction of lower YC and lower OA increased the IVCPD. In the second experiment (diet 2), OA and interaction supplementation increased the IVDMD, while YC alone had no effect on IVDMD. Studies have reported that YC has inconsistent effects on hemicellulose digestibility in ruminants. The addition of YC to the diet did not show a significant impact on hemicellulose digestibility (34). However, another study has shown that YC supplementation could increase hemicellulose digestibility (35). The interaction of YC and OA also improved IVCPD, which is consistent with a report that found feeding different levels of YC increased CP digestibility (36). However, another researcher reported that oxalic acid has no significant effect on CP digestibility in goats (37). This result suggests that the addition of YC and OA may improve nutrient disappearance.

4.2 Rumen fermentation parameters

4.2.1 Ruminal pH

In ruminant nutrition, the optimal ruminal pH is an indicator of the rumen's performance and the overall health of the animal (38). In modern profit-driven sheep production systems, it is common for ruminal pH to decrease and for subacute ruminal acidosis (SARA) to occur. Therefore, it is necessary to buffer and maintain an optimal ruminal pH in an intensive sheep production system. In both diets, YC and OA supplementation significantly increased the pH levels at

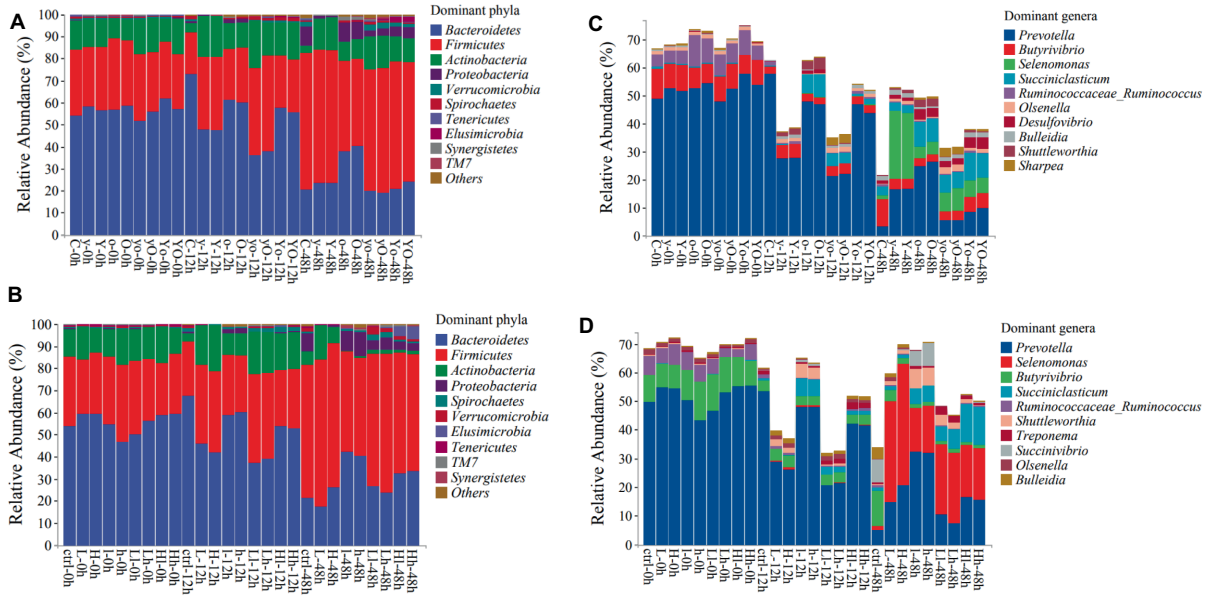


FIGURE 5
The top 10 dominant bacterial phyla and genera in both diets at different fermentation times [Diet 1 phyla **(A)**, Diet 2 phyla **(B)**, Diet 1 genera **(C)**, and diet 2 genera **(D)**]. Where: C = Diet 1 (10.3% hemicellulose); y = diet 1 with low yeast culture; Y = diet 1 with high yeast culture; o = diet 1 with low oxalic acid; O = diet 1 with high oxalic acid; yo = diet 1 with low yeast culture and low oxalic acid; yO = diet 1 with low yeast culture and high oxalic acid; Yo = diet 1 with high yeast culture and low oxalic acid; YO = diet 1 with high yeast culture and high oxalic acid; ctrl = Diet 2 (17% hemicellulose); L = diet 2 with low yeast culture; H = diet 2 with high yeast culture; l = diet 2 with low oxalic acid; h = diet 2 with high oxalic acid; Ll = diet 2 with low yeast culture and low oxalic acid; Lh = diet 2 with low yeast culture and high oxalic acid; Hl = diet 2 with high yeast culture and low oxalic acid; Hh = diet 2 with high yeast culture and high oxalic acid. In each diet 108 samples were used for analysis.

TABLE 6 **Effects of YC and OA supplementation in diet 2 on the dominant bacterial phylum composition (%).**

Phylum	Time	Diet 2	Yeast culture (YC)		Oxalic acid (OA)		Interactions				SEM	*p* value		
		ctrl	L	H	l	h	Ll	Lh	Hl	Hh		YC	OA	YC × OA
Bacteroidetes	0 h	53.89^{abc}	59.20^{c}	59.18^{c}	54.51^{abc}	46.87^{a}	50.16^{ab}	56.08^{bc}	58.70^{c}	59.48^{c}	0.89	<0.001	0.063	0.006
	12 h	67.87^{e}	45.73^{b}	41.83^{ab}	58.99^{d}	60.14^{d}	37.17^{a}	38.87^{a}	53.64^{c}	52.88^{c}	1.71	<0.001	0.112	<0.001
	48 h	21.33^{ab}	17.41^{a}	26.13^{bc}	42.38^{d}	40.65^{d}	26.59^{bc}	23.93^{ab}	32.71^{c}	33.33^{c}	1.43	<0.001	<0.001	<0.001
Firmicutes	0 h	31.12^{ab}	24.42a	27.97^{ab}	30.53^{ab}	34.80^{b}	33.21^{ab}	28.21^{ab}	23.52^{a}	27.26^{ab}	0.87	0.009	0.45	0.04
	12 h	24.15^{a}	35.91^{b}	36.89^{b}	27.37^{a}	25.58^{a}	40.14^{b}	39.14^{b}	25.63^{a}	27.00^{a}	1.08	<0.001	0.115	<0.001
	48 h	60.35^{cd}	66.22^{d}	65.00^{d}	44.94^{a}	44.27^{a}	60.16^{bcd}	62.82^{d}	54.40^{bc}	52.83^{b}	1.38	<0.001	<0.001	0.006
Actinobacteria	0 h	12.85	14.78	10.94	12.87	16.08	14.51	14.21	16.35	11.51	0.64	0.612	0.554	0.282
	12 h	4.06^{a}	17.86^{cd}	20.90^{d}	9.30^{b}	10.22^{b}	19.27^{cd}	18.17^{cd}	16.64^{c}	16.60^{c}	0.92	<0.001	0.331	<0.001
	48 h	5.95^{a}	15.69^{b}	7.50^{a}	0.69^{a}	0.85^{a}	1.64^{a}	1.85^{a}	1.53^{a}	1.67^{a}	0.91	0.014	<0.001	0.026
Proteobacteria	0 h	0.16^{ab}	0.13^{ab}	0.15^{ab}	0.17^{b}	0.11^{ab}	0.12^{ab}	0.09^{ab}	0.05^{a}	0.07^{ab}	0.01	0.028	0.023	0.156
	12 h	0.51^{b}	0.11^{a}	0.11^{a}	2.07^{c}	2.13^{c}	0.17^{a}	0.24^{a}	0.19^{a}	0.18^{a}	0.13	<0.001	<0.001	<0.001
	48 h	8.43^{de}	0.39^{a}	1.08^{ab}	8.76^{de}	10.47^{e}	4.34^{bc}	5.48^{cd}	3.46^{abc}	3.20^{abc}	0.60	<0.001	<0.001	0.105
Spirochaetes	0 h	0.46^{ab}	0.56^{b}	0.47^{ab}	0.48^{ab}	0.44^{ab}	0.40^{ab}	0.24^{a}	0.40^{ab}	0.41^{ab}	0.02	0.511	0.039	0.105
	12 h	1.38^{cde}	0.11^{a}	0.05^{a}	0.86^{bc}	0.68^{ab}	1.20^{bcd}	1.77^{def}	2.40^{f}	1.86^{ef}	0.13	<0.001	<0.001	<0.001
	48 h	0.77^{b}	0.03^{a}	0.04^{a}	1.03^{b}	0.97^{b}	2.88^{c}	2.34^{c}	1.16^{b}	0.99^{b}	0.16	<0.001	<0.001	<0.001
Verrucomicrobia	0 h	0.58	0.43	0.56	0.60	0.65	0.69	0.37	0.38	0.56	0.03	0.245	0.893	0.074
	12 h	1.33^{c}	0.01^{a}	0.01^{a}	0.01^{a}	0.003^{a}	0.65^{b}	0.56^{b}	0.35^{ab}	0.37^{ab}	0.08	0.022	0.147	<0.001
	48 h	1.11^{b}	0.01^{a}	0.01^{a}	0.001^{a}	0.01^{a}	2.82^{d}	1.90^{c}	1.19^{b}	1.28^{b}	0.17	<0.001	<0.001	<0.001

^{a,b,c,d,e,f}Different superscripts in the same row imply that their mean values are significantly different ($p \leq 0.05$). Where: ctrl = Diet 2 (17% hemicellulose); L = diet 2 with low yeast culture; H = diet 2 with high yeast culture; l = diet 2 with low oxalic acid; h = diet 2 with high oxalic acid; Ll = diet 2 with low yeast culture and low oxalic acid; Lh = diet 2 with low yeast culture and high oxalic acid; Hl = diet 2 with high yeast culture and low oxalic acid; and Hh = diet 2 with high yeast culture and high oxalic acid.

TABLE 7 Effects of YC and OA supplementation in diet 1 on ruminal bacterial genus composition (%).

Genus	Time	Diet 1	Yeast culture (YC)		Oxalic acid (OA)		Interactions				SEM	*p* value		
		C	y	Y	o	O	yo	yO	Yo	YO		YC	OA	YC × OA
Prevotella	0 h	49.12	52.77	51.66	52.79	54.50	47.85	52.33	57.84	53.71	0.88	0.265	0.523	0.298
	12 h	57.99^{e}	27.53^{bc}	27.98^{c}	48.03^{d}	46.75^{d}	21.40^{a}	21.83^{ab}	46.99^{d}	43.69^{d}	2.17	<0.001	0.386	<0.001
	48 h	3.43^{a}	16.52^{c}	16.92^{c}	24.89^{d}	26.45^{d}	5.27^{ab}	5.37^{ab}	8.61^{b}	9.99^{b}	1.40	<0.001	0.159	<0.001
Selenomonas	0 h	0.01^{ab}	0.02^{ab}	0.02^{ab}	0.03^{b}	0.02^{ab}	0.01^{ab}	0.01^{ab}	0.00^{a}	0.01^{ab}	0.002	0.067	0.212	0.016
	12 h	0.00^{a}	0.21^{b}	0.15^{ab}	0.48^{c}	0.48^{c}	0.10^{ab}	0.16^{ab}	0.08^{ab}	0.06^{ab}	0.03	<0.001	0.002	<0.001
	48 h	1.32^{a}	24.55^{c}	23.53^{c}	4.18^{ab}	4.29^{ab}	6.98^{ab}	7.94^{b}	5.96^{ab}	5.42^{ab}	1.41	<0.001	<0.001	<0.001
Butyrivibrio	0 h	10.66^{b}	8.64^{ab}	9.30^{ab}	7.17^{ab}	6.92^{a}	9.05^{ab}	9.14^{ab}	6.67^{a}	8.97^{ab}	0.31	0.495	0.021	0.035
	12 h	2.40^{a}	4.65^{d}	4.64^{d}	2.68^{ab}	2.41^{a}	3.38^{bc}	3.92^{cd}	2.79^{ab}	2.99^{ab}	0.15	<0.001	<0.001	<0.001
	48 h	9.70^{c}	3.57^{ab}	3.48^{ab}	2.48^{a}	2.58^{a}	3.10^{a}	3.54^{ab}	5.15^{b}	5.06^{b}	0.37	<0.001	<0.001	<0.001
Succiniclasticum	0 h	0.45^{ab}	0.39^{ab}	0.58^{b}	0.23^{ab}	0.30^{ab}	0.38^{ab}	0.29^{ab}	0.14^{ab}	0.12^{a}	0.03	0.488	0.001	0.064
	12 h	0.47^{ab}	0.15^{a}	0.04^{a}	6.26^{e}	7.92^{e}	4.20^{d}	3.52^{cd}	0.94^{ab}	2.16^{bc}	0.47	<0.001	<0.001	<0.001
	48 h	3.01^{a}	2.79^{a}	2.65^{a}	9.20^{c}	8.52^{c}	6.41^{b}	5.81^{b}	9.98^{c}	8.81^{c}	0.48	<0.001	<0.001	<0.001
Ruminococcaceae_ Ruminococcus	0 h	4.78^{a}	4.50^{a}	4.86^{a}	1.17^{b}	8.75^{ab}	7.64^{ab}	6.98^{ab}	8.95^{ab}	5.25^{a}	0.53	0.086	<0.001	0.412
	12 h	1.12^{e}	0.51^{bc}	0.76^{cd}	0.19^{a}	0.17^{a}	0.35^{ab}	0.43^{ab}	0.94^{de}	0.72^{cd}	0.06	<0.001	<0.001	<0.001
	48 h	0.65^{c}	0.35^{ab}	0.33^{ab}	0.30^{a}	0.29^{a}	0.38^{ab}	0.37^{ab}	0.50^{bc}	0.45^{ab}	0.02	0.117	0.073	<0.001
Shuttleworthia	0 h	0.11^{ab}	0.11^{ab}	0.20^{b}	0.21^{b}	0.21^{b}	0.10^{ab}	0.05^{a}	0.11^{ab}	0.04^{a}	0.01	0.004	0.212	0.006
	12 h	0.01^{a}	1.41^{b}	2.20^{c}	3.11^{d}	4.00^{e}	0.30^{a}	0.40^{a}	0.02^{a}	0.02^{a}	0.24	<0.001	0.004	<0.001
	48 h	0.21^{a}	0.75^{a}	0.79^{a}	2.91^{b}	2.81^{b}	0.32^{a}	0.55^{a}	0.42^{a}	0.37^{a}	0.18	<0.001	<0.001	<0.001

^{a,b,c,d,e}Different superscripts in the same row imply that their mean values are significantly different ($p \leq 0.05$). Where: C = Diet 1 (10.3% hemicellulose); y = diet 1 with low yeast culture; Y = Diet 1 with high yeast culture; o = diet 1 with low oxalic acid; O = diet 1 with high oxalic acid; yo = diet 1 with low yeast culture and low oxalic acid; yO = diet 1 with low yeast culture and high oxalic acid; Yo = diet 1 with high yeast culture and low oxalic acid; and YO = diet 1 with high yeast culture and high oxalic acid.

different fermentation times. Similarly, reports have shown that YC might have a buffering effect on the rumen (39, 40). Nevertheless, studies examining the impact of OA have shown that it does not have a statistically significant influence on rumen pH (41). Therefore, supplementing with YC and OA may help to stabilize the rumen environment. This increase in ruminal pH may imply an improvement in fiber digestion, protein degradation, microbial activities, nutrient absorption, and overall rumen health.

4.2.2 Ammonia nitrogen concentration

Ruminants have a special stomach physiology that enables them to utilize different forms of nitrogen as protein sources. Ammonia nitrogen in the rumen is derived from the degradation of dietary protein and nonprotein nitrogen sources. It serves as an indicator of rumen internal performance, being continuously produced in the rumen, and is essential for microbes to synthesize microbial protein (42). In both diets, YC and OA interactions significantly increased NH_3-N concentrations at different fermentation times. However, supplementing with either YC or OA separately reduced NH_3-N concentrations in both diets. Similarly, Habeeb (43) found that YC supplements could lead to reduced NH_3-N. On the other hand, in lambs fed orchard grass, the addition of YC did not affect rumen NH_3-N (44). Studies have reported that YC supplementation reduces NH_3-N concentration in the rumen (11, 12, 45). This may be because YC supplementation depresses ammonia-producing microbes (46). The current finding, however, suggests that combining YC and OA supplements could have a positive effect on increasing NH_3-N levels, ultimately leading to an increase in microbial protein synthesis in the rumen. Moreover, supplementing YC and OA together might reduce the inhibitory effects of OA on NH_3-N in the rumen, which could be due to the deamination and proteolysis effects of OA (41). The higher ammonia nitrogen concentration resulted in increased microbial protein synthesis, improved nitrogen utilization, and enhanced dietary protein degradation.

4.2.3 Volatile fatty acids production

Volatile fatty acids are the main energy sources and precursors for the production of animal products. They are produced through the bioconversion of diets in the rumen by anaerobic digestion (47). In both diets, supplementation with OA at lower and higher levels as well as the interaction between YC and OA significantly increased the production of acetic acid. This suggests that OA supplementation alone had a greater impact on acetic acid production than YC and their interactions. This indicates that OA may facilitate the degradation of fiber and increase nutrient availability for the host. However, at 24 and 48 h, the control produced more acetic acid. Supplementation with YC and OA showed significantly higher TVFA production compared to the control. Individual supplementation of YC and OA had a greater impact on TVFA production than their interactions. Although there is a lack of reports on the effects of OA, many researchers have reported that YC supplementation has positive effects on increasing acetic, propionic, and butyric acids (43, 48). Therefore, the combination of YC and OA might have a promising effect on boosting essential volatile fatty acid production. Furthermore, YC and

TABLE 8 Effects of supplementation with YC and OA in diet 2 on the dominant genus composition of ruminal bacteria (%).

Genus	Time	Diet 2	Yeast culture (YC)		Oxalic acid (OA)		Interactions				SEM	*p* value		
		ctrl	L	H	l	h	Ll	Lh	Hl	Hh		YC	OA	YC × OA
Prevotella	0 h	49.77^{ab}	54.89^{b}	54.37^{b}	50.53^{ab}	43.24^{a}	46.56^{ab}	53.10^{b}	55.23^{b}	55.47^{b}	0.88	<0.001	0.239	0.01
	12 h	53.47^{e}	29.00^{b}	26.31^{b}	47.91^{d}	48.13^{d}	20.57^{a}	21.47^{a}	42.10^{c}	41.65^{c}	2.01	<0.001	0.542	<0.001
	48 h	4.84^{a}	14.70^{bc}	20.84^{d}	32.20^{e}	32.12^{e}	10.43^{ab}	7.30^{a}	16.73^{cd}	15.55^{bcd}	1.60	<0.001	<0.001	<0.001
Selenomonas	0 h	0.00^{a}	0.02^{ab}	0.01^{ab}	0.02^{b}	0.022^{b}	0.02^{b}	0.02^{b}	0.02^{b}	0.02^{b}	0.00	1.99	<0.001	2.228
	12 h	0.02^{a}	0.35^{bcd}	0.37^{cd}	0.73^{e}	0.56^{de}	0.16^{abc}	0.09^{a}	0.13^{ab}	0.07^{a}	0.04	<0.001	0.021	<0.001
	48 h	1.58^{a}	35.52^{cd}	42.18^{d}	15.37^{ab}	16.17^{b}	24.75^{bc}	24.70^{bc}	17.91^{b}	18.03^{b}	2.08	<0.001	0.009	<0.001
Butyrivibrio	0 h	9.54^{ab}	8.11^{a}	8.26^{a}	10.39^{abc}	13.68^{d}	12.85^{cd}	12.22^{bcd}	10.30^{abc}	8.59^{a}	0.38	<0.001	<0.001	0.002
	12 h	3.61^{abc}	3.93^{bc}	4.40^{c}	3.20^{ab}	3.00^{a}	3.78^{abc}	3.62^{abc}	3.23^{ab}	3.63^{abc}	0.09	0.004	<0.001	0.106
	48 h	12.17^{c}	3.61^{b}	2.04^{a}	1.35^{a}	1.52^{a}	0.85^{a}	1.18^{a}	1.20^{a}	1.24^{a}	0.58	<0.001	<0.001	<0.001
Succiniclasticum	0 h	0.14	0.23	0.14	0.11	0.13	0.18	0.23	0.07	0.22	0.02	0.099	0.203	0.717
	12 h	0.94^{ab}	0.07^{a}	0.07^{a}	6.10^{c}	5.85^{c}	2.50^{b}	1.92^{ab}	1.17^{ab}	1.47^{ab}	0.38	<0.001	<0.001	<0.001
	48 h	1.57^{a}	1.42^{a}	1.25^{a}	5.48^{b}	5.66^{b}	5.31^{b}	7.07^{b}	13.09^{c}	13.04^{c}	0.74	<0.001	<0.001	<0.001
Ruminococcaceae_ Ruminococcus	0 h	6.46	5.45	7.24	6.07	5.77	5.31	3.01	2.72	5.44	0.46	0.396	0.239	0.332
	12 h	1.23^{c}	0.86^{b}	0.80^{b}	0.26^{a}	0.26^{a}	0.51^{a}	0.46^{a}	0.82^{b}	0.88^{b}	0.05	<0.001	<0.001	<0.001
	48 h	0.55^{d}	0.26^{abc}	0.27^{bc}	0.22^{ab}	0.22^{ab}	0.13^{a}	0.21^{ab}	0.36^{c}	0.33^{bc}	0.02	<0.001	<0.001	<0.001
Shuttleworthia	0 h	0.14	0.10	0.13	0.15	0.20	0.25	0.10	0.04	0.18	0.02	0.657	0.785	0.203
	12 h	0.00^{a}	2.45^{b}	1.82^{b}	4.99^{d}	4.06^{c}	0.49^{a}	0.64^{a}	0.02^{a}	0.02^{a}	0.30	<0.001	0.022	<0.001
	48 h	0.29^{a}	0.29^{a}	1.41^{ab}	6.87^{d}	5.94^{cd}	3.76^{bc}	2.03^{ab}	1.33^{ab}	0.38^{a}	0.41	<0.001	<0.001	<0.001

^{a,b,c,d,e}Different superscripts in the same row imply that their mean values are significantly different ($p \leq 0.05$). Where: ctrl = diet 2 (17% hemicellulose); L = diet 2 with low yeast culture; H = diet 2 with high yeast culture; l = diet 2 with low oxalic acid; h = diet 2 with high oxalic acid; Ll = diet 2 with low yeast culture and low oxalic acid; Lh = diet 2 with low yeast culture and high oxalic acid; Hl = diet 2 with high yeast culture and low oxalic acid; and Hh = diet 2 with high yeast culture and high oxalic acid.

OA supplementation significantly increased propionic acid production in both diets. However, the acetic acid to propionic acid ratio was significantly lower in the supplemented treatments in both diets. A study reported that YC supplementation could significantly increase propionic and TVFA production, but reduce acetic acid, resulting in a lower acetic to propionic acid ratio (49). Hence, YC and OA supplementation may enhance VFA production in the rumen by improving rumen functions. Increased volatile fatty acid production may lead to improved energy yield, enhanced rumen efficiency, better nutrient absorption, maintained pH levels, efficient fiber digestion, and increased microbial activities.

4.3 Rumen bacterial community composition

4.3.1 Bacterial species diversity

The rumen ecosystem contains diverse microbes, predominantly bacteria (50). Factors that affect the diversity of bacterial species include diet, dietary supplements, host species, and environmental factors (51). Species diversity can be measured using different alpha diversity indices, such as the Chao1 index and the Shannon and Simpson diversity indices, which quantify the richness of species in a community (52). In diet 1, the addition of OA alone, as well as the interaction of YC and OA, increased species diversity. This aligns with a previous study that found an increase in the richness and diversity of rumen microbiota when organic acids were added to high-grain diets (53). However, this partially contradicts an other study that found oxalic acid reduced the microbial diversity in sheep, but the diversity gradually returned to normal (41). YC and OA supplementation also affected the Shannon and Simpson diversity indices of the bacterial community. Treatments supplemented with YC and OA interactions showed significantly higher Shannon diversity index values. In diet 2, the YC and OA interactions significantly increased the Chao1 index values, indicating higher bacterial species richness. However, YC alone showed significantly lower Chao1 values. Supplementing YC and OA together significantly increased the Shannon and Simpson indices. This implies that YC and OA may have a synergistic effect as a supplement to enhance rumen bacterial richness and diversity, contributing to the overall stability and resilience of the rumen ecosystem. A diverse bacterial community can help the animal cope with different stresses and disturbances by maintaining various functions in the body. Furthermore, diverse bacterial communities may help the animal withstand different diseases and environmental impacts.

4.3.2 Rumen bacterial phylum composition

The composition of ruminal bacterial phyla varies across different parts of the rumen, however, *Bacteroidetes* and *Firmicutes* are the most dominant phyla (54). This result showed that *Bacteroidetes* comprised 45.06%, *Firmicutes* 37.39%, and *Actinobacteria* 11.70% of the total bacterial composition. Similarly, a study found that *Bacteroidetes* (40.95%) and *Firmicutes* (36.36%) are dominant phyla in sheep rumen (55). The main fiber fermenters such as *Prevotella*, *Butyrivibrio*, and

TABLE 9 The correlation between ruminal bacteria community composition (genus) and nutritional disappearance, and rumen fermentation parameters in the two diets.

Item	Genus	Nutritional disappearance and rumen fermentation parameters									
		IVDMD	IVNDFD	IVCPD	pH	NH_3-N	Acetic	Propionic	Butyric	TVFA	A:P
Diet 1	*Prevotella*	0.43*	0.24	−0.02	−0.05	−0.82**	−0.30	0.86**	−0.43*	0.42*	−0.75**
	Selenomonas	0.41*	0.07	−0.36	−0.47*	0.03	−0.49*	0.50*	0.22	0.25	−0.56
	Butyrivibrio	−0.32	−0.68	−0.02	−0.50*	0.22	0.66**	−0.71**	0.46*	−0.12	0.90**
	Succiniclasticum	−0.52	0.37	0.44*	0.66**	−0.37	−0.21	−0.12	−0.82**	−0.49*	−0.06
	Ruminococcaceae_ Ruminococcus	−0.41	−0.64	0.04	−0.34	0.26	0.56*	−0.71**	0.39*	−0.20	0.81**
	Shuttleworthia	0.40	0.24	0.06	0.18	−0.75**	−0.12	0.65**	−0.52*	0.30	−0.53*
	Olsenella	−0.21	0.46*	−0.04	0.45	0.77**	−0.34	−0.23	0.18	−0.29	−0.06
	Bulleidia	−0.39	−0.41*	−0.10	−0.56*	0.40*	0.17	−0.44*	0.48*	−0.13	0.43*
	Treponema	−0.12	0.21	0.35	0.73*	0.29	0.03	−0.32	−0.24	−0.33	0.18
Diet 2	*Prevotella*	−0.19	−0.09	0.13	0.04	−0.78**	0.50*	0.75**	−0.34	0.66**	−0.62**
	Selenomonas	−0.32	−0.12	−0.05	0.10	−0.41*	−0.36	0.35	0.09	0.15	−0.63**
	Butyrivibrio	−0.30	−0.15	−0.57*	−0.77**	0.34	0.13	−0.39	0.49*	−0.18	0.66**
	Succiniclasticum	0.82**	0.27	0.81**	0.74**	0.24	−0.24	−0.36	−0.77**	−0.45*	0.20
	Ruminococcaceae_ Ruminococcus	0.08	−0.15	−0.17	−0.44	0.30	−0.09	−0.57*	−0.03	−0.45*	0.72**
	Shuttleworthia	−0.15	0.05	0.11	0.15	−0.34	0.60**	0.64**	−0.04	0.67**	−0.49**
	Olsenella	−0.56*	−0.13	−0.47*	−0.41	−0.29	−0.28	0.11	0.38	0.03	−0.19
	Bulleidia	−0.38	−0.17	−0.63**	−0.78**	0.27	0.00	−0.36	0.52*	−0.20	0.57*
	Treponema	0.54*	0.25	0.31	0.62**	0.69**	−0.09	−0.34	−0.05	−0.27	0.23

**Indicates that $p < 0.001$, and *indicates when $0.05 \geq p \geq 0.001$. The significance arrangement is columnwise. Where: IVDMD = *in vitro* dry matter disappearance; IVNDFD = *in vitro* neutral detergent fiber disappearance; IVCPD = *in vitro* crude protein disappearance; NH_3-N = ammonia nitrogen; TVFA = total volatile fatty acid, and A:P = acetic to propionic acid ratio.

Pseudobutyrivibrio are members of the phyla *Bacteroidetes* and *Firmicutes* (56), which are also efficient hemicellulose fermenters (50). In the first experiment (diet 1), the relative abundance of *Bacteroidetes* was higher in the control at 12 h; however, at 48 h, OA supplementation significantly increased the abundance, possibly due to the bacteria acclimatizing to oxalic acid. The abundance of *Firmicutes* was not affected. However, supplementation with OA reduced the relative abundance of *Firmicutes*. The interaction between YC and OA increased the abundance of the *Actinobacteria* phylum, which contains the crucial probiotic *Bifidobacterium* genus for gut health (7). Additionally, supplementation with YC and OA interaction significantly increased the abundance of the *Spirochaetes* and *Verrucomicrobia* phyla. The phyla *Spirochaetes* and *Verrucomicrobia* have been identified as possible producers and facilitators of enzymes involved in the degradation of complex polysaccharides such as xyloglucans, peptidoglycans, and pectin (57). In the second experiment (diet 2), supplementation of OA increased the abundance of the *Bacteroidetes* phylum. The YC and OA interaction also significantly increased the composition of this phylum. Both low and high levels of YC supplementation increased the abundance of *Firmicutes*. Furthermore, lower level of YC and higher level of OA, and lower levels of both YC and OA supplementation, also increased the abundance of the *Firmicutes* phylum. The supplementation of YC and OA also increased abundance of the *Actinobacteria* phylum. These results indicate that the interaction of YC and OA enhanced the composition of the rumen bacterial community at the phylum level. Overall, these findings suggest that the supplementation of YC and OA may have a promising effect on increasing the abundance of beneficial bacterial phyla which are primary fermenters of cellulose and hemicellulose (50, 56).

4.3.3 Rumen bacterial genera composition

The dominant genera identified in the recent *in vitro* experiment were *Prevotella, Selenomonas, Butyrivibrio, Succiniclasticum, Ruminococcaceae_Ruminococcus, Shuttleworthia, and Olsenella*. In both diets, YC and OA supplemented treatments increased the abundance of the *Prevotella* genus. Individual supplementation with YC and OA also significantly increased the abundance of this genus. The *Selenomonas* and *Succiniclasticum* genera were dominant and showed a significant increase in the supplemented treatments. However, the *Butyrivibrio* genus was higher in the controls than in the supplemented treatments at 12 and 48 h in both diets. *Prevotella* strains have been found to ferment hemicellulose but exhibit differences in fermentation end products (58). It contains the *Prevotella ruminicola* species which plays a role in nitrogen metabolism and is also a fibrolytic bacteria that possesses carbohydrate esterases, facilitating hemicellulose degradation (59). The *Butyrivibrio* genus can also degrade hemicellulose (arabinoxylans) and pectin and produce butyrate (60). In both diets, the supplementation also showed a higher *Selenomonas* genus at 48 h. It contains the *Selenomonas bovis* species, which could ferment various sugars and produce acetate, propionate, and CO_2 as end products (61, 62). Studies have reported that *Selenomonas* enhances the proliferation of the microbial community in the rumen (63). Additionally, it encompasses several

species that actively participate in the process of nitrate and nitrite reduction inside the rumen (64) and could facilitate propionic acid producing bacteria (65).

This *in vitro* experiment showed that adding YC and OA to hemicellulosic diets significantly altered the composition of beneficial rumen bacterial genera. This could be attributed to the creation of a favorable environment for bacterial growth and performance by the supplemented ingredients. As the pH results indicated, YC and OA supplementation increased the pH level of the ruminal fluid. Additionally, the ammonia nitrogen concentration increased in these experiments, suggesting that bacteria were able to obtain sufficient microbial protein, thereby facilitating their proliferation in the rumen. Hence, this may explain the significant increase in rumen bacteria at the genus and phylum taxonomic levels.

4.3.4 Correlations between rumen fermentation parameters and bacterial genus composition

The correlation between the composition of ruminal bacteria and rumen fermentation parameters provides valuable insights into their roles in the rumen. The dominant genera that were highly abundant in the supplemented groups showed a significant and positive correlation with nutrient disappearance. Specifically, *Prevotella, Selenomonas,* and *RFN20* were among the genera that were greatly increased with YC and OA supplementation and showed significant medium positive correlations with IVDMD in diet 1. Similarly, a report showed that *Prevotella* was positively correlated with digestibility (66). Another study also reported a positive correlation between *Selenomonas* and DM digestibility (67). In diet 2, *Olsenella, Desulfovibrio,* and *Bifidobacterium* showed a significant positive association with the IVNDFD. The IVCPD showed a significant medium positive correlation with *Succiniclasticum* and *Desulfovibrio* genera. The dominant genera also showed a positive correlation with the rumen fermentation parameters. The *Succiniclasticum, Treponema*, *Desulfovibrio*, and *Sharpea* genera showed a highly significant strong positive correlation with pH. Hence, these genera might be responsible for increased ruminal pH by facilitating fiber digestion. *Succiniclasticum dextrinosolvens* and *Succinimonas amylolytica* are prominent species that play a significant role in starch digestion and are responsible for succinate production. This succinate can then be transformed into propionate through the activity of *Selenomonas ruminantium* (68). *Treponema* enhances cellulose and hemicellulose breakdown (69). NH_3-N had a significant and strong positive correlation with the *Olsenella, Bulleidia*, and *Oscillospira* genera. However, it showed a significantly strong negative correlation with the *Prevotella, Shuttleworthia*, and *Bifidobacterium* genera. This correlation analysis showed that changes in the composition of the bacterial community may be responsible for the improved disappearance of nutrients and the production of rumen fermentation products including NH_3-N concentration and volatile fatty acids.

5 Conclusion

Taken together, this study showed that the addition of yeast culture, oxalic acid, or both to hemicellulosic diets can increase the disappearance of dry matter, neutral detergent fiber, and crude protein in the rumen. This supplementation also leads to significant improvement in ruminal pH levels, ammonia nitrogen concentration, acetic acid, propionic acid, and TVFA production. However, it caused a reduction in the acetic to propionic acid ratio. Furthermore, it induced modifications in the diversity, richness, and relative abundance of rumen bacteria across various taxonomic levels. The dominant bacterial genera showed a strong positive correlation with nutrient disappearance and rumen fermentation parameters. Therefore, YC and OA have a great potential to buffer and create a conducive rumen environment and improve rumen fermentation efficiency and hemicellulose digestion.

Data availability statement

The data presented in the study has been deposited in the NCBI Sequence Read Archive (SRA) database (Bio project ID: PRJNA1054784 and PRJNA1054794).

Ethics statement

The Institutional Animal Care Committee of Jilin Agricultural University (JLAU-ACUC2023-003) approved the comprehensive process used in the management of the experimental animals. 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

NA: Data curation, Formal analysis, Methodology, Software, Writing – original draft, Writing – review & editing. LZ: Data curation, Methodology, Writing – original draft. ZW: Data curation, Methodology, Writing – original draft, Software. YX: Data curation, Methodology, Writing – original draft, Software. GY: Data curation, Methodology, Writing – original draft. JD: Data curation, Methodology, Writing – original draft, Software. YZ: Conceptualization, Funding acquisition, Project administration, Resources, Supervision, Validation, Visualization, Writing – review & editing, Data curation. XZ: Conceptualization, Investigation, Resources, Visualization, Writing – review & editing. TW: Conceptualization, Investigation, Resources, Supervision, Validation, Visualization, Writing – review & editing. ZS: Conceptualization, Funding acquisition, Investigation, Project administration, Resources, Supervision, Validation, Writing – review & editing. GQ: Writing – review & editing.

Funding

The author(s) declare financial support was received for the research, authorship, and/or publication of this article. The present work was funded by the Scientific and Technological Developing Scheme of Jilin Province under grant number 20230202075NC.

Conflict of interest

YZ, XZ, TW, ZS, and GQ were employed by Changchun Borui Science and 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.

Publisher's note

All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2023.1330841/full#supplementary-material

References

1. Wang J, Zhao G, Zhuang Y, Chai J, Zhang N. Yeast (*Saccharomyces cerevisiae*) culture promotes the performance of fattening sheep by enhancing nutrients digestibility and rumen development. *Fermentation*. (2022) 8:719. doi: 10.3390/fermentation8120719

2. Hao X, Zhang M, Zhang X, Mu C, Zhang C, Zhao J, et al. Effects of feeding corn bran and soybean hulls on nutrient digestibility, rumen microbial protein synthesis, and growth performance of finishing lambs. *Animal*. (2021) 15:100172. doi: 10.1016/j.animal.2021.100172

3. Holtzapple M. Hemicelluloses In: B Caballero, editor. *Encyclopedia of food science and nutrition*. *2nd* ed. Texas, USA: Academic Press (2003). 3060–71.

4. Weimer P. Degradation of cellulose and hemicellulose by ruminal microorganisms. *Microorganisms*. (2022) 10:2345. doi: 10.3390/microorganisms10122345

5. Perdomo MC, Marsola RS, Favoreto MG, Adesogan A, Staples CR, Santos JEP. Effects of feeding live yeast at 2 dosages on performance and feeding behavior of dairy cows under heat stress. *J Dairy Sci*. (2020) 103:325–39. doi: 10.3168/jds.2019-17303

6. Wang X, Li F, Zhang N, Ungerfeld E, Guo L, Zhang X, et al. Effects of supplementing a yeast culture in a pelleted total mixed ration on fiber degradation, fermentation parameters, and the bacterial community in the rumen of sheep. *Anim Feed Sci Technol*. (2023) 296:115565. doi: 10.1016/j.anifeedsci.2022.115565

7. Liu C, Ma N, Feng Y, Zhou M, Li H, Zhang X, et al. From probiotics to postbiotics: concepts and applications. *Anim ResOne Health*. (2023) 1:92–114. doi: 10.1002/aro2.7

8. Majewska MP, Miltko R, Bełżecki G, Kowalik B. Population of protozoa and carbohydrate-digesting enzymes in the rumen of sheep fed a diet supplemented with yeast *Saccharomyces cerevisiae*. *Small Rumin Res*. (2021) 205:106544. doi: 10.1016/j.smallrumres.2021.106544

9. Shurson GC. Yeast and yeast derivatives in feed additives and ingredients: sources, characteristics, animal responses, and quantification methods. *Anim Feed Sci Technol*. (2018) 235:60–76. doi: 10.1016/j.anifeedsci.2017.11.010

10. Plata F, Bárcena-Gama J. Effect of a yeast culture (*Saccharomyces cerevisiae*) on neutral detergent fiber digestion in steers fed oat straw based diets. *Anim Feed Sci Technol*. (1994) 49:203–10. doi: 10.1016/0377-8401(94)90046-9

11. Dawson KA, Newman KE, Boling JA. Effects of microbial supplements containing yeast and lactobacilli on roughage-fed ruminal microbial activities. *J Anim Sci*. (1990) 68:3392–8. doi: 10.2527/1990.68103392x

12. Lascano GJ, Heinrichs AJ, Tricarico JM. Substitution of starch by soluble fiber and *Saccharomyces cerevisiae* dose response on nutrient digestion and blood metabolites for precision-fed dairy heifers. *J Dairy Sci*. (2012) 95:3298–309. doi: 10.3168/jds.2011-5047

13. Halfen J, Carpinelli N, Del Pino FAB, Chapman JD, Sharman ED, Anderson JL, et al. Effects of yeast culture supplementation on lactation performance and rumen fermentation profile and microbial abundance in mid-lactation Holstein dairy cows. *J Dairy Sci*. (2021) 104:11580–92. doi: 10.3168/jds.2020-19996

14. Duncan AJ, Frutos P, Young S. Rates of oxalic acid degradation in the rumen of sheep and goats in response to different levels of oxalic acid administration. *Anim Sci*. (1997) 65:451–5. doi: 10.1017/S135772980000864X

15. Strobel BW. Influence of vegetation on low-molecular-weight carboxylic acids in soil solution—A review. *Geoderma*. (2001) 99:169–98. doi: 10.1016/S0016-7061(00)00102-6

16. Khampa S, Wanapat MJ. Manipulation of rumen fermentation with organic acids supplementation in ruminants raised in the tropics. *Pak J Nutr*. (2007) 6:20–7. doi: 10.3923/pjn.2007.20.27

17. Palmieri F, Estoppey A, House GL, Lohberger A, Bindschedler S, Chain PSG, et al. Oxalic acid, a molecule at the crossroads of bacterial-fungal interactions. *Adv Appl Microbiol*. (2019) 106:49–77. doi: 10.1016/bs.aambs.2018.10.001

18. Benbati M, Belenguer A, Hervás G, Toral P, Frutos P, Méditerranéens S. Effect of oxalic acid on rumen function and microbiota in sheep fed a low quality diet In: SH Ben, editor. *Options Méditerranéennes: Série A*. Séminaires Méditerranéens: CIHEAM (2013). 133–8.

19. Rahman MM, Nakagawa T, Niimi M, Fukuyama K, Kawamura O. Effects of feeding oxalate containing grass on intake and the concentrations of some minerals and parathyroid hormone in blood of sheep. *Asian Australas J Anim Sci*. (2011) 24:940–5. doi: 10.5713/ajas.2011.10445

20. Chen X. Study on the effective substance of yeast culture regulating rumen function of sheep. [Dissertation]: Jilin Agricultural University, Changchun, China; (2022).

21. Ruminants NRC. *Nutrient requirements of small ruminants: Sheep, goats, cervids, and new world camelids*. USA: The National Academies Press, Washington DC (2007).

22. AOAC. Association of Official Analytical Chemists. *Official methods of analysis. Association of Official Agricultural Chemists (USA)*, Washington, DC (1990). 881–882.

23. Menke K. Estimation of the energetic feed value obtained from chemical analysis and in vitro gas production using rumen fluid. *Anim Res Dev*. (1988) 28:7–55.

24. Huang Z, Urriola PE, Shurson GC. Use of in vitro dry matter digestibility and gas production to predict apparent total tract digestibility of total dietary fiber for growing pigs. *J Anim Sci*. (2017) 95:5474–84. doi: 10.2527/jas2017.1964

25. DeFeo ME, Shampoe KV, Carvalho PHV, Silva FAS, Felix TL. In vitro and in situ techniques yield different estimates of ruminal disappearance of barley. *Transl Anim Sci*. (2020) 4:141–8. doi: 10.1093/tas/txz170

26. Chaney AL, Marbach EP. Modified reagents for determination of urea and ammonia. *Clin Chem*. (1962) 8:130–2. doi: 10.1093/clinchem/8.2.130

27. Kozich JJ, Westcott SL, Baxter NT, Highlander SK, Schloss PD. Development of a dual-index sequencing strategy and curation pipeline for analyzing amplicon sequence data on the MiSeq Illumina sequencing platform. *Appl Environ Microbiol*. (2013) 79:5112–20. doi: 10.1128/AEM.01043-13

28. Nelson A, Stewart CJ. Microbiota analysis using sequencing by synthesis: from library preparation to sequencing. *Methods Mol Biol*. (2020) 2121:165–84. doi: 10.1007/978-1-0716-0338-3_15

29. Chao A. Nonparametric estimation of the number of classes in a population. *Scand J Stat*. (1984) 11:265–70.

30. Shannon CE. A mathematical theory of communication. *Bell Syst Tech J*. (1948) 27:379–423. doi: 10.1002/j.1538-7305.1948.tb01338.x

31. Simpson EH. Measurement of diversity. *Nature*. (1949) 163:688. doi: 10.1038/163688a0

32. R Core Team. *R: A language and environment for statistical computing*. Vienna, Austria: R Foundation for Statistical Computing. (2018).

33. Habeeb AA. Current view of the significance of yeast for ruminants a review 1-role of yeast and modes of action. *Am J Inf Sci Technol*. (2017) 1:8–14. doi: 10.11648/j.ajist.20170101.12

34. Callaway E, Martin SA. Effects of a *Saccharomyces cerevisiae* culture on ruminal bacteria that utilize lactate and digest cellulose. *J Dairy Sci*. (1997) 80:2035–44. doi: 10.3168/jds.S0022-0302(97)76148-4

35. Dias da Silva A, Guedes C, Gomes M, Loureiro N, Brizida E, Mena E, et al. Effects of saccharomyces cerevisciae yeast (CNCM I-1077) on ruminal fermentation and fibre degreadation. Proceedings of the International Symposium for Centre for Animal Nutrition; Role of plant cell walls in dairy cow nutrition; (2010).

36. Zhang J, He H, Yuan Y, Wan K, Li L, Liu A. Effects of yeast culture supplementation on growth performance, nutrient digestibility, blood metabolites, and immune response in geese. *Animals*. (2022) 12:1270. doi: 10.3390/ani12101270

37. Rahman MM, Rahman MR, Niimi M, Khadijah WEW, Akashi R, Abdullah RJ. Effects of different levels of oxalic acid administration on feed intake and nutrient digestibility in goats. *Sains Malays*. (2017) 46:515–9. doi: 10.17576/jsm-2017-4604-01

38. Natnael DA, Tao W, Gui-Xin Q, Yu-Guo Z, Xue-Feng Z, Xue C, et al. Effects of physically effective fiber on rumen and milk parameters in dairy cows: a review. *Indian J Anim Res*. (2020) 54:1317–23. doi: 10.18805/ijar.B-1104

39. Abu El-Kassim M, Abdou S, Hassan E, Abdullah MJ. Effect of macroalgae and yeast culture on body performance, blood metabolites, ruminal fermentation and

digestibility coefficients of Ossimi lambs. *Arch Agri Sci J*. (2021) 4:156–67. doi: 10.21608/aasj.2021.77678.1065

40. Maamouri O, Ben SM. Effect of yeast culture feed supply on growth, ruminal pH, and digestibility of fattening calves. *Food Sci Nutr*. (2021) 9:2762–7. doi: 10.1002/fsn3.2238

41. Belenguer A, Ben Bati M, Hervas G, Toral PG, Yanez-Ruiz DR, Frutos P. Impact of oxalic acid on rumen function and bacterial community in sheep. *Animal*. (2013) 7:940–7. doi: 10.1017/S1751731112002455

42. Shen J, Zheng W, Xu Y, Yu Z. The inhibition of high ammonia to in vitro rumen fermentation is pH dependent. *Front Vet Sci*. (2023) 10:1163021. doi: 10.3389/fvets.2023.1163021

43. Habeeb AA. Importance of yeast in ruminants feeding on production and reproduction. *Ecol Evol Biol*. (2017) 2:49. doi: 10.11648/j.eeb.20170204.11

44. Hernández R, González SS, Pinos-Rodríguez JM, Ortega ME, Hernández A, Bueno G, et al. Effect of a yeast culture on nitrogen balance and digestion in lambs fed early and mature orchard grass. *J Appl Anim Res*. (2009) 35:53–6. doi: 10.1080/09712119.2009.9706984

45. Marden J-P, Julien C, Monteils V, Auclair E, Moncoulon R, Bayourthe C. How does live yeast differ from sodium bicarbonate to stabilize ruminal pH in high-yielding dairy cows? *J Dairy Sci*. (2008) 91:3528–35. doi: 10.3168/jds.2007-0889

46. Bryant RW, Burns ER, Feidler-Cree C, Carlton D, Flythe MD, Martin LJ. Spent craft Brewer's yeast reduces production of methane and ammonia by bovine rumen microbes. *Fanim*. (2021) 2:720646. doi: 10.3389/fanim.2021.720646

47. Nozière P, Glasser F, Sauvant D. In vivo production and molar percentages of volatile fatty acids in the rumen: a quantitative review by an empirical approach. *Animal*. (2011) 5:403–14. doi: 10.1017/S1751731110002016

48. Liu Y-z, Chen X, Zhao W, Lang M, Zhang X-f, Wang T, et al. Effects of yeast culture supplementation and the ratio of non-structural carbohydrate to fat on rumen fermentation parameters and bacterial-community composition in sheep. *Anim Feed Sci Technol*. (2019) 249:62–75. doi: 10.1016/j.anifeedsci.2019.02.003

49. Amin AB, Mao S. Influence of yeast on rumen fermentation, growth performance and quality of products in ruminants: a review. *Anim Nutr*. (2021) 7:31–41. doi: 10.1016/j.aninu.2020.10.005

50. Zhou M, Chen Y, Guan L. Rumen bacteria In: A Puniya, R Singh and D Kamra, editors. *Rumen microbiology: From evolution to revolution*. New Delhi: Springer (2015). 79–95.

51. Mahayri TM, Fliegerová KO, Mattiello S, Celozzi S, Mrázek J, Mekadim C, et al. Host species affects bacterial evenness, but not diversity: comparison of fecal bacteria of cows and goats offered the same diet. *Animals*. (2022) 12:2011. doi: 10.3390/ani12162011

52. Zhang Y, Zhao J, Qin Y, Wang Y, Yu Z, Ning X, et al. Specific alterations of gut microbiota in patients with membranous nephropathy: a systematic review and meta-analysis. *Front Physiol*. (2022) 13:909491. doi: 10.3389/fphys.2022.909491

53. Saleh ASM, Zhang Q, Chen J, Shen Q. Millet grains: nutritional quality, processing, and potential health benefits. *Compr Rev Food Sci F*. (2013) 12:281–95. doi: 10.1111/1541-4337.12012

54. Pinnell L, Reyes A, Wolfe C, Weinroth M, Metcalf J, Delmore R, et al. Bacteroidetes and firmicutes drive differing microbial diversity and community composition between micro-environments in the bovine rumen. *Front Vet Sci*. (2022) 9:897996. doi: 10.3389/fvets.2022.897996

55. Zhang Y, Zhang X, Li F, Li C, Li G, Zhang D, et al. Characterization of the rumen microbiota and its relationship with residual feed intake in sheep. *Animal*. (2021) 15:100161. doi: 10.1016/j.animal.2020.100161

56. Mizrahi I, Wallace RJ, Morais S. The rumen microbiome: balancing food security and environmental impacts. *Nat Rev Microbiol*. (2021) 19:553–66. doi: 10.1038/s41579-021-00543-6

57. Gharechahi J, Vahidi MF, Sharifi G, Ariaeenejad S, Ding XZ, Han JL, et al. Lignocellulose degradation by rumen bacterial communities: new insights from metagenome analyses. *Environ Res*. (2023) 229:115925. doi: 10.1016/j.envres.2023.115925

58. Palevich N, Kelly WJ, Ganesh S, Rakonjac J, Attwood GT. *Butyrivibrio hungatei* MB2003 competes effectively for soluble sugars released by *Butyrivibrio proteoclasticus* B316(T) during growth on Xylan or pectin. *Appl Environ Microbiol*. (2019) 85:e02056–18. doi: 10.1128/AEM.02056-18

59. Kovár L, Savka O, Dušková D, Marounek M. Fermentation of glucose and xylose in *Prevotella ruminicola* AR29. *J Anim Feed Sci*. (1999) 8:115–22. doi: 10.22358/jafs/68814/1999

60. Emerson EL, Weimer PJ. Fermentation of model hemicelluloses by Prevotella strains and *Butyrivibrio fibrisolvens* in pure culture and in ruminal enrichment cultures. *Appl Microbiol Biotechnol*. (2017) 101:4269–78. doi: 10.1007/s00253-017-8150-7

61. Al-Khaldi SF, Durocher LL, Martin SA. Deoxyribonuclease activity in *Selenomonas ruminantium*, Streptococcus bovis, and *Bacteroides ovatus*. *Curr Microbiol*. (2000) 41:182–6. doi: 10.1007/s002840010115

62. Zhang K, Dong X. *Selenomonas bovis* sp. nov., isolated from yak rumen contents. *Int J Syst Evol Microbiol*. (2009) 59:2080–3. doi: 10.1099/ijs.0.007641-0

63. Asanuma N, Yokoyama S, Hino T. Effects of nitrate addition to a diet on fermentation and microbial populations in the rumen of goats, with special reference to S elenomonas ruminantium having the ability to reduce nitrate and nitrite. *Anim Sci J*. (2015) 86:378–84. doi: 10.1111/asj.12307

64. Christodoulou C, Mavrommatis A, Loukovitis D, Symeon G, Dotas V, Kotsampasi B, et al. Effect of Spirulina Dietary Supplementation in Modifying the Rumen Microbiota of Ewes. *Animals*. (2023) 13:740. doi: 10.3390/ani13040740

65. Hobson PN, Mann SO, Smith W. Growth factors for *Selenomonas ruminantium*. *Nature*. (1963) 198:213. doi: 10.1038/198213a0

66. Li S, Zeng H, Wang C, Han Z. Effect of methionine hydroxy Analog on hu sheep digestibility, rumen fermentation, and rumen microbial community in vitro. *Meta*. (2023) 13:169. doi: 10.3390/metabo13020169

67. Sawanon S, Koike S, Kobayashi Y. Evidence for the possible involvement of *Selenomonas ruminantium* in rumen fiber digestion. *FEMS Microbiol Lett*. (2011) 325:170–9. doi: 10.1111/j.1574-6968.2011.02427.x

68. Kumar S, Treloar BP, Teh KH, McKenzie CM, Henderson G, Attwood GT, et al. Sharpea and Kandleria are lactic acid producing rumen bacteria that do not change their fermentation products when co-cultured with a methanogen. *Anaerobe*. (2018) 54:31–8. doi: 10.1016/j.anaerobe.2018.07.008

69. Kudo H, Cheng K-J, Costerton J. Interactions between treponema bryantii and cellulolytic bacteria in the in vitro degradation of straw cellulose. *Can J Microbiol*. (1987) 33:244–8. doi: 10.1139/m87-041

frontiers | Frontiers in Veterinary Science

TYPE Original Research
PUBLISHED 02 February 2024
DOI 10.3389/fvets.2024.1347585

OPEN ACCESS

EDITED BY
Tao Wang,
Jilin Agricultural University, China

REVIEWED BY
Lingyan Li,
Heilongjiang Bayi Agricultural University, China
Lianbin Xu,
Qingdao Agricultural University, China

*CORRESPONDENCE
Yujia Tian
✉ 15810814632@163.com

RECEIVED 01 December 2023
ACCEPTED 10 January 2024
PUBLISHED 02 February 2024

CITATION
Zhao X, Wang Y, Wang L, Sun S, Li C, Zhang X, Chen L and Tian Y (2024) Differences of serum glucose and lipid metabolism and immune parameters and blood metabolomics regarding the transition cows in the antepartum and postpartum period. *Front. Vet. Sci.* 11:1347585. doi: 10.3389/fvets.2024.1347585

Differences of serum glucose and lipid metabolism and immune parameters and blood metabolomics regarding the transition cows in the antepartum and postpartum period

Xinya Zhao[1], Yuxin Wang[1], Luyao Wang[1], Shouqiang Sun[2], Chaoyue Li[2], Xuewei Zhang[1], Long Chen[3] and Yujia Tian[1]*

[1]Tianjin Key Laboratory of Agricultural Animal Breeding and Healthy Husbandry, College of Animal Science and Veterinary Medicine, Tianjin Agricultural University, Tianjin, China, [2]Tianjin Jialihe Animal Husbandry Group Co., Ltd., Tianjin, China, [3]Beijing Dongfang Lianming Technology Development Co., Ltd., Beijing, China

This study aims to investigate differences in metabolism regarding the transition cows. Eight cows were selected for the test. Serum was collected on antepartum days 14th (ap14) and 7th (ap7) and postpartum days 1st (pp1), 7th (pp7), and 14th (pp14) to detect biochemical parameters. The experiment screened out differential metabolites in the antepartum (ap) and postpartum (pp) periods and combined with metabolic pathway analysis to study the relationship and role between metabolites and metabolic abnormalities. Results: (1) The glucose (Glu) levels in ap7 were significantly higher than the other groups ($p < 0.01$). The insulin (Ins) levels of ap7 were significantly higher than pp7 ($p = 0.028$) and pp14 ($p < 0.01$), and pp1 was also significantly higher than pp14 ($p = 0.016$). The insulin resistance (HOMA-IR) levels of ap7 were significantly higher than ap14, pp7, and pp14 ($p < 0.01$). The cholestenone (CHO) levels of ap14 and pp14 were significantly higher than pp1 ($p < 0.01$). The CHO levels of pp14 were significantly higher than pp7 ($p < 0.01$). The high density lipoprotein cholesterol (DHDL) levels of pp1 were significantly lower than ap14 ($p = 0.04$), pp7 ($p < 0.01$), and pp14 ($p < 0.01$), and pp14 was also significantly higher than ap14 and ap7 ($p < 0.01$). (2) The interferon-gamma (IFN-γ) and tumor necrosis factor α (TNF-α) levels of ap7 were significantly higher than pp1 and pp7 ($p < 0.01$); the immunoglobulin A (IgA) levels of pp1 were significantly higher than ap7 and pp7 ($p < 0.01$); the interleukin-4 (IL-4) levels of pp7 were significantly higher than ap7 and pp1 ($p < 0.01$), the interleukin-6 (IL-6) levels of ap7 and pp1 were significantly higher than pp7 ($p < 0.01$). (3) Metabolomics identified differential metabolites mainly involved in metabolic pathways, such as tryptophan metabolism, alpha-linolenic acid metabolism, tyrosine metabolism, and lysine degradation. The main relevant metabolism was concentrated in lipid and lipid-like molecules, organic heterocyclic compounds, organic acids, and their derivatives. The results displayed the metabolic changes in the transition period, which laid a foundation for further exploring the mechanism of metabolic abnormalities in dairy cows in the transition period.

KEYWORDS

transition period, metabolomics, glucose metabolism, lipid metabolism, metabolic pathway

Introduction

During the transition period, cows have to go through complicated physiological processes, such as pregnancy, delivery, and lactation (1). These physiological processes will change the nutritional needs and metabolism of the body. These changes are caused by the increasing nutritional and energy needs at the beginning of milk production, which will result in a negative balance state and even cause metabolic abnormalities, such as insulin resistance and various metabolic diseases (2). The nutritional requirements of peripartum cows, such as carbohydrates, fat, and protein, vary with physiological changes from calving to lactation (3). Large amount of glucose is lost to the breast during pregnancy and lactation, initiating the use of carbohydrate resources (4). Due to the limited supply of glucose in feed energy intake, cows lack carbohydrate intake for a long time, and lactation consumes a large amount of energy, resulting in lower blood glucose. To satisfy energy requirement, the body prioritizes mobilizing fat storage for milk production (5). Triglycerides in adipose tissues are subjected to lipolysis, releasing large amount of non-esterified fatty acids into the blood circulation and esterifying in the liver. However, when the amount of non-esterified fatty acids exceeds the esterification ability of the liver, ketone bodies are formed, resulting in an increase in β-hydroxybutyric acid and the occurrence of ketosis (6). Currently, to prevent and improve the NEB status, nutritional regulation is adopted, for instance, probiotics, prebiotics, dietary lipids, and Chinese medicine compounds, which may also reduce the risk of postpartum disease (7–9). The physiological mechanisms related to the transitional period in dairy cows are not fully understood, and it is unclear whether mutual synergy can better prevent and treat transitional metabolic diseases. Only limited measures can treat metabolic diseases in transition cows, and the investigators try to use metabolomics to address this problem. Metabonomics is helpful to further understand the physiological mechanism of dairy cows in transition period, so as to identify different blood metabolites before and after calving. Thus, providing information is helpful to treat and prevent metabolic diseases of dairy cows in transition period. This method is widely used in the treatment of subclinical mastitis, placenta retention, and ketosis in cows (10–13). Previous studies have assessed the dynamic changes in biological amines, acylcarnitines, glycerophospholipids, and sphingolipids, based on targeted metabolomics. Blood samples at 28 days postpartum showed some increases in AA and sphingomyelin when compared with 7 days postpartum (14). Only the longitudinal changes in the blood metabolome were analyzed to identify new biomarkers. However, few studies clearly indicate that metabolic pathway changes in the whole transition period.

Untargeted metabolomics can detect metabolite differences between the control and test groups (15, 16). Although studies of metabolic diseases in cows have been reported previously, there are still no clearly effective prevention and treatment options in production.

We hypothesized that cows may cause changes in metabolites before and after delivery and may reveal metabolite biomarkers that reflect cows before and after delivery. In this study, biochemical and immune indexes of serum samples from transition cows were determined, and plasma samples from transition cows were analyzed by untargeted metabolomics methods. Moreover, differential metabolites and major metabolic pathways were identified. Meanwhile, biochemical and immune indexes were correlated with differential metabolites. These trials may aid in the prevention and treatment of metabolic abnormalities in cattle and explain the underlying metabolic mechanisms.

Materials and methods

Serum sample collection and biochemical measurements

The present study was carried out in Tianjin Haorui Feng Animal Husbandry Co., Ltd. (Tianjin, China) during February 2022. Twenty transitional cows were randomly selected and eight healthy Holstein cows with similar body condition (3.25 ± 0.5, 5-point scale for body condition score), weight (570 ± 50 kg), and similar pregnancy days were finally selected for testing. The TMR formulas for the pre-partum period and the post-partum period were formulated following the NRC requirements (2001). The cows were fed twice, in the morning and evening, with free water access. Table 1 presents the composition of TMR and the nutritional values. Blood samples were collected from the caudal vein of cows antepartum 14th and 7th days and postpartum 1st, 7th, and 14th days in the morning. Two 10 mL vacuum blood collection tubes and one 10 mL vacuum blood collection tube containing Ethylenediaminetetraacetic acid (EDTA) were used for collection. The whole blood in EDTA tube was directly subpackaged in 2 mL cryovials, and two vacuum blood collection tubes were centrifuged at 3,500 rpm/min for 10 min. The upper serum was collected and subpackaged in 2 mL cryovials, all of which were stored at −80°C refrigerator. Blood samples collected were grouped by time, and serum at antepartum 14th and 7th days and postpartum 1st, 7th, and 14th days was grouped as ap14 and ap7 and pp1, pp7, and pp14, respectively. Moreover, the whole blood in EDTA tube at ap14, ap7, pp1, pp7, and pp14 day was grouped as ap and pp, respectively.

Biochemical and immune measurements

The estimated due date of each cow was estimated, and the glucose levels of the cows were measured using a glucose meter (OGM-161, Harold Beijing Technology Co., Ltd., China) at ap14, ap7, pp1, pp7, and pp14, respectively. In total, 2 mL of frozen storage tubes containing serum was unfrozen.

Measurement method of biochemical indexes: insulin, glycosylated hemoglobin (HbA1c), glucagon (GC), and cholinesterase (AChE) contents of each cow at ap14, ap7, pp1, pp7, and pp14 were measured using enzyme-linked immunosorbent assay (ELISA) kit (Shanghai Jianglai Biotechnology Co., Ltd., China) and Bio-Rad iMark. HOMA-IR (HOMA-IR = Glu (mmol/L) × Ins (mIU/L)/22.5) index was calculated by the steady-state model evaluation method (17, 18). CHO by CHOD-PAP method and triglyceride (TG) by GPO-PAP method, low density lipoprotein cholesterol (DLDL) by direct method-surfactant removal method, and DHDL by direct method-catalase removal method. The kits

TABLE 1 Ingredient composition of diets during the peripartum period.

Items	Prepartum	Postpartum
Ingredient (%)		
Oat hay	25	7
Alfalfa haylage		11
Corn silage	35	29
Cotton seed		4.5
Corn grain ground fine	12	8
Soybean meal	10	5.5
Low erucic acid and low glucoside rapeseed meal	16	15
Haylage		10
Steam corn flakes		4.5
Antifungal agent		0.2
Rumen by pass fats		0.8
Sodium bicarbonate		1.5
Mineral premix	2[1]	3[2]
Chemical analysis[3]		
Net energy for lactation[3], Mcal/kg	1.31	1.74
Dry matter (%)	51.04	53.96
Crude ash (%)	6.21	7.81
Crude protein (%)	18.66	14.83
Crude fiber (%)	1.98	4.28
Neutral detergent fiber (%)	38.44	22.09
Acid detergent fiber (%)	22.16	14.46

[1]Each kilogram of prepartum premix contains vitamin A 32,000 IU, vitamin D3 7,000 IU, dl-a-tocopherol acetate 2,500 mg, and selenium 1.5 mg.
[2]Each kilogram of postpartum premix contains vitamin A 35,000 IU, vitamin D3 10,000 IU, dl-a-tocopherol acetate 2,600 mg, copper 340 mg, manganese 1,500 mg, and zinc 1,500 mg.
[3]Net energy for lactation is the calculated value; the others are the measured values.

were purchased from Zhongsheng Beikong Biotechnology Co., Ltd. The levels of CHO, TG, DLDL, and DHDL were analyzed using a fully automated biochemical analyzer (GLAMOUR 3000, Molecular Devices Co., Ltd., America) at ap14, ap7, pp1, pp7, and pp14 in each cow.

Measurement method of immune indexes: interferon-gamma (IFN-γ), immunoglobulin A (IgA), immunoglobulin G (IgG), immunoglobulin M (IgM), interleukin-1β (IL-1β), interleukin-4 (IL-4), interleukin-6 (IL-6), and tumor necrosis factor α (TNF-α) contents of each cow at ap7, pp1, and pp7 were measured using enzyme-linked immunosorbent assay (ELISA) kit (Shanghai Jianglai Biotechnology Co., Ltd., China) and Bio-Rad iMark.

Untargeted liquid chromatography-MS metabolomic sample measurements

A thawed serum sample (100 μL) was added to a 1.5 mL centrifuge tube, and 400 μL of methanol: acetonitrile (vol/vol = 1:1) was added for extraction. The samples were mixed by vortexing for 30 s and sonicating at 40 kHz and 5°C for 30 min. The samples were allowed to stand at −20°C for 30 min. The supernatants were then centrifuged for 15 min at 13,000 × g and 4°C and dried with nitrogen. To the sample, 120 μL of reconstituted solution (acetonitrile: water =1:1) was added for reconstitution and vortexed again for 30 s. After ultrasonic extraction for 5 min at 40 kHz and 5°C and centrifugation for 10 min at 13,000 × g and 4°C, the supernatants were transferred to autosampled vials and analyzed on the computer. All samples were mixed with 20 μL of supernatant per sample as quality control (QC) samples, and one QC sample was inserted in every 5–15 analysis samples to examine the stability of the overall assay. After the completion of sample pretreatment, the samples were subjected to liquid chromatography-tandem mass spectrometry analysis.

Each sample was analyzed on an Ultra Performance Liquid Chromatography Tandem Fourier Transform Mass Spectrometry (UHPLC-Q Exactive HF-X) system. The system column is ACQUITY UPLC HSS T3 (100 mm × 2.1 mm I.D., 1.8 μm). The column temperature was 40°C. The mobile phase consisted of A (95% water + 5% acetonitrile + 0.1% formic acid) and B (47.5% acetonitrile + 47.5% isopropanol + 5% water + 0.1% formic acid). The gradient elution procedure is presented in Supplementary Table S1, and the injection volume was 3 μL. Samples were ionized by electrospray and collected in positive and negative ion scanning modes (Supplementary Tables S1, S2).

Serum biochemical and immune data processing and statistical analyses

The data of biochemical indexes and immune indexes of cows were analyzed by one-way ANOVA and multiple comparisons with Tukey' s honestly significant difference as *post hoc* test procedure using SPSS software (version 25.0, IBM SPSS). Differences were declared significant at $p < 0.05$ and were declared extremely significant at $p < 0.01$.

Metabolomics data processing and statistical analyses

The raw data were imported into the metabolomics processing software ProgenesisQI (Waters Corporation, Milford, United States) for baseline filtering, peak identification, retention time correction, and peak alignment and finally obtained a data matrix of retention time, mass-charge ratio, and peak intensity. Subsequently, the software was used to identify the feature peak search library and matched the MS and MS/MS mass spectral information with metabolic public database HMDB,[1] Metlin,[2] and Majorbio self-built library. MS mass error was set to less than 10 ppm while metabolites were identified based on second-order MS matching scores. After searching, the matrix data were uploaded to Meiji Biological Cloud Platform[3] for data analysis. Less than 20% of the ion peaks were removed, and the minimum was used to fill the vacancy values and sum normalized, thus reducing sample and instrument errors. The

1 http://www.hmdb.ca/
2 https://metlin.scripps.edu/
3 https://cloud.majorbio.com

TABLE 2 Index related to glucose metabolism at different time in the transition period ($n = 8$).

Index	Time					SEM[4]	p-value
	ap14[3]	ap7	pp1	pp7	pp14		
Glu (mmol/L)	2.56^{b2}	4.78^{a}	3.19^{b}	2.83^{b}	2.99^{b}	0.433	<0.01
Ins (mIU/L)	11.22^{abc}	20.80^{a}	17.07^{ab}	10.06^{bc}	5.59^{c}	3.445	<0.01
HOMA-IR[1]	1.28^{b}	4.52^{a}	2.63^{ab}	1.21^{b}	0.74^{b}	0.697	<0.01
HbA1c (ng/mL)	6.81	8.41	8.53	7.27	6.68	1.236	0.418
GC (pg/mL)	126.44	183.73	167.18	128.16	81.77	39.953	0.118

[1]HOMA-IR (HOMA-IR = Glu (mmol/L) × Ins (mIU/L)/22.5) index was calculated by the steady-state model evaluation method, which is the coefficient of insulin resistance.
[2]a–c Means within a row with different letters differed significantly ($p < 0.05$). Same as below.
[3]The ap is prenatal and the pp is postpartum. Same as below.
[4]SEM is representing the differences between the groups obtained from the multiple comparison method. Same as below.

Variables with relative deviation ≤30% (generally, variables with RSD > 30% fluctuate too much during the experiment) were excluded. Then log10 was used to transform the data, so as to improve the normal distribution of the data structure and reduce the analysis error. The R software package ropls (version 1.6.2) performs orthogonal least squares-discriminant analysis (OPLS-DA) to evaluate the stability of the model. OPLS-DA can eliminate the noise information irrelevant to the classification and can also obtain the relevant metabolite information that leads to significant differences between the two groups. To add a positive exchange calculation over partial least squares-discriminant analysis (PLS-DA), OPLS-DA was used. It filters out the signals irrelevant to the model classification and has interpretation ability. The OPLS-DA model was validated based on Y (R2Y) modeling ability and model (Q2) with 200 iterations and the OPLS-DA displacement test. When the R2Y and Q2 indicators are closer to 1, the more stable and reliable the model is. The Q2 regression line intercept is less than 0, and the model is not overfit. Moreover, Q2 > 0.5 indicates better predictive power of the model (19).

Significant different metabolites were screened based on the variable weight values (VIP) and student's t-test p-values obtained from the OPLS-DA model. Metabolites with VIP >1 and $p < 0.05$ were considered as differential metabolites. Characteristic peak search library identification was performed, and MS information was matched with the metabolic database. MS mass error was set to less than 10 ppm while metabolites were identified based on the secondary MS mating score. The main databases are http://www.hmdb.ca/, https://metlin.scripps.edu/, and other mainstream public databases and self-built databases. In addition, pathways involving differential metabolites were obtained through the metabolic pathway annotation of the KEGG database.[4] Clustering and pathway analysis data were processed and analyzed using the Python software package scipy.stats, to obtain the biological pathway, which was most relevant to experimental processing by Fisher's exact test.

4 https://www.kegg.jp/kegg/pathway.html

Results

Univariate analysis of biochemical indicators

Tables 2, 3 present the results of glucose and lipid metabolism at different times during the transition period. In Table 2, the Glu levels in ap7 were significantly higher than the other time points ($p < 0.01$); the Ins levels of ap7 were significantly higher than pp7 ($p = 0.028$) and pp14 ($p < 0.01$), and pp1 was also significantly higher than pp14 ($p = 0.016$); HOMA-IR levels of ap7 were significantly higher than ap14, pp7, and pp14 ($p < 0.01$). In Table 3, the CHO levels of ap14 and pp14 were significantly higher than pp1 ($p < 0.01$), and the CHO levels of pp14 were significantly higher than pp7 ($p < 0.01$); DHDL levels of pp1 were significantly lower than ap14 ($p = 0.041$), pp7 ($p < 0.01$), and pp14 ($p < 0.01$), and pp14 was also significantly higher than ap14 and ap7 ($p < 0.01$).

Univariate analysis of immune indicators

Table 4 present the results of immune performance at different times during the transition period. The IFN-γ and TNF-α levels of ap7 were significantly higher than pp1 and pp7 ($p < 0.01$); the IgA levels of pp1 were significantly higher than ap7 and pp7 ($p < 0.01$); the IL-4 levels of pp7 were significantly higher than ap7 and pp1 ($p < 0.01$), the IL-6 levels of ap7 and pp1 were significantly higher than pp7 ($p < 0.01$).

Analysis of metabolite profiles of plasma

The results of the RSD represent the degree of dispersion of the data. A lower RSD represents a data point closer to the mean. The standard deviation of the data set is less than 30% of the mean, confirming the consistency and stability of the data. For the overall data, if the QC sample assessment map is RSD <0.3 and the

TABLE 3 Index related to lipid metabolism at different time in the transition period ($n = 8$).

Index	Time					SEM	*p*-value
	ap14	ap7	pp1	pp7	pp14		
AChE (nmol/L)	62.09	66.20	57.21	47.53	41.15	15.645	0.498
TG (mmol/L)	0.50	0.48	1.18	0.34	0.47	0.433	0.377
CHO (mmol/L)	2.17^{ab}	2^{abc}	1.45^{c}	1.78^{bc}	2.54^{a}	0.206	<0.01
DLDL (mmol/L)	0.22	0.2	0.13	0.22	0.16	0.059	0.489
DHDL (mmol/L)	1.21^{b}	1.13^{bc}	0.85^{c}	1.36^{ab}	1.67^{a}	0.121	<0.01

TABLE 4 Index related to immune performance at different time in the transition period ($n = 8$).

Index	Time			SEM	*p*-value
	ap7	pp1	pp7		
IFN-γ (mIU/L)	607.43^{a}	310.64^{b}	332.25^{b}	29.933	<0.01
IgA (ng/mL)	315.75^{b}	906.06^{a}	282.31^{b}	61.575	<0.01
IgG (pg/mL)	2.80	2.61	2.14	0.159	0.228
IgM (mmol/L)	550.25	583.06	558.06	13.833	0.620
IL-1β (mIU/L)	237.73	260.17	190.01	12.900	0.070
IL-4 (ng/mL)	2.64^{b}	1.80^{b}	11.42^{a}	0.925	<0.01
IL-6 (pg/mL)	86.97^{a}	81.43^{a}	58.42^{b}	4.192	<0.01
TNF-α (pg/mL)	18.75^{a}	15.57^{b}	14.92^{b}	0.519	<0.01

cumulative proportion of peaks is >70%, the overall data are qualified (Figure 1).

R2Y (*cum*) and Q2 (*cum*) are used to express the evaluation on the success of OPLS-DA model establishment. The model reliability is expressed as R2Y& Q2 >0.5 (19). The closer to 1, the stronger the reliability is. In the positive ion mode of the OPLS-DA score plot, R2Y = 0.995 and Q2 = 0.928, whereas in the negative ion mode, R2Y = 0.992 and Q2 = 0.93. Both R2Y and Q2 values were greater than 0.5, indicating that the model was stable and reliable (Figures 2A,B). The Q2 intercept values were less than 0.05 (20), indicating that there was no overfitting (Figures 3A,B). The above data indicated that the OPLS-DA model in the ap group and pp group had strong prediction ability, the model was successfully established, and the experimental data were reliable.

Analysis of differential metabolite

A total of 641 differential metabolites were identified, of which, 308 metabolites were upregulated and 333 were downregulated in prenatal compared with postpartum (Figure 4). The cluster heatmap showed that similar metabolites were located in adjacent locations, and the dendrogram showed that the samples from the ap and pp groups can be separated (Figures 5A,B).

Subsequently, the differential metabolites in the Kyoto Encyclopedia of Genes and Genomes pathway database were queried. Metabolic pathways were visualized using iPath 3.0.[5] The nodes in the

5 http://pathways.embl.de

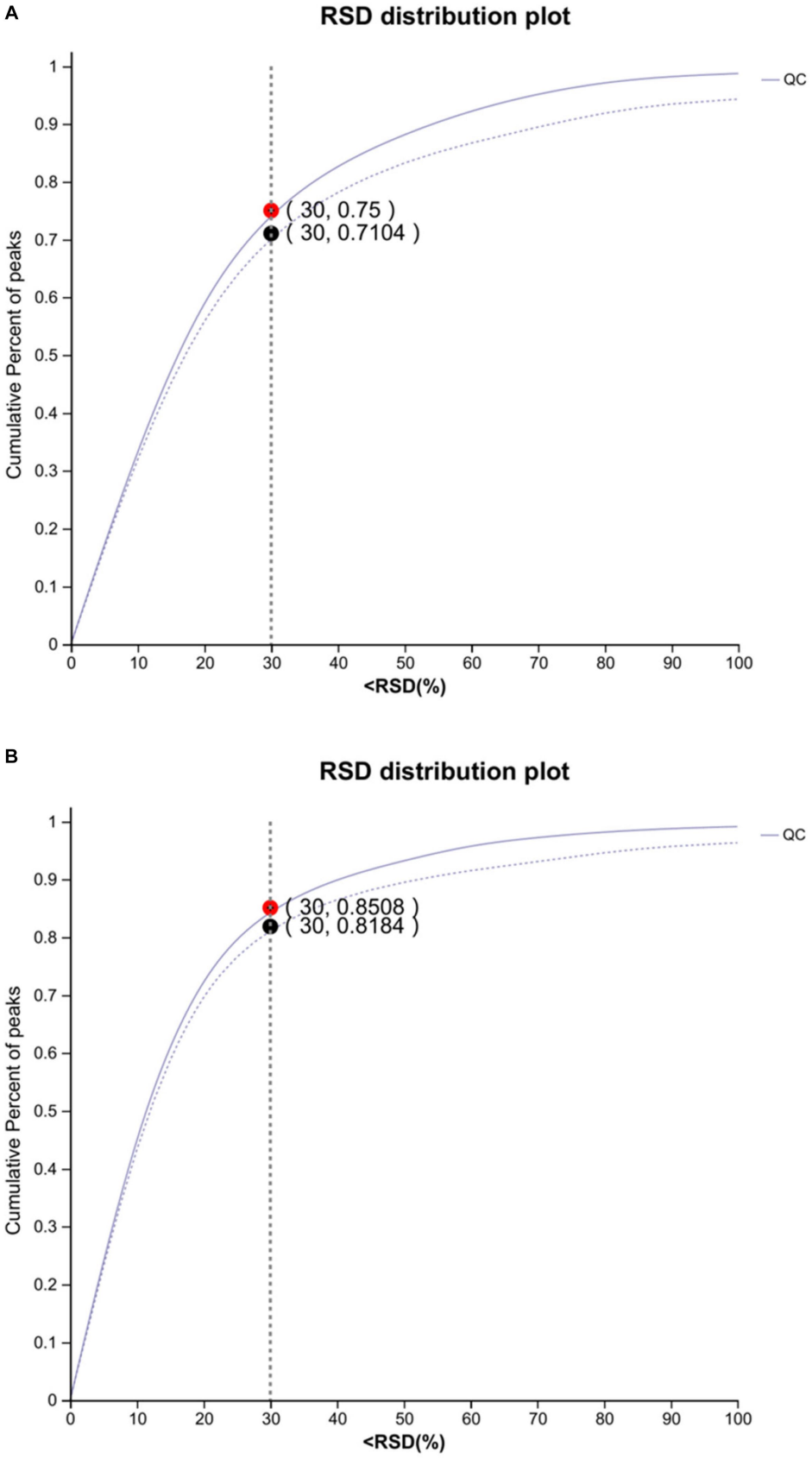


FIGURE 1
Plot of anion **(A)** and cation **(B)** assessment of QC samples (n = 8). The abscissa is the RSD (%) value, the standard deviation/mean, and the ordinate is the cumulative proportion of the ion peaks. (The dotted line indicates before pretreatment and the solid line indicates after pretreatment).

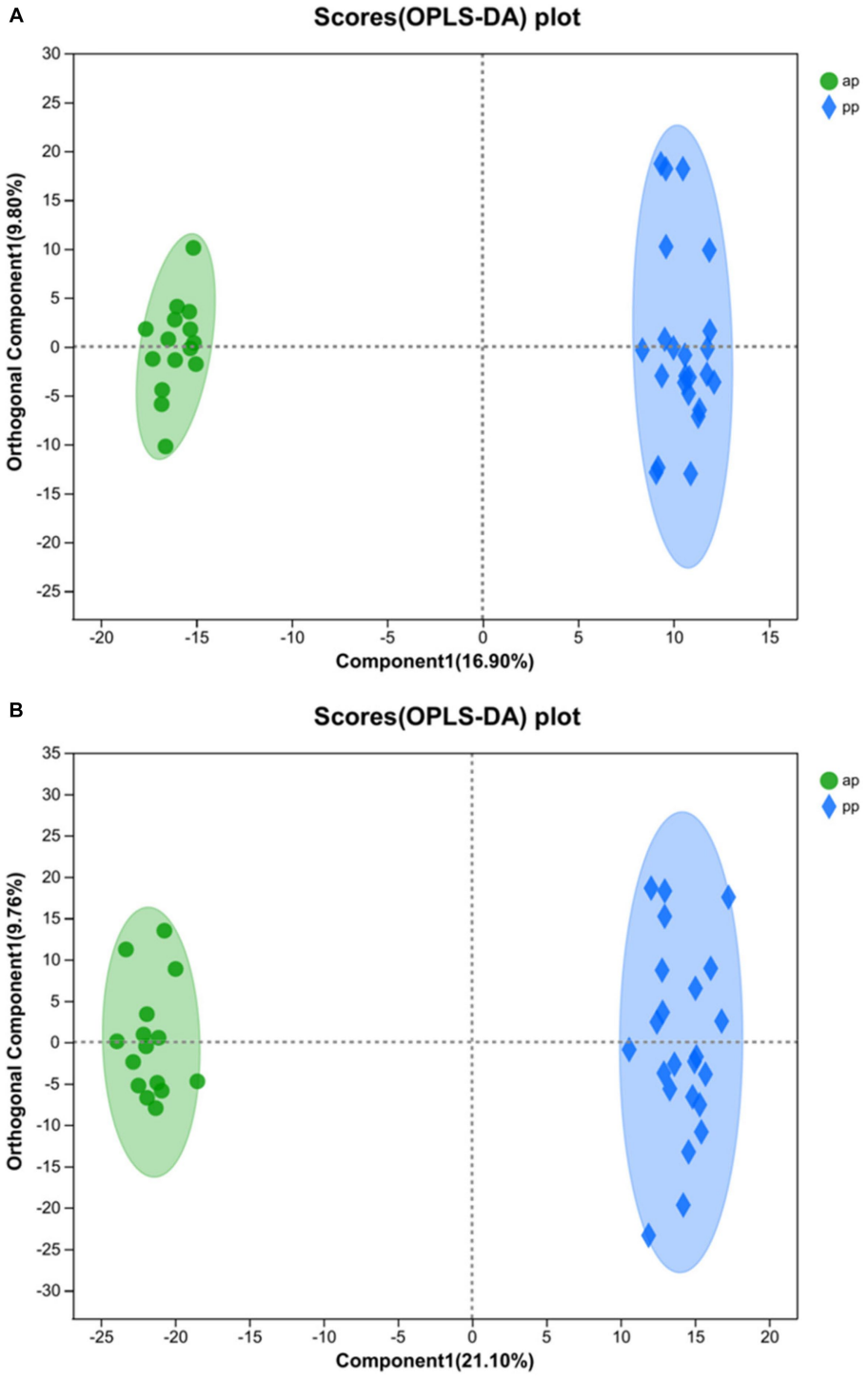


FIGURE 2
OPLS-DA score chart of ap and pp in serum samples for cation **(A)** and anion **(B)** pattern analysis ($n = 8$). Comp1 first predicted principal component interpretation degree, orthogonal Comp1 first orthogonal component interpretation degree. And ap, antenatal sampling time; pp, postnatal sampling time. Same as below.

global overview map represent different compounds, and the boundaries represent different enzymatic reactions (Figure 6). The identified global metabolic pathways were mainly involved in AA metabolism, lipid metabolism, and carbohydrate metabolism. To further screen the pathways, metabolic pathway analysis using metabolic analysis software were performed, and the key pathways with the highest correlation with metabolite differences were found. In total, four major metabolic pathways were affected >0.2, $p < 0.05$. They were tryptophan metabolism, alpha-linolenic acid metabolism, tyrosine metabolism, and lysine degradation (Figure 7).

10.3389/fvets.2024.1347585

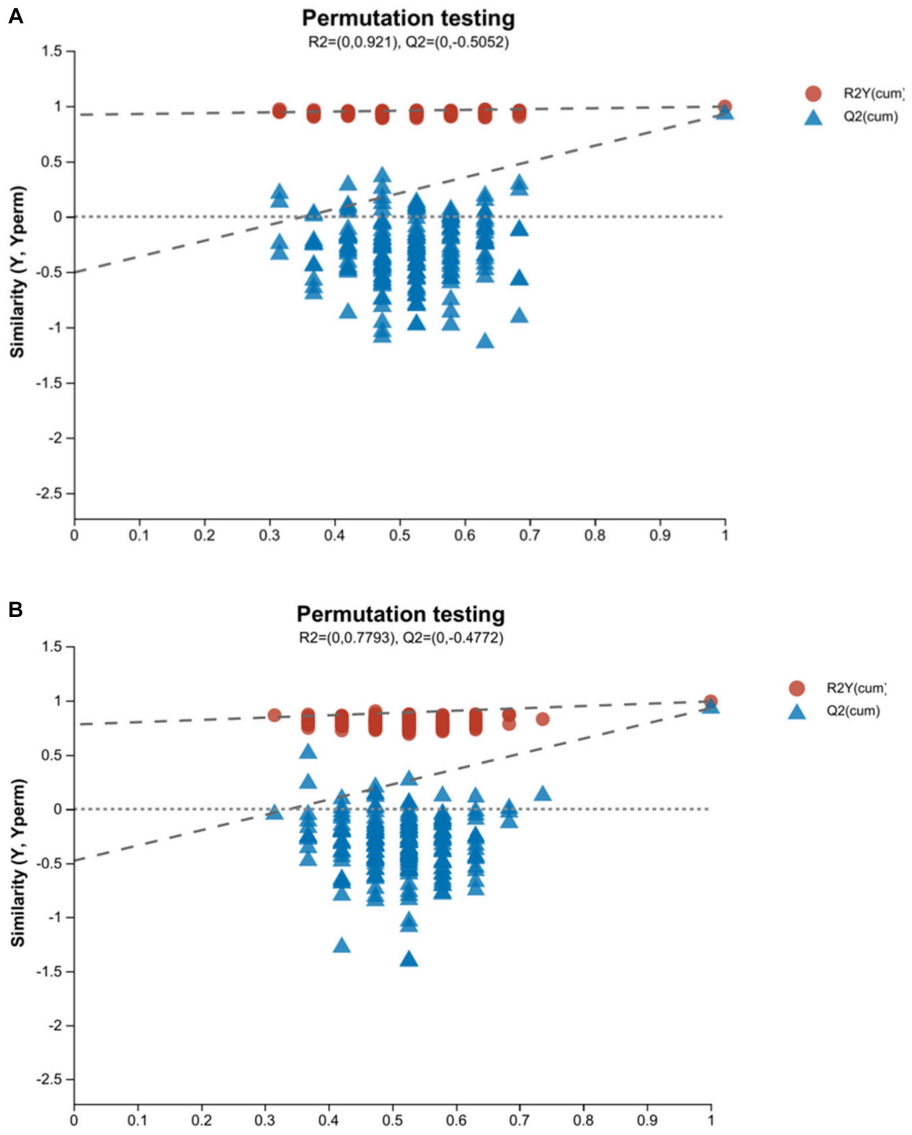


FIGURE 3
OPLS-DA model validation plots of ap and pp in serum samples for cation **(A)** and anion **(B)** pattern analysis ($n = 8$). In this model, R2 is the model interpretation rate, and Q2 is the prediction ability of the model. The abscissa represents the displacement retention degree of the displacement test (the proportion consistent with the order of the *Y* variable in the original model. The point where the displacement retention degree is equal to 1 is the R2 and Q2 values of the original model). The ordinate represents the values of R2 and Q2. The blue dot represents the R2 value obtained by the displacement test. The red square point represents the Q2 value obtained by the displacement test. The two dotted lines, respectively, represent the regression lines of R2 and Q2.

Association analysis between metabolites and biochemical indicators

Metabolites with glucose metabolism index and lipid metabolism index were correlated, respectively. The top 20 abundance metabolites were selected, and the heatmap of their top 50 abundance correlation features is presented in Figure 8. In Figure 8A, Ins levels were compared with 3a, 7b, 12a-trihydroxyoxocholanyl-glycine, glycocholic acid, 4-ethylamino-6-isopropylamino-1, and 3,5-triazin-2-ol, which showed a significant negative correlation. Moreover, Glu level was significantly negatively associated with 3a, 7b, 12a-trihydroxyoxocholanyl-glycine, glycocholic acid, 4-ethylamino-6-isopropylamino-1, 3,5-triazin-2-ol, deoxycholylglycine, deoxycholic acid glycine conjugate, and ergocornine. HOMA-IR showed significant negative correlation with 3a, 7b, 12a-trihydroxyoxocholanyl-glycine, glycocholic acid, 4-ethylamino-6-isopropylamino-1, 3,5-triazin-2-ol, deoxycholylglycine, alpha-muricholic acid, and cholic Acid. HbA1C level showed significant negative correlation with L-tryptophan and indoleacrylic acid. The GC levels showed a significant negative correlation with 3a, 7b, 12a-trihydroxyoxocholanyl-glycine, glycocholic acid, 4-ethylamino-6-isopropylamino-1, 3,5-triazin-2-ol, and deoxycholylglycine.

As shown in Figure 8B, AChE levels showed a significant negative correlation with PC (16:0/0:0) and a significant positive correlation with triacetic acid. The TG levels showed a significant negative correlation with hippuric acid, L-leucine, and

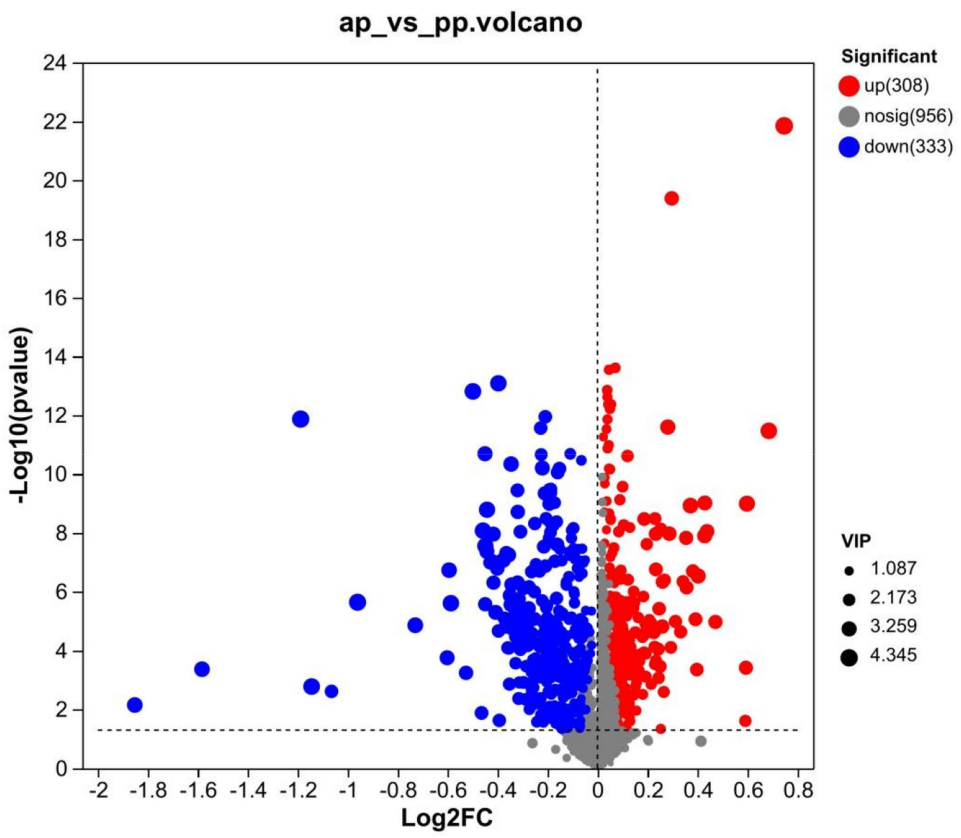


FIGURE 4
Volcano map of differential metabolite screening for groups ap and pp ($n = 8$). Each point in the volcanic map represents a metabolite. The abscissa represents the multiple change (the base-2 log) of the group against each substance, and the ordinate represents the p-value (the base-10 log) of student's t-test. The scatter colors represented the final screening results, with significantly different upregulated metabolites indicated in red, significantly different downregulated metabolites indicated in green, and non-significantly different metabolites indicated in grey.

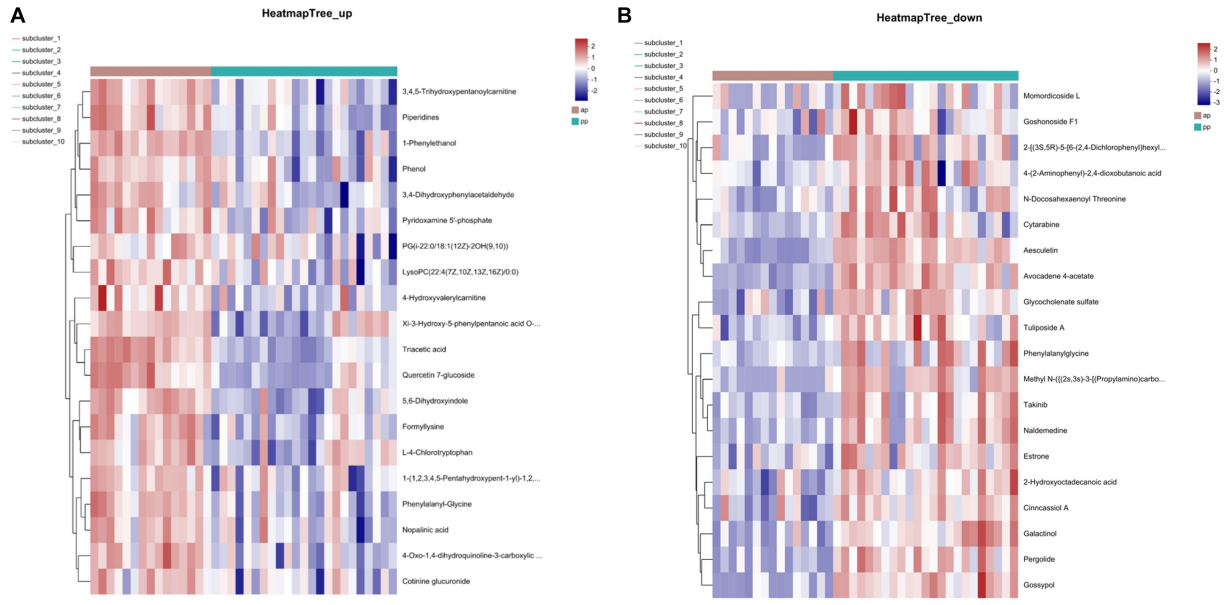


FIGURE 5
Heatmap of the top 20 differential metabolites identified in abundances in serum samples for cation **(A)** and anion **(B)** pattern analysis ($n = 8$).

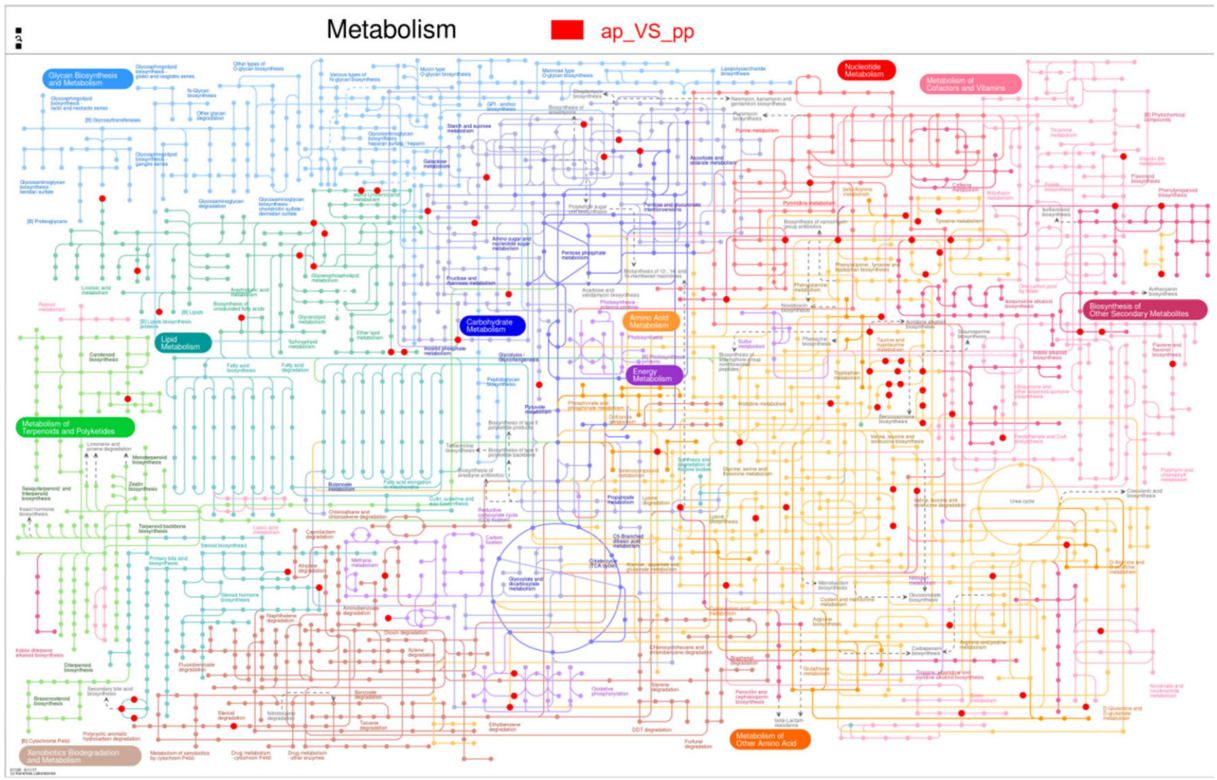


FIGURE 6
Differential metabolites are annotated to the global overview map (n = 8). The pictures represent the pathways annotated by the metabolic set, and one or two metabolic sets can be analyzed; when two metabolic sets are analyzed, different colors represent the pathways annotated by the metabolites in different metabolic sets, and blue represents the pathways jointly annotated by the two metabolic sets. Red dots are the metabolites.

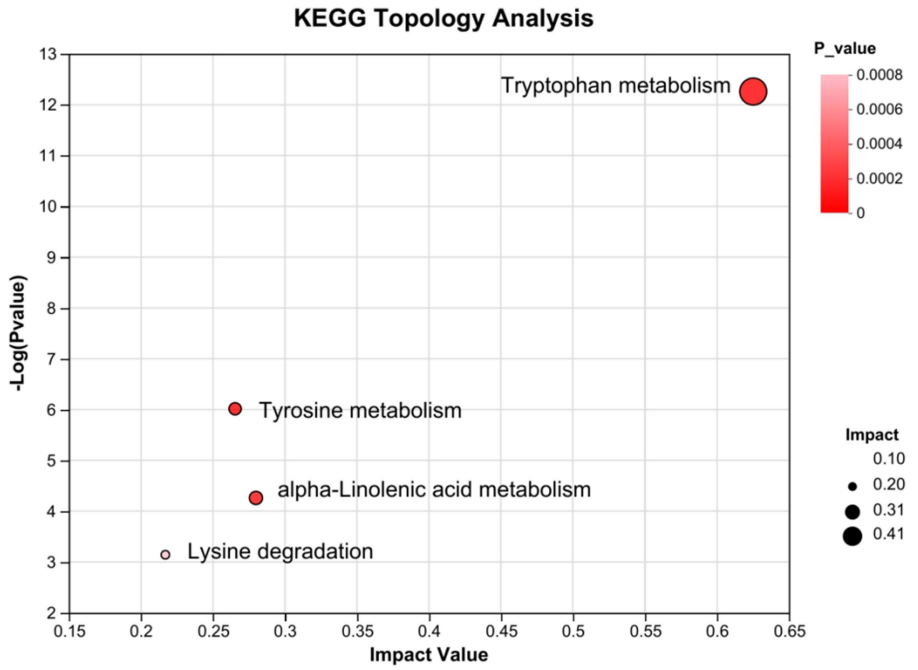


FIGURE 7
KEGG pathway enrichment bubble map by topology analysis (n = 8). Each bubble in the figure represents a KEGG pathway; the horizontal axis indicates the relative importance of the metabolites in the pathway impact value; the vertical axis indicates the significance of the metabolites in the pathway-log10 (p-value); the bubble size represents the impact value; the larger the bubble, the greater the importance of the pathway.

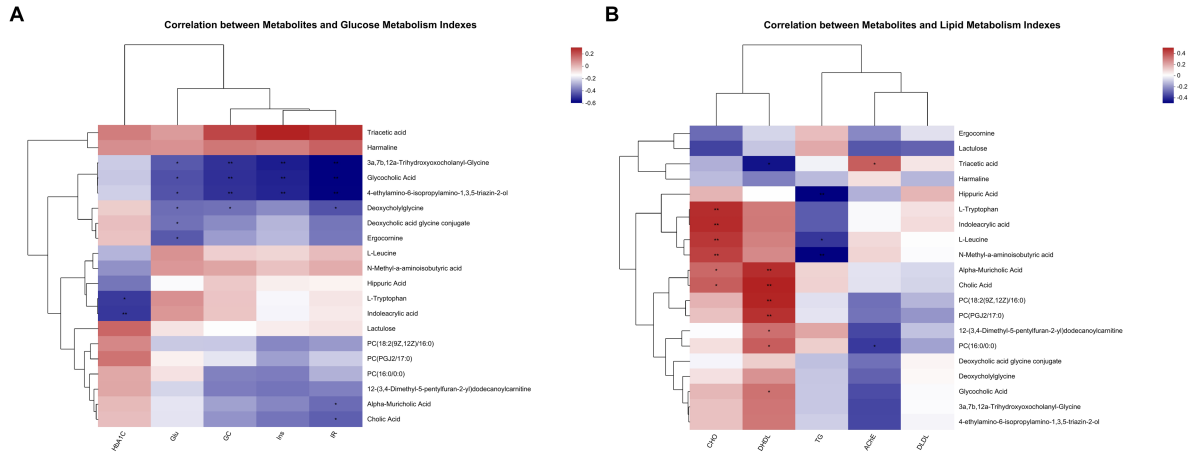


FIGURE 8
(A,B) Shows how the correlation of metabolites with glucose index and the correlation of metabolites with lipid index, respectively (n = 8).

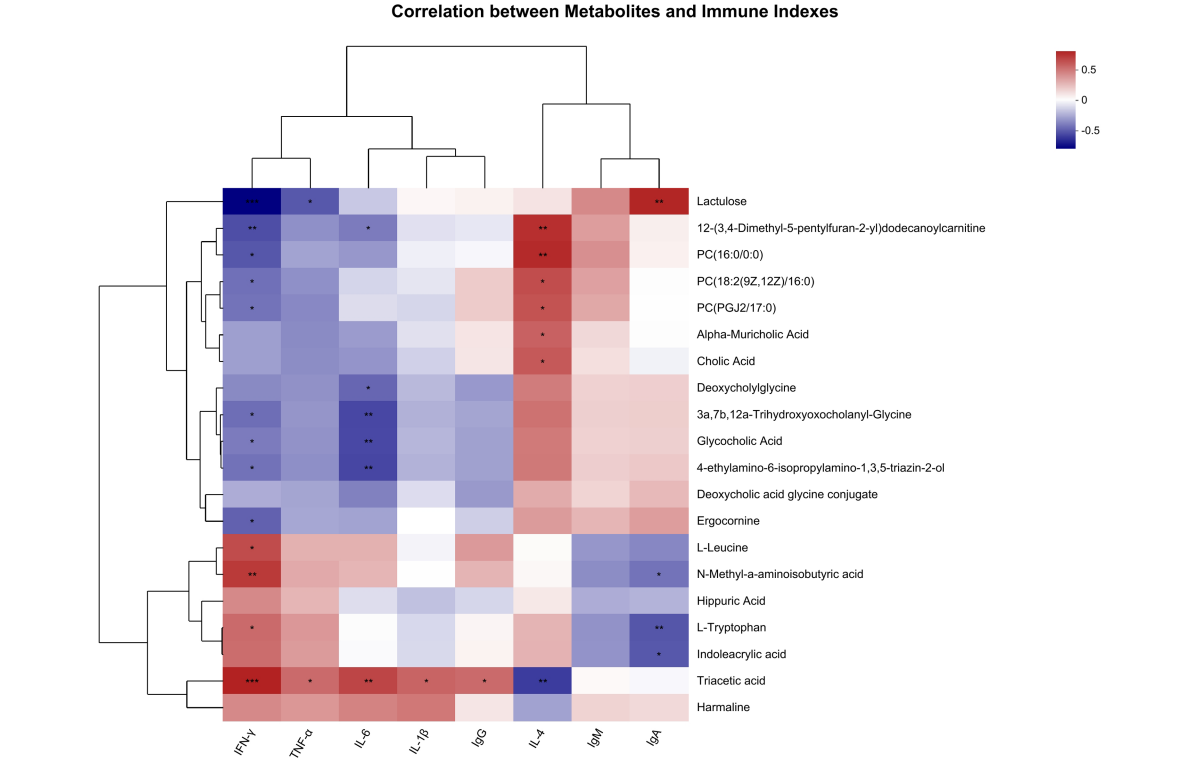


FIGURE 9
Correlation of metabolites with immune index (n = 8).

N-methyl-a-aminoisobutyric acid and significant positive correlation of CHO levels with L-tryptophan, indoleacrylic acid, L-leucine, N-methyl-a-aminoisobutyric acid, alpha-muricholic acid, and cholic Acid. DHDL levels showed significant positive correlation with alpha-muricholic acid, cholic acid, PC (18:2 (9Z, 12Z)/16:0), PC (PGJ 2/17:0), 12-(3,4-dimethyl-5-pentylfuran-2-yl) dodecanoylcarnitine, PC (16:0/0:0), and glycocholic acid and significant negative correlation with triacetic acid.

Association analysis between metabolites and immune indicators

Metabolites with immune indicators were correlated. The top 20 abundance metabolites were selected, and the heatmap of their top 50 abundance correlation features is presented in Figure 9. The IFN-γ levels were compared with triacetic acid, L-leucine, N-methyl-a-aminoisobutyric acid, and L-tryptophan showed a significant positive

correlation and compared with lactulose, ergocomine, glycocholic acid, 3a, 7b, 12a-trihydroxyoxocholanyl-glycine, 4-ethylamino-6-isopropylamino-1, 3,5-triazin-2-ol, 12-(3,4-dimethyl-5-pentylfuran-2-yl) dodecanoylcarnitine, PC (16:0/0:0), PC (18:2 (9Z, 12Z)/16:0), and PC (PGJ 2/17:0) showed a significant negative correlation. TNF-α levels were compared with triacetic acid showed a significant positive correlation and compared with lactulose showed a significant negative correlation. IL-6 levels were compared with triacetic acid showed a significant positive correlation and compared with deoxycholylglycine, glycocholic acid, glycocholic acid, 3a, 7b, 12a-trihydroxyoxocholanyl-glycine, 4-ethylamino-6-isopropylamino-1, 3,5-triazin-2-ol, 12-(3,4-dimethyl-5-pentylfuran-2-yl) dodecanoylcarnitine showed a significant negative correlation. IL-1β and IgG levels were compared with triacetic acid showed a significant positive correlation. IL-4 levels were compared with 12-(3,4-dimethyl-5-pentylfuran-2-yl) dodecanoylcarnitine, PC (16:0/0:0), alpha-muricholic acid, cholic acid, PC [18:2 (9Z, 12Z)/16:0], and PC (PGJ 2/17:0) showed a significant positive correlation and compared with triacetic acid showed a significant negative correlation. IgA levels were compared with lactulose showed a significant positive correlation and compared with N-methyl-a-aminoisobutyric acid, L-tryptophan, indoleacrylic acid showed a significant negative correlation.

Discussion

Changes in perinatal biochemical indicators

During the transition period, plasma Glu levels were significantly associated with the NEB status in ruminants (21). Glu is critical for promoting maximal milk synthesis (22). As a precursor of lactose synthesis, it is also the main energy donor of living cells and an intermediate product of metabolism (3). In this study, the concentration of Glu was the highest at ap7, which may be a large consumption of its own energy materials before and after calving, the feed intake cannot satisfy the nutritional needs of the body, and the body will increase the production of endogenous glucose to maintain glucose homeostasis (8, 23). Ins is a key regulator of maintaining glycemic balance and inhibiting triglyceride lipolysis in adipocytes. Ins has a key role in maintaining the balance of glucose metabolism, with Ins concentration decreases before and after calving (24). In this experiment, the increase in Ins concentration before and after delivery may disrupt the balance of glucose metabolism. This result may be due to the fact that 1 ap7 and pp are in negative energy equilibrium. Moreover, the cows had the potential to develop IR symptoms before and after calving.

Compared with normal condition cows, transition cows secrete various hormones acting against Ins and show greater insulin secretion capacity with greater peak insulin (25). In this study, HOMA-IR at ap7 was significantly higher than at ap14, pp7, and pp14. With fetal growth, insulin resistance significantly increased.

The detection of CHO level in the blood is important for the diagnosis of postpartum health of cows. CHO is mainly synthesized by the liver, and lipid metabolism is mainly completed in the liver. In healthy conditions, fat mobilization accelerates after calving, and cholesterol content will continue to rise. In this study, the CHO content gradually decreased from ap7 and reached the lowest value on the day of delivery and gradually increased later. Consistent with healthy conditions in which fat mobilization accelerates after calving, the cholesterol content will continue to rise. DHDL, as the main lipoprotein for transporting cholesterol, may be an indicator to test whether cows enter NEB (26). This study found that the concentrations of DHDL and DLDL decreased from ap14 to pp1, with the lowest concentration at pp1. It is possibly due to fetal growth and increased maternal demand for cholesterol at the end of pregnancy (27).

Changes in perinatal immune indicators

Immunoglobulin has antibody activity, and its increased content can improve the immune capacity of the animal body. Cytokines are multifunctional protein molecules produced by immune cells and certain non-immune cells after stimulation, which play important roles in immune defense and inflammatory processes (28–30). Immunoglobulin and cytokines jointly participate in the immune process and enhance the body's defense ability. Due to stress, such as pregnancy and deliver, the tissue metabolism of cows during the transition period changes, resulting in the neuroendocrine and immune status, which reduces the immunity (31).

Studies have found that IFN-γ is reduced in the breast before delivery, indicating that susceptibility to disease is related to immune cytokines. Cytokines have important roles in regulating fat mobilization. It can serve as a marker of lipid metabolism (32). The highest IFN-γ levels were tested in ap7, consistent with the findings of Sordillo. This provides further evidence that the transitional disease susceptibility is known through immune cytokines. IgA prevents bacteria or viruses on the mucosal surface and attaches to the mucosa for antibacterial and antiviral protective effects (33).

IL-4 is an anti-inflammatory factor, and the highest level of IL-4 is found in pp7, which may be related to the occurrence of deliver stress. TNF-α has tumor cell-killing and immunomodulatory functions. It can stimulate the synthesis of IL-6, IL-8, and other interleukins, affecting lipid metabolism and sugar metabolism processes (34). IL-6 is dominated by humoral immunity and is a key component in inflammatory cytokines. Studies have shown that prenatal serum TNF-α and IL-6 in transitional cows can cause disturbance of immune function (35). The increase in TNF-α can make the body obtain a strong anti-inflammatory effect and accelerate the body's response to inflammation of TNF-α, and IL-6 levels were minimized at pp7 probably because of the lack of carbohydrate intake after delivery, mobilizing large amount of fat for energy, affecting lipid metabolism and glucose metabolism processes, and causing immune dysfunction.

Overall metabolic pathways mainly in amino acid metabolism and lipid metabolism

The physiological mechanisms associated with delivery in cows are still unclear. Most previous studies have focused on the metabolic regulation of cows by dietary additives, while the metabolism of ruminants itself before and after delivery is rare (36, 37). Therefore, our study analyzed the changes in the prenatal and postnatal blood metabolome. There were two main types of enriched metabolic

metabolites: one is amino acid metabolism, the main metabolic pathways were tryptophan metabolism, tyrosine metabolism, and lysine degradation; the second is lipid metabolism, the main metabolic pathway was alpha-linolenic acid metabolism.

Amino acids are the final absorbed form of the protein. Catabolism of amino acids is also a necessary process to participate in energy production under the negative energy balance. Tryptophan is an important substrate for protein biosynthesis that reduces animal consumption and requires maintenance (38, 39). Tryptophan deficiency can hinder the accumulation of fat in the body. The catabolic pathway of tryptophan is catabolized through the canine urine pathway, promoting the production of antimicrobial peptides to alleviate intestinal inflammatory response and regulating intestinal immune tolerance (40, 41). Tyrosine can be used in the synthesis of adrenaline and thyroxine and regulate glucose metabolism and fat metabolism in the body. The results of this test showed that the tryptophan and tyrosine metabolic pathways were upregulated in prenatal cows than in postnatal metabolism, indicating that the breakdown of tryptophan and tyrosine is promoted, which is not conducive to lipid synthesis in cows. Lysine is one of the ketogenic amino acids when the lack of available carbohydrate can participate in the formation of ketone body and glucose metabolism. A precursor of the synthesis of botulinum alkali is involved in fat metabolism. The significant upregulation of lysine indicates that the ketogenic pathway is more active before delivery than after delivery. Most of glucose which needs in transtion period is provided by liver gluconeogenesis (42). Thus, glycogen coneogenesis is significantly upregulated before delivery to maintain the stable Glu levels (43).

Strengthening lipid metabolism can relieve the body in negative energy balance due to calving and lactation. Alpha-linolenic acid is an essential unsaturated fatty acid that constitutes the fat of the animal body. It is mainly the direct deposition of fatty acids obtained from the diet or the *de novo* synthesis of fatty acids in the body. The results of this test showed that alpha-linolenic acid was downregulated in postpartum cows compared with prenatal production, which may be related to the involvement of fatty acids in lipid synthesis in blood.

Correlation mainly concentrated in lipid and lipid-like molecules, organic heterocyclic compounds, organic acids, and their derivatives

To explore deeply, the top 20 metabolites were associated with glucose metabolism indicators, lipid metabolism indicators, and immune indicators and found that the metabolites were mainly concentrated in lipid and lipid-like molecules, organic heterocyclic compounds, organic acids, and their derivatives. Moreover, these metabolites were upregulated relative to the prenatal period. Bile acids and their derivatives were negatively correlated with glucose metabolism indicators and positively correlated with lipid metabolism indicators. Bile acids come from the catabolism of cholesterol and are a physiological detergent that can promote the digestion and absorption of fat in the intestine and liver (44). DHDL can carry cholesterol in the tissue into bile acid (45). Deoxycholic acid glycine conjugate can dissolve fat for absorption and is absorbed. Cholic acid is the primary bile acid produced by the liver. Moreover, when the content is particularly high, it will damage the liver (46). According to the test results, fat mobilization was accelerated, insulin content and glucose content decreased, total cholesterol content increased, and fat metabolism was more vigorous after calving, possibly because bile acid promotes liver glycogen synthesis (47, 48). In addition, they can also regulate bile flow and lipid secretion and are involved in all key enzyme regulation of cholesterol homeostasis (49–51). According to the test results, bile acids and their derivatives were negatively correlated with IL-4 and were positively correlated with IL-6. Through its correlation, it can improve the body's immune function and maintain its physical health (52–54).

L-tryptophan and indoleacrylic acid belong to indao and its derivatives, and these metabolites were downregulated relative to the prenatal period. The increased absorption capacity of sugars will increase the concentration of HbA1c, which will then affect the tryptophan absorption. Tryptophan is a precursor of serotonin. Serotonin plays a key role in regulating energy metabolism, locomotor activity, and dietary behavior. In turn, the effect of serotonin on metabolic processes is through the activation of the signaling pathway in hypothalamic neurons (55). The metabolism of tryptophan to serotonin requires nutrients such as vitamin B6 and niacin. Niacin is synthesized by canine urine and quinolinic acid. Two tryptophan anabolites were obtained by the canine urine pathway, and canine uric acid, indao, and its derivatives were negatively correlated with IgA and were positively correlated with IFN-γ. It is possible that indoleacrylic acid promotes intestinal epithelial barrier function, stimulates the production of butyryl acrylic acid, and subsequently alleviates the inflammatory response (56).

L-Leucine is a branched-chain amino acid that participates in energy and muscle metabolism. Leucine is only used in the metabolic pathway of ketogenic fat, and the metabolic end products are acetyl-coenzyme A and acetoacetate. Leucine, like other branched-chain amino acids, is implicated in insulin resistance (57). Postnatal L-leucine showed downregulation, positively associated with insulin resistance and IFN-γ, probably because leucine stimulates insulin release and promotes protein biosynthesis (58). This suggests that IFN-γ levels can indirectly indicate an association with the development of insulin resistance.

Strengths and limitations of the study

Although there have been many studies on transitional metabolic disorders, there is still no clear and effective prevention and treatment options in production. Moreover, the results and effects of previous studies are more for other animals, which has great limitations for cows. Whether they are able to better prevent and ameliorate cow in the transition period metabolic disorders through synergistic interaction is not known. This experiment used the metabolic mechanism of cows in the transition period as the entry point and then reveal the different metabolic mechanism and mechanisms of blood metabolites and metabolic pathways at different times in the transition period. These results provided evidence for further exploration of the mechanisms of transitional metabolic abnormalities in dairy cows and could help in the development of new metabolic strategies.

Twenty transitional cows were randomly selected and eight healthy Holstein cows with similar body condition (3.25 ± 0.5, five-point scale for body condition score), weight (570 ± 50 kg), and

similar pregnancy days were finally selected for testing. The sample size of eight cows is quite small. But it still laid a certain foundation for other investigators.

Conclusion

In this study, the results of measuring biochemical indicators and immune indicators found that the tissue metabolism of dairy cows changed during the transition period due to stress, such as pregnancy and deliver. The body mobilized a large amount of fat energy, affecting the lipid metabolism and glucose metabolism processes. It potentially develops both IR and NEB, leading to immune dysfunction. Metabolic changes in transition cows were investigated using an untargeted LC-MS-based metabolomics approach. The global metabolic pathways identified by differential metabolites were mainly involved in amino acid metabolism, lipid metabolism, and carbohydrate metabolism; the key pathways with the highest correlation with metabolites include tryptophan metabolism, alpha-linolenic acid metabolism, tyrosine metabolism, and lysine degradation. These illustrated the metabolic changes in lipids and amino acids during the transition period. Lipid metabolism should be enhanced after delivery to relieve the NEB produced by the body. These data will provide a better understanding of the mechanisms of metabolic disorders during the transition. These may lay the stage for proposing new preventive and control strategies to regulate metabolic disorders, metabolic health, and immune function during the transition period.

Epilogue

The experiment conducted the study of blood glucose metabolic indicators, serum metabolites and serum metabolic pathways at different times in the transition period and to explain the potential metabolic mechanism. In the next trial, we will increase the sample size and evaluate the key parameters of metabolic changes in lactation performance. Further determine the representativeness of the results of this study and strive to provide practical and effective prevention programs for production pastures.

Data availability statement

The original contributions presented in the study are publicly available. This data can be found at: www.ebi.ac.uk/metabolights/MTBLS9144 (9, 59).

Ethics statement

The experimental procedures were approved by College of Animal Science and Veterinary Medicine, Tianjin Agricultural University, Tianjin, China. The Animal Experiment Scheme (protocol number 2021LLSC27) has been audited by the Experimental Animal Ethics Committee, which conforms to the principles of animal protection, animal welfare and ethics, and conforms to the relevant provisions of the national experimental animal welfare ethics.

Author contributions

XiZ: Writing – original draft, Data curation, Investigation, Methodology, Software. YW: Data curation, Project administration, Software, Validation, Writing – review & editing. LW: Conceptualization, Investigation, Validation, Writing – review & editing. SS: Formal analysis, Supervision, Validation, Writing – review & editing. CL: Resources, Validation, Visualization, Writing – review & editing. XuZ: Conceptualization, Methodology, Supervision, Validation, Writing – review & editing. LC: Formal analysis, Funding acquisition, Visualization, Writing – review & editing. YT: Funding acquisition, Methodology, Project administration, Writing – review & editing.

Funding

The author(s) declare financial support was received for the research, authorship, and/or publication of this article. This study was funded by the Research Project of the Regulatory Mechanism of Transition Cows with Type II Ketosis by Compound Nutritional Regulators (2021XY023) and Innovation Team of Tianjin Dairy (Sheep) Research System (ITTCRS2021000). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Acknowledgments

The authors express our appreciation to the Shanghai Meiji Biomedical Technology Co., Ltd. (Shanghai, China) for running the metabolomic analysis and providing helpful insight and guidance for the statistical analysis.

Conflict of interest

SS and CL was employed by Tianjin Jialihe Animal Husbandry Group Co., Ltd. LC was employed by Beijing Dongfang Lianming Technology 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.

Publisher's note

Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2024.1347585/full#supplementary-material

References

1. Mezzetti M, Bionaz M, Trevisi E. Interaction between inflammation and metabolism in periparturient dairy cows. *J Anim Sci*. (2020) 98:S155–74. doi: 10.1093/jas/skaa134

2. Kreipe L, Vernay MC, Oppliger A, Wellnitz O, Bruckmaier RM, van Dorland HA. Induced hypoglycemia for 48 hours indicates differential glucose and insulin effects on liver metabolism in dairy cows. *J Dairy Sci*. (2011) 94:5435–48. doi: 10.3168/jds.2011-4208

3. Loncke C, Nozière P, Vernet J, Lapierre H, Bahloul L, Al-Jammas M, et al. Net hepatic release of glucose from precursor supply in ruminants: a meta-analysis. *Animal*. (2020) 14:1422–37. doi: 10.1017/S1751731119003410

4. Wankhade PR, Manimaran A, Kumaresan A, Jeyakumar S, Ramesha KP, Sejian V, et al. Metabolic and immunological changes in transition dairy cows: a review. *Vet World*. (2017) 10:1367–77. doi: 10.14202/vetworld.2017.1367-1377

5. Wathes DC, Clempson AM, Pollott GE. Associations between lipid metabolism and fertility in the dairy cow. *Reprod Fertil Dev*. (2012) 25:48–61. doi: 10.1071/RD12272

6. Zhang F, Nan X, Wang H, Guo Y, Xiong B. Research on the applications of calcium propionate in dairy cows: a review. *Animals*. (2020) 10:1336. doi: 10.3390/ani10081336

7. Lopreiato V, Mezzetti M, Cattaneo L, Ferronato G, Minuti A, Trevisi E. Role of nutraceuticals during the transition period of dairy cows: a review. *J Anim Sci Biotechnol*. (2020) 11:96. doi: 10.1186/s40104-020-00501-x

8. Luo ZZ, Shen LH, Jiang J, Huang YX, Bai LP, Yu SM, et al. Plasma metabolite changes in dairy cows during parturition identified using untargeted metabolomics. *J Dairy Sci*. (2019) 102:4639–50. doi: 10.3168/jds.2018-15601

9. Thakkar NV, Patel JA. Pharmacological evaluation of Glyoherb: a polyherbal formulation on streptozotocin-induced diabetic rats. *Int. J. Diabetes Dev. Ctries.* (2010) 30:1–7. doi: 10.4103/0973-3930.60001

10. Li Y, Zhao Z, Yu Y, Liang X, Wang S, Wang L, et al. Plasma metabolomics reveals pathogenesis of retained placenta in dairy cows. *Front Vet Sci*. (2021) 8:697789. doi: 10.3389/fvets.2021.697789

11. Li Y, Xu C, Xia C, Zhang H, Sun L, Gao Y. Plasma metabolic profiling of dairy cows affected with clinical ketosis using LC/MS technology. *Vet Q*. (2014) 34:152–8. doi: 10.1080/01652176.2014.962116

12. Zhang G, Tobolski D, Zwierzchowski G, Mandal R, Wishart DS, Ametaj BN. A targeted serum metabolomics GC-MS approach identifies predictive blood biomarkers for retained placenta in Holstein dairy cows. *Meta*. (2021) 11:633. doi: 10.3390/metabo11090633

13. Zhang G, Tobolski D, Zwierzchowski G, Mandal R, Wishart DS, Ametaj BN. Identification of serum-predictive biomarkers for subclinical mastitis in dairy cows and new insights into the pathobiology of the disease. *J Agric Food Chem*. (2022) 70:1724–46. doi: 10.1021/acs.jafc.1c07281

14. Schären M, Riefke B, Slopianka M, Keck M, Gruendemann S, Wichard J, et al. Aspects of transition cow metabolomics-part III: alterations in the metabolome of liver and blood throughout the transition period in cows with different liver metabotypes. *J Dairy Sci*. (2021) 104:9245–62. doi: 10.3168/jds.2020-19056

15. Li S, Wang Q, Lin X, Jin X, Liu L, Wang C, et al. The use of "omics" in lactation research in dairy cows. *Int J Mol Sci*. (2017) 18:983. doi: 10.3390/ijms18050983

16. Schrimpe-Rutledge AC, Codreanu SG, Sherrod SD, McLean JA. Untargeted metabolomics strategies-challenges and emerging directions. *J Am Soc Mass Spectrom*. (2016) 27:1897–905. doi: 10.1007/s13361-016-1469-y

17. Katsuki A, Sumida Y, Gabazza EC, Murashima S, Furuta M, Araki-Sasaki R, et al. Homeostasis model assessment is a reliable indicator of insulin resistance during follow-up of patients with type 2 diabetes. *Diabetes Care*. (2001) 24:362–5. doi: 10.2337/diacare.24.2.362

18. Matthews DR, Hosker JP, Rudenski AS, Naylor BA, Treacher DF, Turner RC. Homeostasis model assessment: insulin resistance and beta-cell function from fasting plasma glucose and insulin concentrations in man. *Diabetologia*. (1985) 28:412–9. doi: 10.1007/BF00280883

19. Xiong QQ, Shen TH, Zhong L, Zhu CL, Peng XS, He XP, et al. Comprehensive metabolomic, proteomic and physiological analyses of grain yield reduction in rice under abrupt drought-flood alternation stress. *Physiol Plant*. (2019) 167, 564–84. doi: 10.1111/ppl.12901

20. Zhang L, Li M, Zhan L, Lu X, Liang L, Su B, et al. Plasma metabolomic profiling of patients with diabetes-associated cognitive decline. *PLoS One*. (2015) 10:e0126952. doi: 10.1371/journal.pone.0126952

21. Duffield TF, Lissemore KD, McBride BW, Leslie KE. Impact of hyperketonemia in early lactation dairy cows on health and production. *J Dairy Sci*. (2009) 92:571–80. doi: 10.3168/jds.2008-1507

22. McCarthy CS, Dooley BC, Branstad EH, Kramer AJ, Horst EA, Mayorga EJ, et al. Energetic metabolism, milk production, and inflammatory response of transition dairy cows fed rumen-protected glucose. *J Dairy Sci*. (2020) 103:7451–61. doi: 10.3168/jds.2020-18151

23. Ghaffari MH, Jahanbekam A, Sadri H, Schuh K, Dusel G, Prehn C, et al. Metabolomics meets machine learning: longitudinal metabolite profiling in serum of normal versus over conditioned cows and pathway analysis. *J Dairy Sci*. (2019) 102:11561–85. doi: 10.3168/jds.2019-17114

24. Petersen MC, Shulman GI. Mechanisms of insulin action and insulin resistance. *Physiol Rev*. (2018) 98:2133–223. doi: 10.1152/physrev.00063

25. Pascottini OB, Leroy JLMR, Opsomer G. Metabolic stress in the transition period of dairy cows: focusing on the prepartum period. *Animals*. (2020) 10:1419. doi: 10.3390/ani10081419

26. Pamir N, Pan C, Plubell DL, Hutchins PM, Tang C, Wimberger J, et al. Genetic control of the mouse HDL proteome defines HDL traits, function, and heterogeneity. *J Lipid Res*. (2019) 60:594–608. doi: 10.1194/jlr.M090555

27. Takahashi T, Mori A, Oda H, Murayama I, Kouno M, Sako T. Comparison of cholesterol levels among lipoprotein fractions separated by anion-exchange high-performance liquid chromatography in periparturient Holstein–Friesian dairy cows. *J Vet Med Sci*. (2021) 83:260–6. doi: 10.1292/jvms.20-0361

28. Fan HK, Cook JA. Molecular mechanisms of endotoxin tolerance. *J Endotoxin Res*. (2004) 10:71–84. doi: 10.1179/096805104225003997

29. Tanaka T, Narazaki M. Kishimoto T.IL-6 in inflammation, immunity, and disease. *Cold Spring Harb Perspect Biol*. (2014) 6:a016295. doi: 10.1101/cshperspect.a016295

30. Zhang J, Liu XQ, Wan CY, Liu Y, Wang YQ, Meng CD, et al. NLRP3 inflammasome mediates M1 macrophage polarization and IL-1β production in inflammatory root resorption. *J Clin Periodontol*. (2020) 47:451–60. doi: 10.1111/jcpe.13258

31. Goff JP, Horst RL. Physiological changes at parturition and their relationship to metabolic disorders. *J Dairy Sci*. (1997) 80:1260–8. doi: 10.3168/jds.S0022-0302(97)76055-7

32. Sordillo L M, Redmond M J, Campos M, Warren L, Babiuk LA. Cytokine activity in bovine mammary gland secretions during the periparturient period. *Can J Vet Res* (1991) 55:298–301.

33. Heybeli C, Oktan MA, Yildiz S, Arda HÜ, Ünlü M, Çavdar C, et al. Clinical significance of mesangial IgM deposition in patients with IgA nephropathy. *Clin Exp Nephrol*. (2019) 23:371–9. doi: 10.1007/s10157-018-1651-6

34. Zanini M, Meyer E, Simon S. Pulp inflammation diagnosis from clinical to inflammatory mediators: a systematic review. *J Endod*. (2017) 43:1033–51. doi: 10.1016/j.joen.2017.02.009

35. Wathes DC, Fenwick M, Cheng Z, Bourne N, Llewellyn S, Morris DG, et al. Influence of negative energy balance on cyclicity and fertility in the high producing dairy cow. *Theriogenology*. (2007) 68:S232–41. doi: 10.1016/j.theriogenology.2007.04.006

36. Añez-Osuna F, Penner GB, Campbell J, Dugan MER, Fitzsimmons CJ, Jefferson PG, et al. Level and source of fat in the diet of gestating beef cows: I. Effects on the prepartum performance of the dam and birth weight of the progeny1. *J Anim Sci*. (2019) 97:3103–19. doi: 10.1093/jas/skz171

37. Mann S, Leal Yepes FA, Duplessis M, Wakshlag JJ, Overton TR, Cummings BP, et al. Dry period plane of energy: effects on glucose tolerance in transition dairy cows. *J Dairy Sci*. (2016) 99:701–17. doi: 10.3168/jds.2015-9908

38. Antonia Álvarez-Fernández M, Fernández-Cruz E, Valero E, Troncoso AM, Carmen G-PM. Efficiency of three intracellular extraction methods in the determination of metabolites related to tryptophan and tyrosine in winemaking yeast's metabolism by LC-HRMS. *Food Chem*. (2019) 297:124924. doi: 10.1016/j.foodchem.2019.05.198

39. Nicholls T, Nitsos I, Walker DW. Tryptophan metabolism in pregnant sheep: increased fetal kynurenine production in response to maternal tryptophan loading. *Am J Obstet Gynecol*. (1999) 181:1452–60. doi: 10.1016/s0002-9378(99)70391-1

40. Hashimoto T, Perlot T, Rehman A, Trichereau J, Ishiguro H, Paolino M, et al. ACE2 links amino acid malnutrition to microbial ecology and intestinal inflammation. *Nature*. (2012) 487:477–81. doi: 10.1038/nature11228

41. Moffett JR, Namboodiri MA. Tryptophan and the immune response. *Immunol Cell Biol*. (2003) 81:247–65. doi: 10.1046/j.1440-1711.2003.t01-1-01177.x

42. Aschenbach JR, Kristensen NB, Donkin SS, Hammon HM, Penner GB. Gluconeogenesis in dairy cows: the secret of making sweet milk from sour dough. *IUBMB Life*. (2010) 62:869–77. doi: 10.1002/iub.400

43. Greenfield RB, Cecava MJ, Donkin SS. Changes in mRNA expression for gluconeogenic enzymes in liver of dairy cattle during the transition to lactation. *J Dairy Sci*. (2000) 83:1228–36. doi: 10.3168/jds.S0022-0302(00)74989-7

44. de Aguiar Vallim TQ, Tarling EJ, Edwards PA. Pleiotropic roles of bile acids in metabolism. *Cell Metab*. (2013) 17:657–69. doi: 10.1016/j.cmet.2013.03.013

45. Schweigert FJ. Effect of gestation and lactation on lipoprotein pattern and composition in dairy cows1. *J Anim Physiol Anim Nutr*. (1990) 63:75–83. doi: 10.1111/j.1439-0396.1990.tb00119.x

46. Delzenne NM, Calderon PB, Taper HS, Roberfroid MB. Comparative hepatotoxicity of cholic acid, deoxycholic acid and lithocholic acid in the rat: *in vivo* and *in vitro* studies. *Toxicol Lett*. (1992) 61:291–304. doi: 10.1016/0378-4274(92)90156-E

47. Lambert G, Amar MJ, Guo G, Brewer HBJ, Gonzalez FJ, Sinal CJ. The farnesoid X-receptor is an essential regulator of cholesterol homeostasis. *J Biol Chem*. (2003) 278:2563–70. doi: 10.1074/jbc.M209525200

48. Potthoff MJ, Boney-Montoya J, Choi M, He T, Sunny NE, Satapati S, et al. FGF15/19 regulates hepatic glucose metabolism by inhibiting the CREB-PGC-1α pathway. *Cell Metab*. (2011) 13:729–38. doi: 10.1016/j.cmet.2011.03.019

49. Chiang JY. Bile acid regulation of hepatic physiology: III. Bile acids and nuclear receptors. *Am J Physiol Gastrointest Liver Physiol*. (2003) 284:G349–56. doi: 10.1152/ajpgi.00417.2002

50. Claudel T, Staels B, Kuipers F. The farnesoid X receptor: a molecular link between bile acid and lipid and glucose metabolism. *Arterioscler Thromb Vasc Biol*. (2005) 25:2020–30. doi: 10.1161/01.ATV.0000178994.21828.a7

51. St-Pierre MV, Kullak-Ublick GA, Hagenbuch B, Meier PJ. Transport of bile acids in hepatic and non-hepatic tissues. *J Exp Biol*. (2001) 204:1673–86. doi: 10.1242/jeb.204.10.1673

52. Huang S, Ma S, Ning M, Yang W, Ye Y, Zhang L, et al. TGR5 agonist ameliorates insulin resistance in the skeletal muscles and improves glucose homeostasis in diabetic mice. *Metabolism*. (2019) 99:45–56. doi: 10.1016/j.metabol.2019.07.003

53. Lazarević S, Đanić M, Goločorbin-Kon S, Al-Salami H, Mikov M. Semisynthetic bile acids: a new therapeutic option for metabolic syndrome. *Pharmacol Res*. (2019) 146:104333. doi: 10.1016/j.phrs.2019.104333

54. Stanimirov B, Stankov K, Mikov M. Bile acid signaling through farnesoid X and TGR5 receptors in hepatobiliary and intestinal diseases. *Hepatobiliary Pancreat Dis Int*. (2015) 14:18–33. doi: 10.1016/s1499-3872(14)60307-6

55. Burke LK, Heisler LK. 5-hydroxytryptamine medications for the treatment of obesity. *J Neuroendocrinol*. (2015) 27:389–98. doi: 10.1111/jne.12287

56. Wlodarska M, Luo C, Kolde R, d'Hennezel E, Annand JW, Heim CE, et al. Indoleacrylic acid produced by commensal *Peptostreptococcus* species suppresses inflammation. *Cell Host Microbe*. (2017) 22:25–37.e6. doi: 10.1016/j.chom.2017.06.007

57. Lynch CJ, Adams SH. Branched-chain amino acids in metabolic signalling and insulin resistance. *Nat Rev Endocrinol*. (2014) 10:723–36. doi: 10.1038/nrendo.2014.171

58. Etzel MR. Manufacture and use of dairy protein fractions. *J Nutr*. (2004) 134:996S–1002S. doi: 10.1093/jn/134.4.996S

59. Haug K, Cochrane K, Nainala VC, Williams M, Chang J, Jayaseelan KV, et al. MetaboLights: a resource evolving in response to the needs of its scientificcommunity. *Nucleic Acids Res*. (2020) 48:D440–4. doi: 10.1093/nar/gkz1019

frontiers | Frontiers in Veterinary Science

TYPE Original Research
PUBLISHED 16 February 2024
DOI 10.3389/fvets.2024.1359234

OPEN ACCESS

EDITED BY
Kai Wang,
Chinese Academy of Agricultural Sciences (CAAS), China

REVIEWED BY
Yongqing Guo,
South China Agricultural University, China
Shoukun Ji,
Hebei Agricultural University, China

*CORRESPONDENCE
Tao Wang
✉ cagewang@163.com
Yuguo Zhen
✉ nickzhen@263.net

†These authors have contributed equally to this work

RECEIVED 21 December 2023
ACCEPTED 01 February 2024
PUBLISHED 16 February 2024

CITATION
Yin G, Sun Z, Wang Z, Xia Y, Cheng L, Qin G, Aschalew ND, Liu H, Zhang X, Wu Q, Zhang W, Zhao W, Wang T and Zhen Y (2024) Mechanistic insights into inositol-mediated rumen function promotion and metabolic alteration using *in vitro* and *in vivo* models. *Front. Vet. Sci.* 11:1359234. doi: 10.3389/fvets.2024.1359234

Mechanistic insights into inositol-mediated rumen function promotion and metabolic alteration using *in vitro* and *in vivo* models

Guopei Yin[1†], Zhe Sun[1,2,3†], Zhanqing Wang[1], Yuanhong Xia[1], Long Cheng[1], Guixin Qin[1], Natnael D. Aschalew[1,4], Hongyun Liu[5], Xuefeng Zhang[1], Qilu Wu[2], Weigang Zhang[3], Wei Zhao[3], Tao Wang[1,3]* and Yuguo Zhen[1,3]*

[1]Key Laboratory of Animal Nutrition and Feed Science of Jilin Province, Key Laboratory of Animal Production Product Quality and Security Ministry of Education, JLAU-Borui Dairy Science and Technology R&D Center, College of Animal Science and Technology, Jilin Agricultural University, Changchun, China, [2]College of Life Sciences, Engineering Research Center of Bioreactor and Pharmaceutical Development, Ministry of Education, Jilin Agricultural University, Changchun, China, [3]Postdoctoral Scientific Research Workstation, Feed Engineering Technology Research Center of Jilin Province, Changchun Borui Science and Technology Co., Ltd., Changchun, China, [4]College of Agriculture and Environmental Science, Dilla University, Dila, Ethiopia, [5]College of Animal Sciences, Zhejiang University, Hangzhou, China

Inositol is a bioactive factor that is widely found in nature; however, there are few studies on its use in ruminant nutrition. This study investigated the effects of different inositol doses and fermentation times on rumen fermentation and microbial diversity, as well as the levels of rumen and blood metabolites in sheep. Rumen fermentation parameters, microbial diversity, and metabolites after different inositol doses were determined *in vitro*. According to the *in vitro* results, six small-tailed Han sheep fitted with permanent rumen fistulas were used in a 3 × 3 Latin square feeding experiment where inositol was injected into the rumen twice a day and rumen fluid and blood samples were collected. The *in vitro* results showed that inositol could increase *in vitro* dry matter digestibility, *in vitro* crude protein digestibility, NH_3-N, acetic acid, propionic acid, and rumen microbial diversity and affect rumen metabolic pathways ($p < 0.05$). The feeding experiment results showed that inositol increased the blood concentration of high-density lipoprotein and IgG, IgM, and IL-4 levels. The rumen microbial composition was significantly affected ($p < 0.05$). Differential metabolites in the rumen were mainly involved in ABC transporters, biotin metabolism, and phenylalanine metabolism, whereas those in the blood were mainly involved in arginine biosynthesis and glutathione and tyrosine metabolism. In conclusion, inositol improves rumen function, affects rumen microorganisms and rumen and blood metabolites and may reduce inflammation, improving animal health.

KEYWORDS

inositol, sheep, rumen fermentation, rumen microorganisms, metabolomics

1 Introduction

Research on ruminant feed nutrition can improve in livestock production (1–4). The rumen is a unique digestive organ that contains many microorganisms living in symbiosis with the host. These bacteria, which are involved in the decomposition and utilization of feeds, produce corresponding metabolites that further promote the growth of the host (5). This complex internal environment makes the nutritional regulation of ruminants difficult; therefore, an environmentally friendly and non-harmful feed additive is imperative to improve rumen fermentation capacity, regulate rumen microorganisms and metabolites, enhance feed-utilization efficiency of ruminants and boost the economic benefits of the farms.

Inositol is a cyclic sugar alcohol widely abundant in fresh fruits, vegetables, and cereals. It is involved in the metabolic activities of cells (6) and is regarded as an active substance essential for the growth of living organisms (7). Our group has previously determined that inositol is a potentially potent metabolite in yeast cultures and is involved in glycine, serine, and threonine metabolic pathways (8). Inositol has many biological functions and plays essential roles in cellular signaling, intracellular transport, and osmoregulation (9, 10). Cellular deficiency of inositol affects phospholipid metabolism and related gene expression and causes cell death (11), demonstrating its essential role in the maintenance of regular cellular activity and metabolic functions. Inositol is often used as a feed additive for aquatic animal production, where it promotes growth and enhances antioxidant capacity (12, 13); however, despite numerous studies in different fields, few have investigated the effects of inositol in ruminants. In this study, the effects of inositol on rumen fermentation function, rumen microbial diversity, blood indices, and rumen and blood metabolites in sheep were investigated. This study provides a reference for the application of inositol in ruminant production.

2 Materials and methods

2.1 Animal ethics

The experiments were conducted at the College of Animal Science and Technology, Jilin Agricultural University. All experimental procedures were performed by the Guidelines for the Care and Use of Experimental Animals of the Jilin Agricultural University (JLAU-ACUC2022-006).

2.2 Experimental design

The experiment involved an *in vitro* and a Latin square feeding experiment. An appropriate dose of inositol was selected for the feeding experiment based on the *in vitro* results. The experimental results were combined to preliminarily investigate the effects of inositol on rumen function, blood indices, and metabolites (Figure 1).

In vitro experiments were designed based on two factors. Factor1 was supplemental inositol dose (Feed grade inositol, 99%; Anhui Yuanzheng Biological, Anhui, China). The experiment included control (0 mg/kg DM), low-dose inositol-supplemented (50, 100, or 200 mg/kg DM), and high-dose inositol-supplemented (400, 800, or 1,600 mg/kg DM) groups. Factor 2 was fermentation time (3, 6, 12, 24, or 48 h). There were 12 replicates in each group (four replicates per experiment; the experiment was repeated three times).

The 3 × 3 Latin square experiment used six experimental animals divided into three groups, 0, 50, or 100 mg/kg DM inositol, with two replicates per group. Each experimental period consisted of 21 days, divided into 14 days for adaptation to the diets and 7 days for sampling. During the sampling period, inositol was injected into the rumen using a disposable syringe every day before feeding. Inositol was diluted with ultra-pure water in advance to the required concentration. The samples were collected on days 1, 4, and 7 for further analysis.

Male small-tail Han sheep (40 ± 1.5 kg) were kept in individual cages and fed twice daily (08:00 and 20:00) at a ratio of 4:6 concentrate (pellet feed; Table 1) to roughage (oat hay), according to the feeding standard of Feed standard of meat-producing sheep and goats (NY/T816-2004). The content of inositol in the diet had been tested by a professional testing company before the experiment, and no inositol content was detected in all the diets.

2.3 *In vitro* experiment

After multi-layer gauze filtration, the rumen fluid was mixed with buffer at a ratio of 1:2 and placed into a fermentation flask containing substrate and inositol. Once the mixture was homogeneous, carbon dioxide was continuously injected into the vessel, and the vessel was placed in a gas-bath oscillator bed for a constant-temperature oscillatory incubation (39°C, 80 rpm). The amount of gas production was recorded with the ANKOM^{RF} gas production system (ANKOM Technology, Macedon, NY, United States). Buffer configuration was based on the method described by Menke and Steingass (14).

2.4 Sample collection

A storage container was pre-warmed and filled with CO_2 to ensure an anaerobic environment for the survival of rumen microorganisms. Rumen fluid was collected from different parts of the rumen of sheep with permanent rumen fistulas on the day of the experiment and mixed before being returned to the laboratory and filtered with multi-layer gauze. During this period, the external ambient temperature (39–41°C) of the rumen fluid was maintained, and CO_2 was continuously injected. Blood was collected from the neck vein into a coagulant tube. The supernatant was collected by centrifugation at 3,000 × g at 4°C for 10 min and stored at −80°C for later use.

2.5 Determination of rumen fermentation parameters

The pH was measured by a Sanxin MP523-04 portable pH meter (Shanghai Sanxin Instrument Co., Ltd., Shanghai, China). The concentration of ammonia nitrogen (NH_3-N) was determined by colourimetric analysis using an ultraviolet spectrophotometer (UV-1201; Shimadzu, Tokyo, Japan). The VFAs were determined by meteorological chromatography (Agilent 7890B, Agilent Technologies, Santa Clara, CA, United States). Total gas production during *in vitro* fermentation was measured using an ANKOM^{RF} gas generator and GPM software (ANKOM Technology). The formula for calculating gas

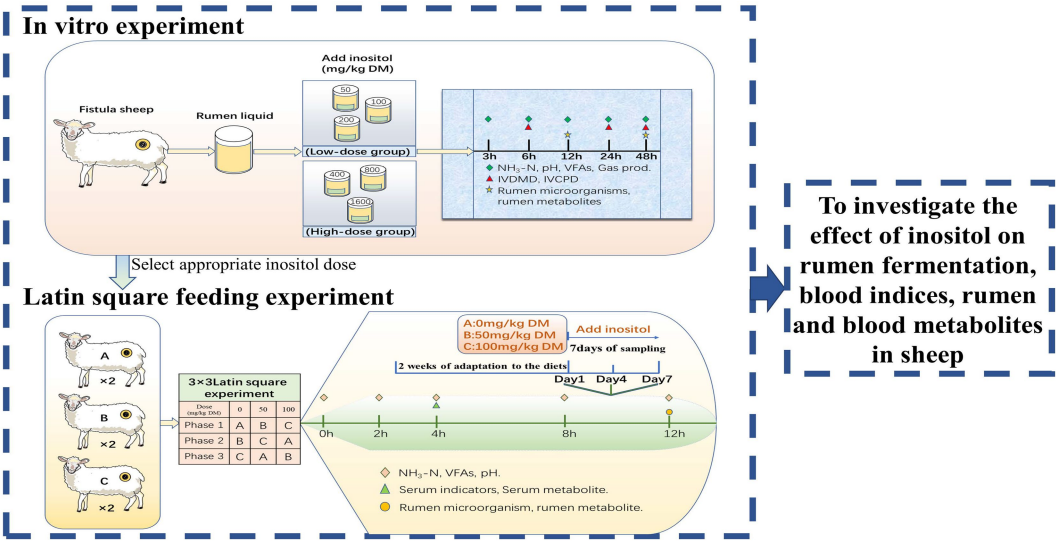


FIGURE 1
Experimental design.

TABLE 1 Ingredients and nutritional composition of pellet feed (dry matter basis, %).

Item	Content
Ingredients	%
Corn	37.0
Brown rice mixture	6.0
Soybean meal	28.5
Cotton meal	1.0
Sesame meal	3.0
Distillers dried grains with soluble	6.0
Spouting corn bran	6.0
Corn bran	6.0
Bentonite	1.0
Molasses	1.5
Premix[1]	4.0
Amount	100
Nutritional composition	%
Metabolic energy (MJ/kg)[2]	11.3
Crude protein	23.8
Neutral detergent fiber	15.0
Acid detergent fiber	7.2
Ether extract	3.9
Ash	11.4
Sugar	6.5
Starch	35.9

[1]Premix composition (per kilogram): $FeSO_4$. 179 mg; $CuSO_4$•$5H_2O$. 23 mg; $ZnSO_4$•$5H_2O$. 92 mg; $MnSO_4$. 70 mg; Vitamin A. 16000 IU; Vitamin D. 111000 IU; Vitamin E. 915 IU.
[2]ME was calculated according to NRC (2001).

production was where V_y is the volume of gas produced in mL; V_x represents the volume of gas in the upper space inside each sample bottle in mL; Ppsi is the cumulative pressure at a certain time of fermentation, expressed in psi. After *in vitro* fermentation, the substrate was dried at 105°C until a constant weight was achieved, and the *in vitro* dry matter digestibility (IVDMD) was calculated. *In vitro* crude protein digestibility (IVCPD) was calculated by mixing the same fermentation substrate and determining the crude protein (15).

2.6 Blood index determination

Total protein, albumin, globulin, total cholesterol, blood glucose, high-density lipoprotein, low-density lipoprotein, triglyceride, urea, free fatty acid, and β-hydroxybutyrate, were determined using a Mindray BS-400 automatic blood biochemical analyzer (Shenzhen, China). Insulin levels were determined using the Insulin Enzyme-Linked Immunoassay Kit (HY-D0001; Beijing Huaying Biological, Beijing, China).

Sheep immunoglobulin A (IgA), Sheep Immunoglobulin G (IgG), Sheep Immunoglobulin M (IgM), and the levels of sheep IL-2, IL-4, IL-6, IL-10, soluble leukocyte differentiation antigen 14 (sCD14), and TNF-α were determined using reagents provided by Shanghai Jining Biological Institute Box. The T-AOC, total oxidant status, total antioxidant status (TAS), GSH-Px, MDA, and SOD were determined using kits provided by the Nanjing Jiancheng Institute of Biotechnology, according to the manufacturer's instructions.

2.7 Determination of rumen microbial diversity

On days 1, 4, and 7, sheep rumen fluid was collected 12 h after morning feeding for microbiological analysis. Total DNA from rumen

microorganisms was extracted using an E.Z.N.A. Soil DNA Kit (D5625-01; Omega Bio-Tek, Norcross, GA, United States), and nucleic acids were quantified using an ultraviolet spectrophotometer. The highly variable V3–V4 region of the bacterial 16S rDNA gene with a length of about 468 bp was selected for sequencing. The V3–V4 region-specific primers F: ACTCCTACGGGAGGCAGCA and R: GGACTACHVGGGTWTCTAAT were selected for PCR amplification. Each primer contained a 7 bp oligonucleotide sequence to distinguish different samples from the same library. Q5 DNA high-fidelity polymerase was used for the PCR (New England Biolabs, Ipswich, MA, United States). The PCR products were quantified on an FLx800 microplate reader (BioTek, Winooski, VT, United States) using the Quant-iT PicoGreen dsDNA Assay Kit, Quantification of the PCR products was performed on an FLx800 microplate reader (BioTek, Winooski, VT, United States) using the Quant-iT PicoGreen dsDNA Assay Kit, and the samples were then normalized. An Illumina TruSeq Nano DNA LT Library Prep Kit (San Diego, CA, USA) was used for library preparation, and an Agilent Sensitivity DNA Kit was used to conduct library quality inspection on an Agilent Bioanalyzer. Paired-end sequencing of the libraries was then performed on an Illumina NovaSeq using the NovaSeq 6000 SP Reagent Kit (500 cycles). The DADA2 plug-in was used to remove primer sequences, filter, denoise, and remove chimeric sequences from the raw data (16).

2.8 Determination of rumen metabolites

One hundred microliters of rumen fluid was placed in a 2 mL centrifuge tube and 400 µL of pre-cooled methanol was added. The samples were vortexed for 30 s and sonicated in a water bath for 15 min, after which they were centrifuged at 13,800 × g for 15 min at 4°C. The supernatants were then concentrated and dried in a lyophilizer. Thirty microlitres of methylammonium salt reagent (1 mL of pyridine per 20 mg of methylammonium hydrochloride) were added to the concentrated samples, which were gently shaken and mixed well before incubating at 80°C for 30 min. Forty microlitres of BSTFA (containing 1% TMCS, v/v, REGIS Technologies) was then added, and the samples were incubated for 1.5 h at 70°C. After incubation, the samples were cooled down to 25°C, and 5 µL of saturated fatty acid methyl ester (FAMEs, dissolved in chloroform) was added. The samples were then tested by gas chromatography (7890B, Agilent) coupled with a time-of-flight mass spectrometer (PEGASUS HT, LECO, GC-TOF-MS).

2.9 Determination of blood metabolites

One hundred microliters of blood sample were placed in a 1.5 mL centrifuge tube, 400 µL of extraction solution (methanol: acetonitrile = 1:1 (V/V), including internal standard) was then added, the sample vortexed for 30 s, sonicated for 10 min in an ice-water bath, and incubated for 1 h at −40°C to precipitate proteins, then centrifuged at 13,800 × g for 15 min at 4°C. The supernatant was carefully withdrawn into a feed bottle. The quality control (QC) sample was prepared by mixing an equal aliquot of the supernatants from all of the samples. LC–MS/MS analyses were performed using a UHPLC system (Vanquish, Thermo Fisher Scientific) with a UPLC BEH Amide column (2.1 mm × 100 mm, 1.7 µm) coupled to Orbitrap Exploris 120 mass spectrometer (Orbitrap MS, Thermo) (17). The mobile phase consisted of 25 mmol/L ammonium acetate and 25 mmol/L ammonium hydroxide in water (pH = 9.75) (A) and acetonitrile (B). The auto-sampler temperature was 4°C, and the injection volume was 2 µL.

2.10 Statistical analysis

The results were analyzed using a general linear model for two-factor trials with IBM SPSS Statistics 23 software (IBM Corp., Armonk, NY, United States). Duncan's multiple comparisons were used to test the level of significance of factors 1 and 2 and their interactions (Factor 1 × Factor 2). p-values with $p < 0.05$ were considered significant differences, and those with $p < 0.01$ were considered highly significant differences.

The rumen metabolome data were analyzed using ChromaTOF v 4.3 software (LECO Corp., St. Joseph, MI, United States) for peak extraction, baseline correction, deconvolution, peak integration, and peak alignment of the mass spectrometry data. The LECO-Fiehn Rtx5 database was used for the substance characterization, including mass spectrum and retention time index matching. Peaks detected in less than half of QC samples or RSD > 30% in QC samples were removed (18).

After the raw data of blood metabolites were converted into mzXML format by ProteoWizard software (Palo Alto, CA, United States), the identified peak maps were processed (peak identification, peak extraction, peak alignment, and integration) using an in-house written R language package with kernel XMCS, and then matched with the secondary mass spectrometry database for substance annotation. The Cutoff value for algorithm scoring was set to 0.3.

3 Results

3.1 Rumen fermentation parameters *in vitro*

The results showed that different inositol doses and fermentation times had highly significant effects on IVDMD and IVCPD ($p < 0.01$). Gas production, NH_3-N, pH, and concentrations of total volatile fatty acid (TVFA), acetic acid, isobutyric acid, butyric acid, isovaleric acid, and valeric acid were significantly affected by the reciprocal effect between the inositol dose and the fermentation time ($p < 0.01$; Table 2). The low-dose inositol groups improved rumen fermentation function, promoted the digestion of nutrients, and increased acid production during rumen fermentation relative to other experimental groups.

3.2 Microbial diversity *in vitro*

Based on the results of the *in vitro* rumen fermentation parameters, the low-dose inositol treatments were selected for rumen microbial diversity analysis at 12 and 48 h. A total of 3,813,300 original sequences were identified in all rumen samples, and 2,258,127 high-quality sequences were obtained.

In all groups, the four most abundant phyla were Bacteroidetes, Firmicutes, Actinobacteria, and Proteobacteria, and the seven most

TABLE 2 *In vitro* rumen fermentation parameters at different inositol doses[1].

		Dose (Inositol mg/kg DM)								*p*-value		
Items	Time (h)	0	50	100	200	400	800	1,600	Sem	Dose	Time	D × T
IVDMD, %	6	24.27^{c}	38.66^{a}	33.01^{ab}	31.94^{b}	17.55^{d}	17.42^{d}	20.11^{cd}	0.01	<0.01	<0.01	0.93
	24	37.76^{ab}	45.23^{a}	44.49^{a}	40.08^{ab}	31.41^{cd}	29.4^{d}	32.6^{bcd}	0.01			
	48	44.59^{bc}	57.56^{a}	54.77^{a}	50.18^{ab}	37.24^{c}	38.28^{c}	40.75^{c}	0.01			
IVCPD, %	6	18^{cd}	26.04^{a}	19.15^{bcd}	25^{ab}	14.55^{d}	19.49^{bcd}	23.22^{abc}	0.009	<0.01	<0.01	<0.01
	24	36.95^{a}	32.03^{ab}	37.08^{a}	31.53^{b}	25.94^{b}	32.42^{ab}	29.6^{ab}	0.009			
	48	45.33^{a}	49.42^{a}	52.72^{a}	52.6^{a}	32.84^{b}	36.76^{b}	32.77^{b}	0.011			
Gas PROD, mL	3	43.81^{ab}	47.41^{ab}	35.23^{bc}	49.57^{a}	43.49^{ab}	24.93^{c}	34.81^{bc}	1.82	<0.01	<0.01	<0.01
	6	84.02^{ab}	78.25^{abc}	71.08^{bc}	75.31^{bc}	94.45^{a}	50.34^{d}	61.78^{cd}	2.49			
	12	126.6^{ab}	149.91^{a}	110.73^{b}	122.37^{ab}	147.88^{a}	73.91^{c}	106.44^{b}	4.3			
	24	134.9^{cd}	246.66^{a}	174.61^{bc}	188.98^{b}	200.71^{b}	127.59^{d}	124^{d}	6.99			
	48	192.36^{bc}	312.59^{a}	259.39^{ab}	257.35^{ab}	272.65^{b}	147.04^{d}	142.7^{d}	11.33			
NH_3-N, mg/dL	3	16.14^{a}	16.69^{a}	18.02^{a}	16.3^{a}	13.06^{b}	10.9^{b}	12.27^{b}	0.47	<0.01	<0.01	<0.01
	6	19.14^{a}	19.9^{a}	19.89^{a}	16.77^{a}	13.22^{b}	12.55^{b}	11.77^{b}	0.58			
	12	27.28^{a}	26.32^{a}	28.12^{a}	25.5^{a}	17.67^{b}	14.5^{b}	13.9^{b}	0.94			
	24	36.35^{a}	31.48^{ab}	30.89^{abc}	35.56^{a}	22.92^{c}	25.78^{bc}	26.61^{bc}	1.11			
	48	65.1^{a}	65.37^{a}	63.12^{a}	63.53^{a}	41.24^{b}	41.58^{b}	44.53^{b}	1.91			
pH value	3	7.19^{ab}	6.97^{cd}	6.88^{d}	7.07^{bc}	7.09^{bc}	7.25^{a}	7.23^{a}	0.02	<0.01	<0.01	<0.01
	6	7.13^{ab}	6.86^{c}	6.80^{c}	7.04^{b}	7.09^{b}	7.21^{a}	7.19^{a}	0.02			
	12	7.09^{ab}	6.58^{c}	6.61^{c}	6.98^{b}	6.98^{b}	7.18^{a}	7.13^{a}	0.03			
	24	6.29^{b}	6.39^{b}	6.47^{b}	6.26^{b}	6.86^{a}	6.79^{a}	6.81^{a}	0.04			
	48	6.22^{b}	6.25^{b}	6.27^{b}	6.14^{b}	6.34^{ab}	6.52^{a}	6.53^{a}	0.03			
TVFA, mmol/L	3	24.70^{bc}	38.91^{a}	41.19^{a}	29.61^{b}	29.33^{b}	17.94^{c}	21.84^{c}	1.24	<0.01	<0.01	<0.01
	6	33.06^{cd}	42.65^{abc}	48.65^{a}	46.01^{ab}	37.89^{bc}	22.37^{e}	25.71^{de}	1.58			
	12	37.96^{c}	59.15^{ab}	65.11^{a}	64.01^{ab}	52.97^{b}	30.67^{c}	35.96^{c}	2.06			
	24	65.08^{b}	85.17^{a}	79.45^{a}	74.70^{b}	69.82^{a}	73.87^{a}	77.59^{a}	4.2			
	48	120.1^{bc}	126.18^{bc}	125.41^{bc}	115.21^{c}	177.58^{a}	154.18^{ab}	137.09^{bc}	4.63			
Acetate acid, mmol/L	3	14.85^{cd}	19.67^{ab}	21.09^{a}	15.34^{c}	16.67^{bc}	11.66^{d}	13.81^{cd}	0.55	<0.01	<0.01	<0.01
	6	19.53^{bc}	21.22^{ab}	24.34^{a}	22.67^{ab}	20.88^{ab}	14.33^{d}	16.25^{cd}	0.66			
	12	22.51^{b}	28.68^{a}	31.24^{a}	32.06^{a}	29.41^{a}	18.96^{b}	21.76^{b}	0.87			
	24	32.32^{b}	50.54^{a}	48.47^{a}	34.85^{b}	44.29^{a}	45.53^{a}	47.70^{a}	1.24			
	48	61.57^{cd}	63.6^{cd}	63.39^{cd}	55.95^{d}	97.76^{a}	87.22^{ab}	77.63^{bc}	2.64			
Propionate acid, mmol/L	3	5.46^{cd}	11.86^{a}	12.62^{a}	8.53^{b}	6.57^{bc}	3.69^{d}	4.92^{cd}	0.48	<0.01	<0.01	<0.01
	6	7.61^{bc}	13.21^{a}	15.34^{a}	14.57^{a}	8.81^{b}	4.87^{c}	5.90^{bc}	0.62			
	12	8.74^{bc}	18.64^{a}	21.22^{a}	18.88^{a}	11.85^{b}	7.37^{c}	9.00^{bc}	0.79			
	24	18.81^{ab}	21.20^{ab}	18.64^{ab}	23.69^{a}	16.36^{b}	16.78^{b}	18.17^{b}	0.66			
	48	32.00^{b}	33.92^{ab}	35.36^{ab}	34.52^{ab}	43.33^{a}	36.46^{ab}	32.30^{b}	3.88			
Isobutyric acid, mmol/L	3	0.49^{abc}	0.59^{a}	0.61^{a}	0.61^{a}	0.57^{ab}	0.41^{c}	0.44^{bc}	0.02	<0.01	<0.01	<0.01
	6	0.66^{ab}	0.66^{ab}	0.78^{a}	0.79^{a}	0.76^{a}	0.48^{b}	0.49^{b}	0.03			
	12	0.75^{bc}	1.00^{ab}	1.14^{a}	1.23^{a}	1.09^{ab}	0.60^{c}	0.65^{c}	0.05			
	24	1.00^{b}	1.15^{ab}	1.30^{ab}	1.34^{a}	1.24^{ab}	1.37^{a}	1.40^{a}	0.04			
	48	1.96^{b}	2.32^{ab}	2.26^{ab}	2.04^{ab}	2.73^{a}	2.35^{ab}	2.23^{ab}	0.08			

(Continued)

TABLE 2 (Continued)

Items	Time (h)	Dose (Inositol mg/kg DM)							Sem	*p*-value		
		0	50	100	200	400	800	1,600		Dose	Time	D × T
Butyric acid, mmol/L	3	2.78^{cd}	4.85^{a}	4.64^{a}	3.42^{bc}	4.10^{ab}	1.40^{e}	1.79^{de}	0.2	<0.01	<0.01	<0.01
	6	3.70^{b}	5.39^{a}	5.40^{a}	5.25^{a}	5.47^{a}	1.71^{c}	2.05^{c}	0.26			
	12	4.06^{b}	7.36^{a}	7.12^{a}	7.76^{a}	7.59^{a}	2.41^{b}	3.07^{b}	0.33			
	24	8.09^{a}	8.13^{a}	7.24^{ab}	8.30^{a}	4.96^{b}	6.19^{ab}	6.33^{ab}	0.32			
	48	14.75^{b}	15.76^{b}	13.83^{b}	12.61^{b}	21.16^{a}	16.74^{ab}	15.52^{b}	0.69			
Isovaleric acid, mmol/L	3	0.74^{ab}	0.87^{a}	0.89^{a}	0.85^{a}	0.89^{a}	0.55^{c}	0.58^{bc}	0.03	<0.01	<0.01	<0.01
	6	1.02^{ab}	0.99^{ab}	1.17^{a}	1.16^{a}	1.27^{a}	0.68^{b}	0.67^{b}	0.05			
	12	1.21^{bc}	1.68^{ab}	1.83^{a}	1.99^{a}	1.97^{a}	0.90^{c}	0.95^{c}	0.08			
	24	1.99	2.24	2.32	2.37	1.79	2.42	2.2	0.11			
	48	4.15^{b}	4.92^{ab}	4.38^{ab}	3.74^{b}	6.03^{a}	5.17^{ab}	4.55^{ab}	0.22			
Valerate acid, mmol/L	3	0.40^{d}	1.07^{ab}	1.34^{a}	0.85^{bc}	0.52^{cd}	0.23^{d}	0.29^{d}	0.06	<0.01	<0.01	<0.01
	6	0.53^{c}	1.17^{b}	1.63^{a}	1.57^{ab}	0.69^{c}	0.30^{c}	0.35^{c}	0.08			
	12	0.69^{c}	1.8^{b}	2.57^{a}	2.09^{ab}	1.07^{c}	0.44^{c}	0.53^{c}	0.12			
	24	2.86^{b}	1.91^{c}	1.48^{c}	3.72^{a}	1.19^{c}	1.58^{c}	1.79^{c}	0.15			
	48	5.69	5.66	6.19	6.36	6.57	6.23	4.86	0.25			
A/P ratio	3	3.01^{a}	1.73^{b}	1.66^{b}	2.23^{b}	3.06^{a}	3.20^{a}	2.81^{a}	0.1	<0.01	<0.01	<0.01
	6	2.82^{a}	1.68^{b}	1.67^{b}	1.74^{b}	2.89^{a}	2.99^{a}	2.76^{a}	0.09			
	12	2.74^{a}	1.63^{b}	1.56^{b}	1.84^{b}	2.85^{a}	2.65^{a}	2.40^{a}	0.08			
	24	0.85^{c}	2.40^{b}	2.63^{a}	0.74^{c}	2.72^{a}	2.72^{a}	2.64^{a}	0.1			
	48	1.97^{bc}	1.97^{bc}	1.86^{c}	1.63^{c}	2.29^{ab}	2.42^{a}	2.49^{a}	0.06			

Gas PROD = gas production; A/P ratio = acetate-to-propionate ratio; SEM = standard error of mean; Dose = different inositol doses; Time = *In vitro* rumen fermentation time; D × T = interaction of different inositol doses and *in vitro* rumen fermentation time. [1]0 mg/kg DM represents basal diet; 50 mg/kg DM, 100 mg/kg DM, 200 mg/kg DM, 400 mg/kg DM, 800 mg/kg DM and 1,600 mg/kg DM represent basal diet supplemented with 50, 100, 200, 400, 800, and 1,600 mg/kg DM inositol, respectively. [a, b, c, d] Means in the same row with different superscripts are significantly different ($p < 0.05$).

abundant genera were *Prevotella*, *Olsenella*, *Succiniclasticum*, *Pseudomonadaceae_Pseudomonas*, *Selenomonas*, *Bulleidia*, and *Desulfovibrio* (Figures 2A,B). At the phylum level, different inositol doses significantly affected the abundance of Firmicutes and Spirochaetes ($p < 0.05$) and Proteobacteria, Tenericutes, and TM7 ($p < 0.01$). Different fermentation times significantly affected the abundance of Firmicutes, Actinobacteria, and TM7 ($p < 0.05$) and Proteobacteria, Synergistetes, Verrucomicrobia, Tenericutes, and Lentisphaerae ($p < 0.01$). The abundance of Bacteroidetes, Proteobacteria, and Synergistetes was significantly affected by the reciprocal effect between the inositol dose and fermentation time ($p < 0.01$). At the genus level, different inositol doses significantly affected the abundance of *Ruminococcus* ($p < 0.05$) and *Prevotella*, *Succiniclasticum*, and *Butyrivibrio* ($p < 0.01$). Different fermentation times significantly affected the abundance of *Prevotella*, *Succiniclasticum*, *Pseudomonadaceae_Pseudomonas*, *Selenomonas*, *Bulleidia*, *Desulfovibrio*, *Butyrivibrio*, and *Oscillospira* ($p < 0.01$). The abundance of *Selenomonas* and *Butyrivibrio* was significantly affected by the reciprocal effect between the inositol dose and fermentation time ($p < 0.01$).

The results for the rumen microbial alpha diversity indices showed that different inositol doses significantly affected the Chao1, Observed_species, Shannon, Simpson, and Goods_coverage indices *in vitro* at 12 h ($p < 0.05$; Figure 2C) and the Shannon indices at 48 h ($p < 0.01$; Figure 2D). In the principal coordinate analysis (PCoA), clustering was distinct between different groups, and there was a clear tendency for dispersion between groups, suggesting that the addition of inositol affected the structure of the rumen microbial communities (Figures 2E,F).

3.3 Rumen metabolites *in vitro*

Rumen metabolite analysis was performed in the low-dose inositol groups at 12 and 48 h. A total of 162 metabolites were identified, of which organic acids and their derivatives accounted for the highest proportion (29.012%) of the total metabolites, lipids and lipid molecules; organic oxygen compounds; benzene ring compounds; polyketones of phenylpropane; nucleosides, nucleotides, and their analogs; organic nitrogen compounds; homogeneous non-metallic compounds; and hydrocarbons accounted for 15.432, 15.432, 8.642, 8.025, 2.469%; 1.235, 0.617, and 0.617%, respectively (Figure 3A).

Principal component analysis (PCA) was performed on all experimental groups. The first principal component (PC1) could explain 12.1% of the variance in the original data set. The PC1 results showed that groups with different fermentation times were separated, indicating that treatment time causes significant changes in

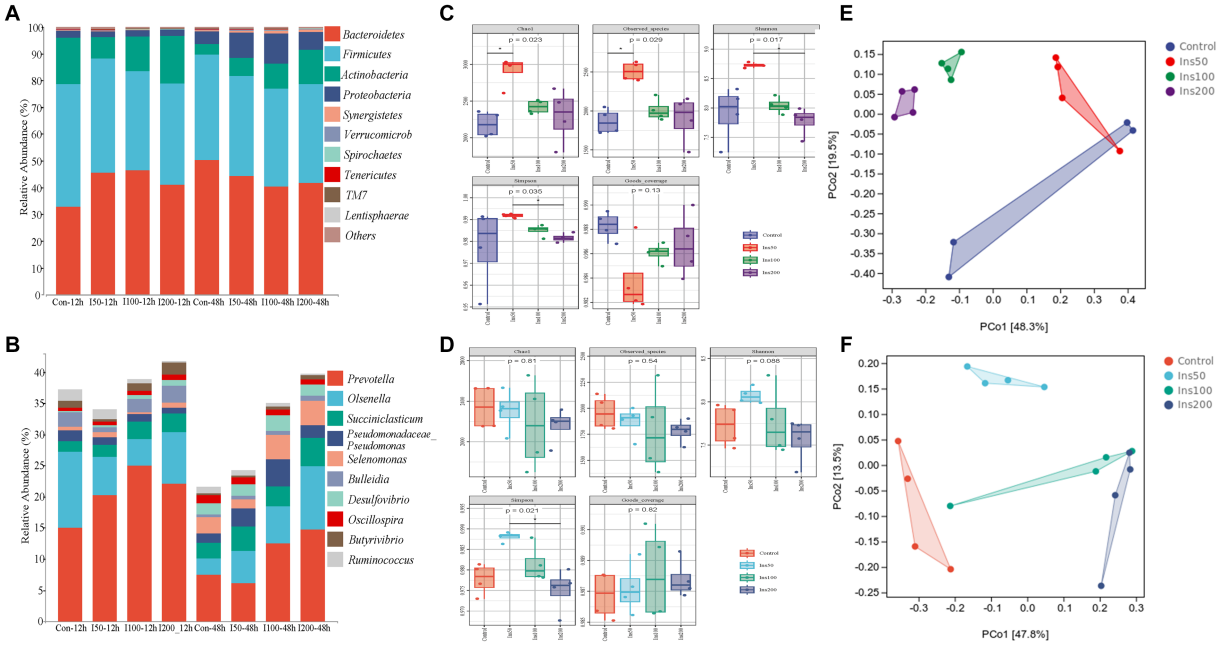


FIGURE 2
Effect of inositol supplementation on ruminal bacterial community *in vitro*. At the **(A)** phylum and **(B)** genus levels. Control-12 h, basal diet. Ins50-12 h, Ins100-12 h, and Ins200-12 h represent basal diet supplemented with 50, 100, and 200 mg/kg DM inositol, respectively, fermented *in vitro* for 12 h; Control-48 h, basal diet; Ins50-48 h, Ins100-48 h, and Ins200-48 h represent basal diet supplemented with 50, 100, and 200 mg/kg DM inositol, respectively, fermented *in vitro* for 48 h. The alpha diversity indices of the ruminal bacterial community. At **(C)** 12 and **(D)** 48 h. The beta diversity was estimated by principal coordinate analysis at **(E)** 12 and **(F)** 48 h. Control represents basal diet. Ins50, Ins100, and Ins200 represent basal diets supplemented with 50, 100, and 200 mg/kg DM inositol, respectively.

metabolites in the samples. Samples in the different regions exhibited unique metabolic profiles. The second principal component explained 8.2% of the variance, and there was a clear separation between the control and the inositol addition groups at 12 h, indicating that the inositol addition treatment had an impact on the metabolites at this time point (Figure 3B).

Volcano plots were used to visualize the differential metabolite data between the inositol-supplemented and control groups at different fermentation times (Figures 3C–H). Multiple differential metabolite screening criteria, such as fold change, *p*-value, and variable importance in the projection (VIP) obtained using the OPLS-DA analysis model, were integrated and analyzed in multiple dimensions to obtain the most accurate differential metabolite analysis.

3.4 Rumen fermentation parameters and blood indices *in vivo*

Based on the results of the *in vitro* experiment, inositol doses of 0, 50, and 100 mg/kg DM were selected for the feeding experiment. The experimental results showed that different concentrations of inositol had no significant effect on volatile fatty acids (VFA) and NH_3-N in the rumen, and none of the rumen fermentation parameters were affected by the interaction between inositol and experimental time points ($p > 0.05$; Supplementary Table S1).

The blood indices showed that there were no significant differences in total protein, albumin, globulin, total cholesterol, blood glucose, high-density lipoprotein, low-density lipoprotein, triglyceride, urea, free fatty acid, and β-hydroxybutyrate after inositol supplementation ($p > 0.05$). On day four, blood insulin levels in the inositol-supplemented group were significantly higher than those in the control ($p < 0.05$; $p < 0.01$; Table 3). Blood immune and oxidative stress indices showed no significant difference between the different doses of inositol, but IL-10, and superoxide varied after inositol supplementation and showed an increasing trend with the increasing days of treatment (Table 4). At each sampling time, blood HDL levels in the 50 mg/kg DM inositol group showed an increase compared to the control (Table 4). The addition of inositol increased glutathione peroxidase (GSH-Px) and decreased MDA (Table 4).

3.5 Microbial diversity in the rumen of sheep

A total of 3,102,488 original sequences were identified, and after removing low-quality sequences 2,690,978 valid sequences were obtained. After clustering and removing chimeras, a total of 2,513,273 high-quality sequences were analyzed. Bacteroidetes, Firmicutes, Actinobacteria, TM7, Tenericutes, Spirochaetes, Proteobacteria, and Verrucomicrobia were the most abundant phyla; and *Prevotella*, *RFN20*, *Butyrivibrio*, *Ruminococcus*, *CF231*, *BF311*, *Treponema*, *Clostridium*, and *Succiniclasticum* were the nine most abundant bacterial genera (Figures 4A,B).

The interaction between the inositol dose and the time of addition had significant effects on each phylum, except for Spirochaetes. The abundance of Bacteroidetes in the control was significantly higher than that in the 50 mg/kg DM ($p < 0.05$) and 100 mg/kg DM groups ($p < 0.01$). The abundance of Firmicutes in the 100 mg/kg DM group

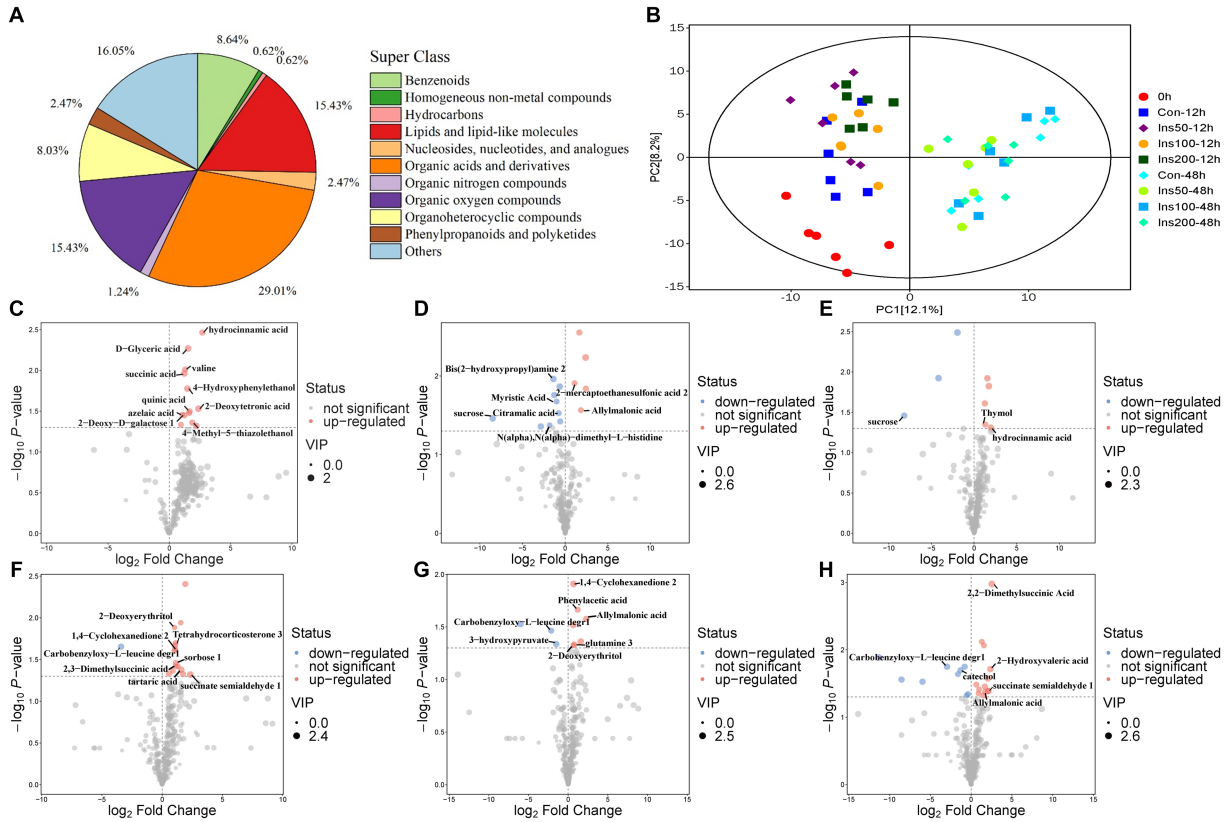


FIGURE 3
Rumen metabolites *in vitro*. Classification statistics of rumen metabolites *in vitro* **(A)**. Principal component analysis of rumen metabolites *in vitro* **(B)**. 0 h, rumen fluid not fermented *in vitro*. Con-12 h, basal diet. Ins50-12 h, Ins100-12 h, and Ins200-12 h represent basal diets supplemented with 50, 100, and 200 mg/kg DM inositol, respectively, fermented *in vitro* for 12 h. Con-48 h, basal diet. Ins50-48 h, Ins100-48 h, and Ins200-48 h represent basal diet supplemented with 50, 100, and 200 mg/kg DM inositol, respectively, fermented *in vitro* for 48 h. **(C–H)** Rumen differential metabolites in the *in vitro* experiment. Differential metabolites identified between control and **(C)** 50 mg/kg DM, **(D)** 100 mg/kg DM, and **(E)** 200 mg/kg DM groups at 12 h. Differential metabolites identified between control and **(F)** 50 mg/kg DM, **(G)** 100 mg/kg DM, and **(H)** 200 mg/kg DM groups at 48 h. Each dot represents a single metabolite. Red and blue scatter points represent significant upregulation and downregulation, respectively. Gray points represent no significant differences. Unnamed scatter points with significance represent substances that were not matched in the current database.

TABLE 3 Blood biochemical indicators at different levels of inositol supplementation on different experimental days[1].

	Treatments (inositol mg/kg DM)										*p*-value		
Item	D1-I0	D1-I50	D1-I100	D4-I0	D4-I50	D4-I100	D7-I0	D7-I50	D7-I100	SEM	Ins	Day	I × D
TP, g/L	73.04	75.38	72.63	73.63	72.80	72.60	71.23	73.93	72.43	0.71	0.67	0.82	0.93
ALB, g/L	21.99	23.66	23.58	23.47	24.33	23.23	22.98	23.48	23.32	0.39	0.61	0.83	0.95
GLB, g/L	51.06	51.71	49.05	50.17	48.47	49.37	48.25	50.45	49.12	0.70	0.85	0.69	0.87
TC, mmol/L	1.68	1.85	1.63	1.76	1.94	1.61	1.62	1.74	1.60	0.06	0.37	0.79	0.99
GLU, mmol/L	3.28	3.23	3.38	2.95	3.13	3.07	3.06	3.18	3.14	0.07	0.84	0.36	0.98
HDL-C, mmol/L	0.92	0.97	0.85	0.95	1.02	0.83	0.88	0.96	0.89	0.04	0.40	0.97	0.99
LDL-C, mmol/L	0.45	0.56	0.46	0.53	0.54	0.46	0.47	0.46	0.46	0.02	0.56	0.69	0.87
TG, mmol/L	0.34	0.29	0.32	0.31	0.31	0.35	0.35	0.33	0.34	0.01	0.64	0.67	0.89
Urea, mmol/L	4.49	4.23	4.33	4.49	4.21	4.46	4.20	4.20	4.43	0.13	0.83	0.95	0.99
NEFA, mmol/L	0.13	0.33	0.24	0.22	0.28	0.15	0.21	0.18	0.17	0.02	0.33	0.77	0.51
BHBA, mmol/L	0.44	0.40	0.41	0.46	0.42	0.48	0.43	0.48	0.49	0.01	0.60	0.19	0.52
INS, μIU/mL	12.74	13.08	13.04	13.17^{b}	14.92^{a}	14.34^{a}	14.04	13.77	15.18	0.22	0.21	<0.05	0.38

TP = total Protein; ALB = albumin; GLB = globulin; TC = total cholesterol; GLU = glucose; HDL-C = high density lipoprotein; LDL-C = low density lipoprotein; TG = triglyceride; NEFA = non-esterified fatty acid; BHBA = β- Hydroxybutyric acid; INS = insulin. SEM = standard error of mean; Ins = different inositol doses; Day = different sampling days; D × T = interaction of different inositol doses and different sampling days. [1]D1, 4, and 7 represent the first, fourth, and seventh days of the sampling period, respectively; I0, basal diet. I50, I100 represent basal diet supplemented with 50, 100 mg/kg DM inositol, respectively. ^{a,b} Means in the same sampling day with different superscripts are significantly different ($p < 0.05$).

TABLE 4 Blood immune and oxidative stress indicators at different levels of inositol supplementation on different experimental days[1].

	Treatments (inositol mg/kg DM)										*p*-value		
Item	D1-I0	D1-I50	D1-I100	D4-I0	D4-I50	D4-I100	D7-I0	D7-I50	D7-I100	SEM	Ins	Day	I × D
IgA, μg/mL	244.48	242.07	242.26	251.21	244.48	242.70	255.92	244.86	245.40	2.31	0.41	0.62	0.98
IgG, mg/mL	75.12	74.83	76.19	72.28	79.84	78.82	79.40	79.79	83.18	0.96	0.26	0.06	0.62
IgM, μg/mL	2428.13	2550.51	2267.21	2553.66	2734.14	2697.90	2510.39	2616.23	2945.10	57.36	0.53	0.12	0.39
IL-2, pg./mL	1009.96	1027.10	1008.24	1094.32	1087.96	1016.86	1072.56	1135.95	1081.11	13.93	0.36	0.07	0.80
IL-4, pg./mL	54.48	51.89	51.44	48.77	47.13	52.93	49.36	53.86	52.64	1.60	0.92	0.74	0.88
IL-6, pg./mL	149.62	140.39	142.14	139.64	144.97	147.19	143.48	149.82	147.17	1.86	0.97	0.78	0.60
IL-10, pg./mL	141.61	138.38	140.50	152.74	146.78	150.30	151.33	153.54	155.34	1.70	0.79	<0.01	0.94
sCD14, ng/mL	21.65	20.75	21.17	23.15	21.11	21.31	20.29	21.27	21.55	0.24	0.54	0.32	0.19
TOS, U/mL	38.23	42.01	43.34	38.89	41.27	38.73	40.01	40.54	40.28	0.59	0.30	0.58	0.57
GSH-Px, pg./mL	1885.00	1948.50	1761.57	1716.87	1785.54	1769.20	1752.78	1927.63	1946.81	33.52	0.47	0.28	0.64
TAS, mmol/L	2.11	1.96	2.09	2.10	2.05	2.02	1.91	2.17	2.22	0.04	0.72	0.85	0.28
CAT, ng/mL	183.91	177.74	190.98	195.01	188.72	190.31	194.59	191.38	195.41	2.16	0.46	0.21	0.89
TNF-α, pg./mL	193.89	216.07	194.05	204.15	199.42	209.52	196.31	209.61	203.47	2.67	0.31	0.90	0.33
SOD, ng/mL	16.21	16.77	15.00	17.30	15.95	16.25	17.61	17.22	17.64	0.19	0.26	<0.01	0.20
MDA, nmol/mL	3.69	3.79	3.91	3.87	3.76	3.98	4.39	4.11	4.20	0.06	0.55	<0.01	0.74
T-AOC, U/mL	8.73	8.75	8.87	9.74	9.79	9.54	8.44	8.42	9.22	0.14	0.70	<0.01	0.65

IgA = immunoglobulin A; IgG = immunoglobulin G; IgM = immunoglobulin M; IL-2 = interleukin 2; IL-4 = interleukin 4; IL-6 = interleukin 6; IL-10 = interleukin 10; sCD14 = soluble cluster of differentiation14; TOS = total oxidant state; GSH-Px = glutathione peroxidase; TAS = total antioxidant status; CAT = catalase; TNF-α = tumor necrosis factor alpha; SOD = superoxide dismutase; MDA = malondialdehyde; T-AOC = total antioxidant capacity; SEM = standard error of mean; Ins = different inositol doses; Day = different sampling days; D × T = interaction of different inositol doses and different sampling days. [1]D1, 4, and 7 represent the first, fourth, and seventh days of the sampling period, respectively; I0, basal diet. I50, I100 represent basal diet supplemented with 50, 100 mg/kg DM inositol, respectively.

was significantly higher than that of the 50 mg/kg DM ($p < 0.05$) and control groups ($p < 0.01$). The abundance of Actinobacteria in the 50 mg/kg DM group was significantly higher than that of the 100 mg/kg DM ($p < 0.05$) and control groups ($p < 0.01$).

The abundance of *Prevotella*, *RFN20*, and *Succiniclasticum* in the 50 mg/kg DM group was significantly higher than that in the control ($p < 0.05$) and 100 mg/kg DM groups ($p < 0.01$). The abundance of *Butyrivibrio* in the 100 mg/kg DM group was significantly lower than in the other experimental groups ($p < 0.05$). The abundance of *Ruminococcus* in the 100 mg/kg DM group was significantly higher than that in the 50 mg/kg DM ($p < 0.05$) and control groups ($p < 0.01$). The abundance of *BF311* in the 50 mg/kg DM group was significantly higher than that in the 100 mg/kg DM ($p < 0.05$) and control groups ($p < 0.01$). *CF231*, *BF311*, *Treponema*, and *Succiniclasticum* had significantly lower abundances on day one than the other time points ($p < 0.05$). The abundance of *Clostridium* on day seven was significantly higher than that on day one ($p < 0.05$) and day four ($p < 0.01$).

Significant differences in alpha diversity were observed at different inositol concentrations, sampling times, and their interactions (Figures 4C–E). The Chao1 index of the 50 mg/kg DM group was significantly lower than that of the control group ($p < 0.05$) and was significantly higher on the first and fourth days of sampling compared to that on the seventh day ($p < 0.05$). The Shannon and Simpson indices in the 100 mg/kg DM group were significantly higher than those in the control ($p < 0.05$) and 50 mg/kg DM groups ($p < 0.01$).

The results of the beta diversity PCoA analysis showed that groups with the same inositol dose were clustered. PCoA1 explained 24% of the features, and groups with different doses of inositol addition had significant separation (Figure 4F). PCoA2 explained 21.2% of the features, and there was a significant separation between the control and other experimental groups, indicating differences in the microbial diversity between these groups.

3.6 Rumen metabolites of sheep

A total of 63 metabolites were identified (Figure 5A). Among the secondary classifications in the HMDB (Human Metabolome Database), organic acids and their derivatives accounted for the highest proportion (30.159%) of the total metabolites.

On day one, the expression of proline and D-α-glycerol 1-phosphate was downregulated in the 50 mg/kg DM group compared to the control (Figure 5B). No differential metabolites were identified in the 100 mg/kg DM group. On the fourth day of inositol supplementation, lysine and L-tyrosine 1 were downregulated in the 100 mg/kg DM compared to the control group (Figure 5C). No differential metabolites were identified in the 50 mg/kg DM group. On day seven of inositol supplementation, the expression of phenylacetic acid in the 100 mg/kg DM group was upregulated compared to the control group (Figure 5D). No differential metabolites were identified in the 50 mg/kg DM group.

KEGG enrichment analysis of the differential metabolites showed that on the first day of inositol supplementation, the choline metabolism in the cancer pathway was most enriched in the 50 mg/kg DM vs. control comparison, and enrichment in the ABC transporter pathway was the most significant (Figure 5E). On the fourth day of

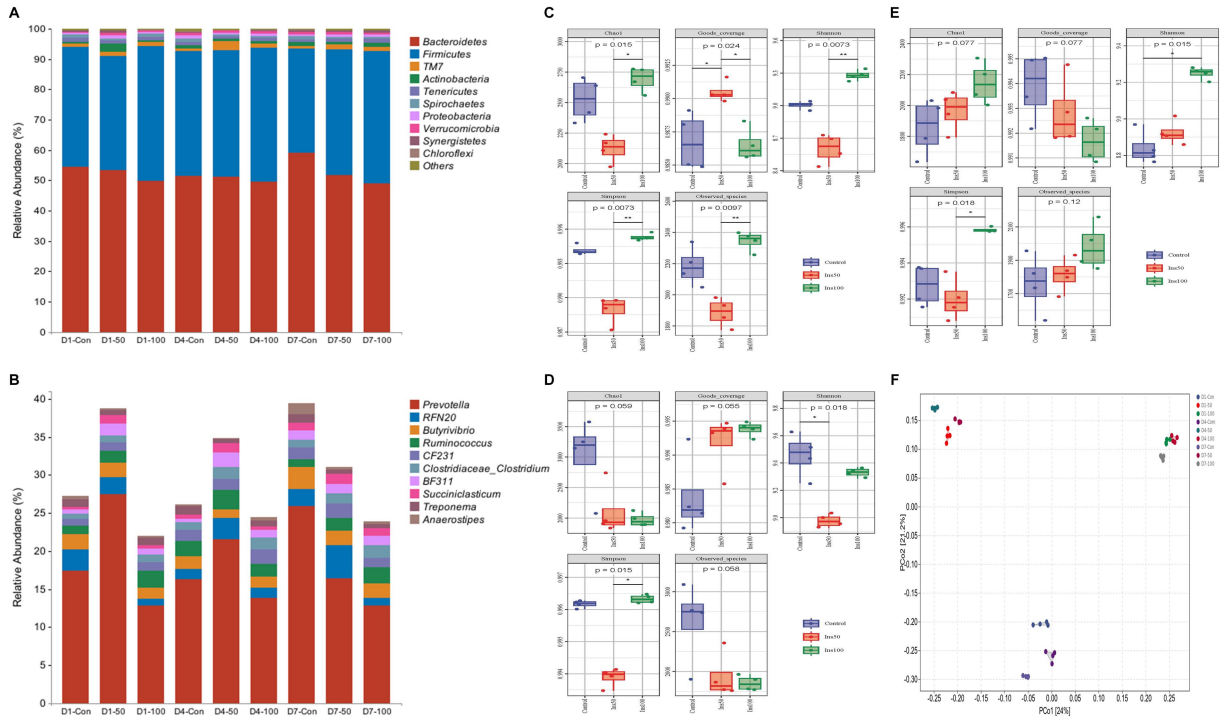


FIGURE 4
Effect of inositol supplementation on ruminal bacterial community. At the **(A)** phylum and **(B)** genus levels. D1-Con, basal diet. D1-50 and D1-100 represent basal diet supplemented with 50 and 100 mg/kg DM inositol, respectively, on the first day of sampling. D4-Con, basal diet. D4-50 and D4-100 represent basal diet supplemented with 50 and 100 mg/kg DM inositol, respectively, on the fourth day of sampling. D7-Con, basal diet. D7-50 and D7-100 represent basal diet supplemented with 50 and 100 mg/kg DM inositol, respectively, on the seventh day of sampling. The alpha diversity of the ruminal bacterial community. Estimated on the **(C)** first, **(D)** fourth, and **(E)** seventh day of the sampling period. **(F)** The beta diversity was estimated by principal coordinate analysis on the first, fourth, and seventh days of the sampling period.

inositol supplementation, the biotin metabolism pathway showed the highest enrichment and was the most significant in the 100 mg/kg DM vs. control comparison (Figure 5F). On the seventh day of inositol supplementation, only the phenylalanine metabolism pathway was enriched in the 100 mg/kg DM vs. control comparison (Figure 5G).

3.7 Blood metabolites

A total of 454 blood metabolites were identified after sampling on days one, four, and seven. Among the secondary classifications in the HMDB database, lipids and lipid-like molecules constituted the highest proportions (Figure 6A).

On day one of sampling, 5Z-Dodecenoic acid (10E,12Z)-(9S)-9-hydroperoxyoctadeca-10,12-dienoic acid, and palmitoleic acid were significantly upregulated in the 50 mg/kg DM (Figure 6B) compared to the control group ($p < 0.05$), whereas 9,10-DHOME was significantly upregulated in the 100 mg/kg DM (Figure 6C) compared to the control group ($p < 0.05$). On day four of sampling, 3-Butyl-1(3H)-isobenzofuranone and inosine were significantly upregulated in the 50 mg/kg DM (Figure 6D) compared to the control group ($p < 0.05$). No significant differential metabolites were found on day four in the 100 mg/kg DM vs. control group comparison. On day seven of sampling, glycohyocholic acid was significantly downregulated in the 50 mg/kg DM (Figure 6E) compared to the control group ($p < 0.05$), and 4-Hydroxy-3-methoxymandelic acid, glycohyocholic acid, 3,4-Dihydroxymandelic acid, and acadesine were significantly downregulated in the 100 mg/kg DM (Figure 6F) compared with the control group ($p < 0.05$).

KEGG enrichment analysis of the blood differential metabolites showed that on day one of sampling, the 50 mg/kg DM group was enriched in the biosynthesis of secondary metabolites compared to the control group (Figure 6G), with the highest number of metabolites in metabolic pathways and the most significant enrichment in fatty acid biosynthesis. The 100 mg/kg DM group was mainly enriched in metabolic pathways compared to the control group (Figure 6H), with the most significant enrichment in arginine biosynthesis. On day four, the 50 mg/kg DM group was mainly enriched in the biosynthesis of secondary metabolites compared to the control group (Figure 6I), with the most significant enrichment in glutathione metabolism. No differential metabolites were identified in the 100 mg/kg DM group compared with the control. On day seven, no differential metabolic pathways were identified in the 50 mg/kg DM group compared to the control. The 100 mg/kg DM group was mainly enriched in metabolic pathways compared with the control and was significantly enriched in tyrosine metabolism (Figure 6J).

4 Discussion

The rumen is a natural fermenter in which a variety of rumen microorganisms live in symbiosis with the host and produce VFAs,

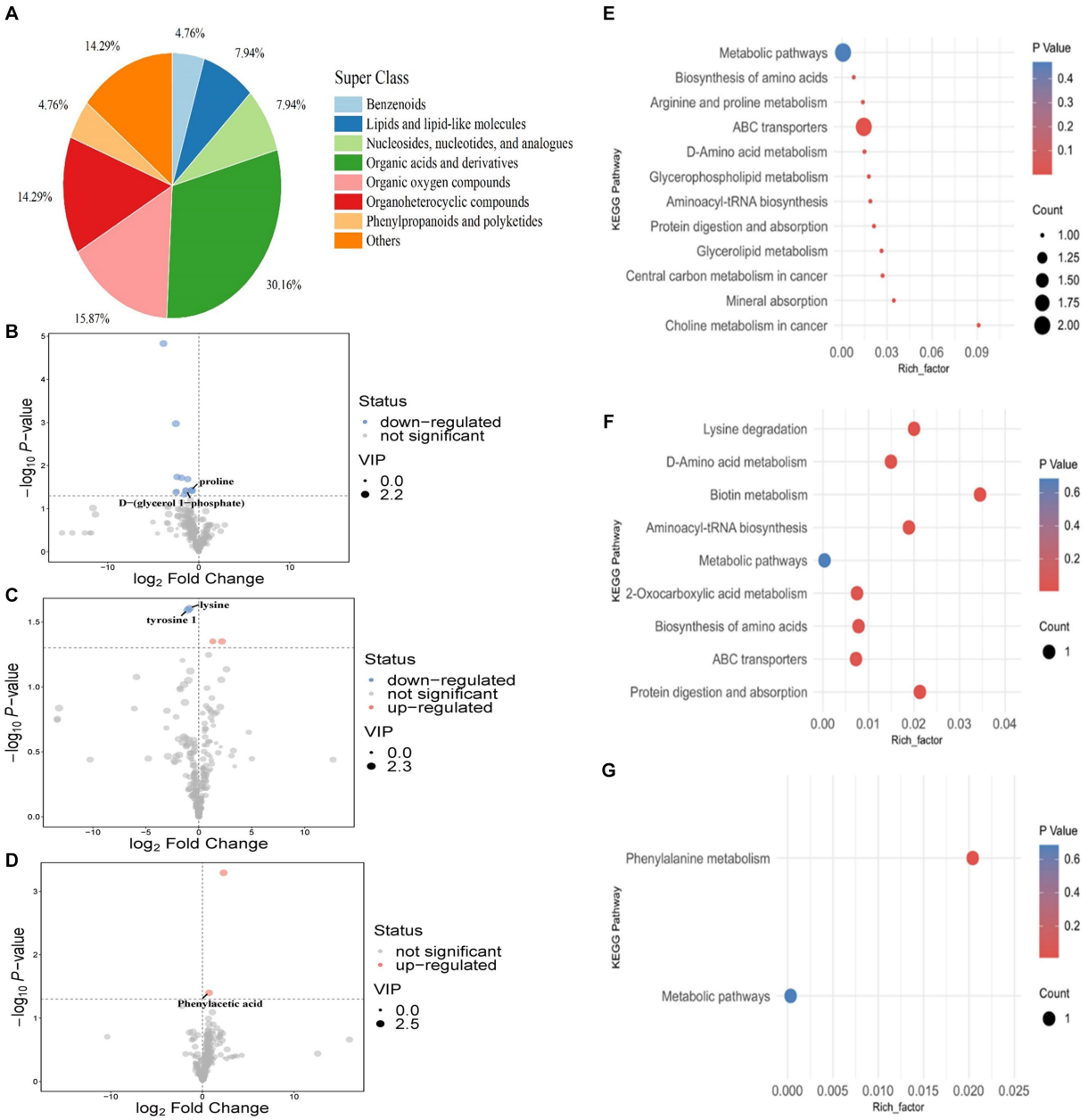


FIGURE 5
Rumen metabolites of sheep. Classification statistics of rumen metabolites **(A)**. Rumen differential metabolites after different lengths of inositol supplementation, first **(B)**, fourth **(C)**, and seventh **(D)** day of sampling. KEGG enrichment analysis of rumen differentiated metabolites in the feeding experiment **(E–G)**. Each point in the diagram represents a metabolic pathway; red and blue scatter points represent significant upregulation and downregulation, respectively. The horizontal coordinate where the points are located and the point size indicate the influence factor size of the path in topology analysis. The larger the size, the larger the influence factor.

microbial proteins, and vitamins for host growth and development (19). Rumen function is reflected by its fermentation parameters and is closely related to its microbial diversity. In this experiment, IVDMD, IVCPD, and NH_3-N concentrations were higher in the low-dose than the high-dose inositol group. IVDMD and IVCPD reflect the simulated rumen utilization of feed *in vitro*, and the higher levels in the low-dose group indicate that a low dose of inositol in ruminant diets can promote the decomposition of feed carbohydrates and proteins by rumen microorganisms and the enrichment and colonization of the corresponding microbial community. NH_3-N and free amino acids in the rumen are the primary precursors of microbial protein synthesis. A lower concentration of NH_3-N affects the synthesis of microbial proteins, whereas a higher concentration leads to nitrogen loss. The higher concentration of NH_3-N in the low-dose group may be due to an increase in the abundance of bacteria associated with protein decomposition, which converts feed proteins into oligopeptides and amino acids, with further degradation producing ammonia nitrogen. Gas production can be used to evaluate the ruminal fermentation efficiency and microbial activity. The 50 mg/kg DM group had the highest gas production, indicating that this dose

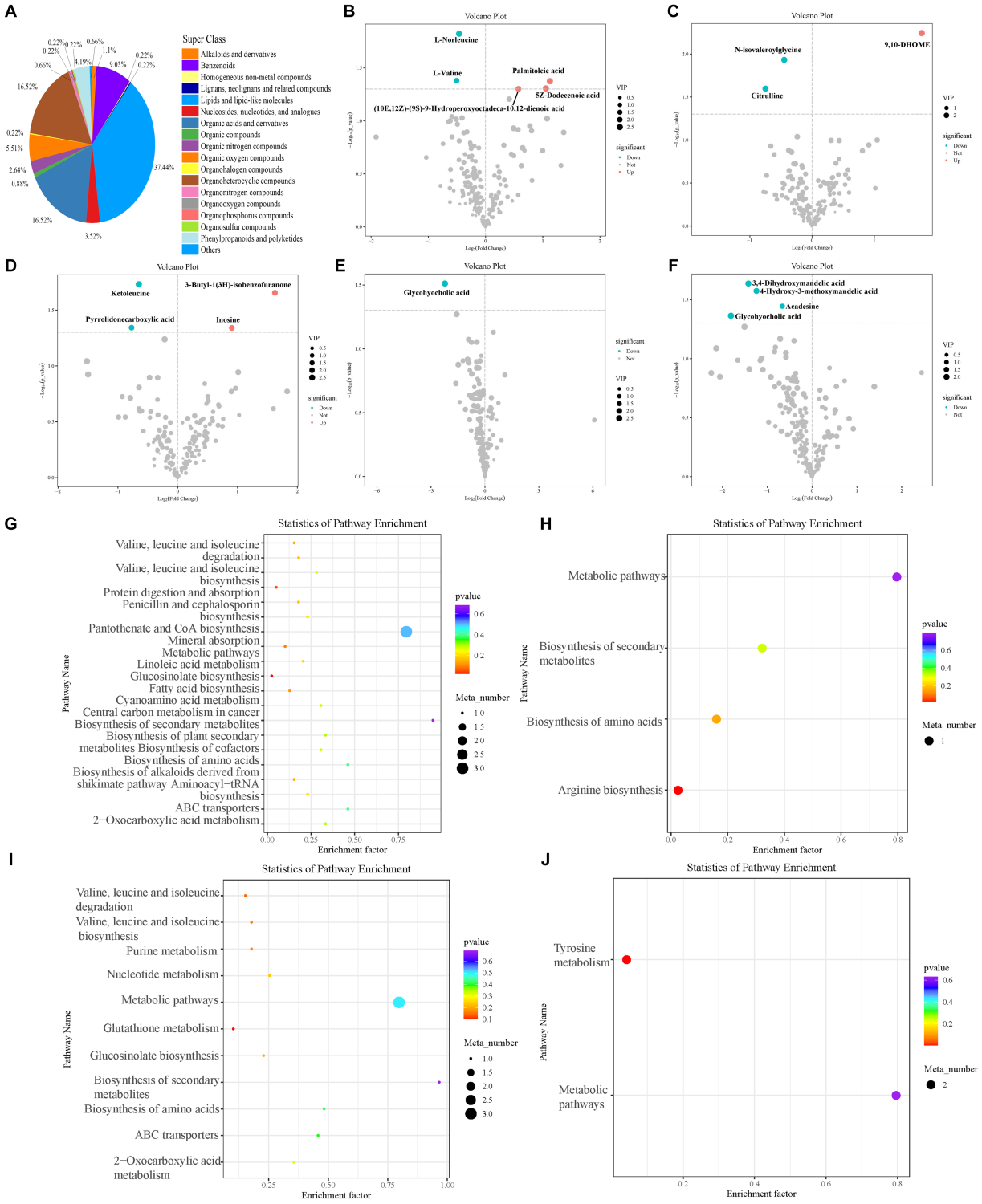


FIGURE 6
Blood metabolites of sheep. Classification statistics of blood metabolites **(A)**. Blood differential metabolites in the feeding experiment **(B–F)**. Differential metabolites between control and **(B)** 50 mg/kg DM and **(C)** 100 mg/kg DM groups on the first day of sampling. Differential metabolites between control and **(D)** 50 mg/kg DM groups on the fourth day of sampling. Differential metabolites between control and **(E)** 50 mg/kg DM and **(F)** 100 mg/kg DM groups on the seventh day of sampling. **(G–J)** KEGG enrichment analysis of blood differential metabolites in the feeding experiment. Enriched pathways in the control and **(G)** 50 mg/kg DM and **(H)** 100 mg/kg DM group comparisons on the first day of sampling. Enriched pathways in the control and **(I)** 50 mg/kg DM group comparison on the fourth day of sampling. Enriched pathways in the control and **(J)** 100 mg/kg DM group comparison on the seventh day of sampling.

is most efficient in regulating microbial activity in the rumen and promoting the fermentation of carbohydrates. VFAs, such as acetic acid, propionic acid, and butyric acid, are the main source of energy for ruminants and are the end products of feed carbohydrates. *In vitro*, the low-dose group had a higher concentration of TVFA at 3, 6, and 12 h. The concentrations of acetic acid, propionic acid, and butyric

acid were significantly higher than those in the control and high-dose groups, and they also had a lower acetic acid/propionic acid (A/P) ratio. Acetic acid is produced by the degradation of cellulose and hemicellulose and is one of the main products of rumen microorganisms. It has the highest proportion in the rumen and can be used to synthesize milk fat together with butyric acid. Propionic acid can synthesize glucose, which is essential for the physiological activities of ruminants, by participating in the gluconeogenesis pathway and can be utilized by the mammary glands to produce lactose. The A/P ratio reflects the fermentation pattern of the rumen, and a decrease in this ratio indicates an increase in the efficiency of energy utilization in the animals' diet. The 100 mg/kg DM dose significantly increased the production efficiency of valeric acid, a type of branched-chain volatile fatty acid; promoted the growth of fiber-degrading bacteria; and increased the efficiency of feed fiber degradation (20).

The rumen environment is a complex microbial system that is highly adaptive and competitive. Various microbial communities in the rumen produce enzymes involved in the breakdown of carbohydrates, nitrogenous compounds, and lipid compounds. Inositol addition influenced both the abundance and diversity of microorganisms in the rumen. Bacteroidetes and Firmicutes were the most abundant phyla, demonstrating the stability of the rumen's internal environment. The bacteroidetes promote structural polysaccharide catabolism, and Firmicutes contain a large number of fiber-decomposing genera (21). *In vitro*, the level of Bacteroidetes in the inositol-supplemented group was significantly lower compared to the control at 48 h. This may be attributed to the higher accumulation of VFAs, resulting in a lower pH, leading to the death of some microorganisms intolerant of acid. Similar results were observed in the feeding experiment, where Bacteroidetes were significantly lower in abundance compared to the control group, whereas *Firmicutes* were all significantly higher than the control group. This suggests that in the inositol-supplemented group, the proportion of Firmicutes: Bacteroidetes increased relative to the control and that this represented an increase in the number of bacterial species that can ferment carbohydrates. Studies have shown that improving the ratio of Firmicutes: Bacteroidetes in the gut enables animals to absorb more energy from their diets (21).

Our results showed that the inositol supplementation increased the abundance of *Prevotella*, which is an important feed protein- and carbohydrate-degrading bacteria (22) that can improve branched-chain amino acid synthesis (23). Its abundance is affected by the composition of the diet and nutrition level (24) and positively correlates with dietary concentrate and protein levels (25). Inositol may promote the catabolic efficiency of feed proteins and enhance feed utilization in ruminants. Inositol increased the abundance of *Pseudomonadaceae_Pseudomonas in vitro*, a genus of uratolytic bacteria capable of hydrolysing urea to ammonia by producing urease, which is used for the synthesis of amino acids and microbial proteins (26). In the feeding experiment, inositol also increased the abundance of *RFN20*, *BF311*, *Treponema*, *Succiniclasticum*, and *Ruminococcus*. *RFN20* is a rumen-specific genus that is more closely associated with average daily gain in beef cattle (27). There are limited studies on the genus *BF311*, but its metabolic capacity may be similar to that of *Prevotella* spp. and it is involved in VFA-related metabolism (28). *Treponema* is a soluble fiber-degrading bacterium commonly found in the rumen that is capable of degrading plant polysaccharides (29). Succinic acid is an intermediate metabolite of the tricarboxylic acid cycle which can be produced or utilized by rumen microorganisms. *Succiniclasticum* is a Gram-negative genus that produces propionic acid by decarboxylation of succinate acid (30, 31) and is important in rumen microbial fermentation. *Ruminococcus* is an important intestinal commensal genus that helps the host degrade dietary phytopolysaccharides and has a great impact on both host health and the development of potential biological functions (32).

Inositol supplementation affected rumen metabolites, including those that regulate rumen function and maintain the health of its internal environment. Organic acids and their derivatives were the major metabolites detected. *In vitro*, the pathways of differential metabolites at 12 h were enriched in valine, leucine, and isoleucine metabolism and significantly enriched in the glycerol ester metabolism pathway. Inositol significantly upregulated the levels of succinic acid, hydrocinnamic acid, 4-Hydroxyphenylethanol, quinic acid, and azelaic acid metabolites. Studies have shown that 3-phenylpropionic acid and quinic acid inhibit bacterial virulence (33, 34), and azelaic acid can reduce intestinal inflammation (35). Inositol may improve the internal rumen environment by regulating these metabolites. Sucrose metabolism was significantly downregulated, suggesting that inositol may promote the efficiency of sucrose catabolism and enhance the efficiency of carbohydrate utilization in the rumen. After 48 h, the differential metabolic pathways were mainly enriched in the alanine, aspartic acid, and glutamic acid, and the differential metabolites were those that provide substrates for rumen microbial synthesis.

In the feeding experiment, 50 mg/kg DM inositol addition on day one resulted in the downregulation of proline and D-(glycerol 1-phosphate). Proline is an amino acid with complex biological functions, including cellular osmoregulation and protein synthesis. As a nutrient, it can promote the pathogenicity of different organisms, and disruption of proline metabolism can significantly attenuate the damage caused by some pathogens. For example, host restriction of nutrient supply is an important defense mechanism against *Brucella*, which is an important pathogenic bacterium in ruminants that requires proline to be supplied by the host for its activity (36). Inhibition of proline metabolism may be an effective strategy for the treatment of damage caused by some pathogens (37). The tumor-suppressive effect of inositol and its derivatives may be due to inhibition of the ERK–MAPK or P13K–Akt pathways (38), but the validation of the ability of inositol addition to modulate the levels of choline compounds is still lacking. On the fourth day of inositol supplementation, lysine and tyrosine expression were downregulated in the 100 mg/kg DM inositol group compared to the control; mass spectrometry results from previous experiments indicate that organophosphorus agents can form covalent bonds with lysines and tyrosines in proteins without a serine active site (39), inositol is metabolized in animals to form inositol phosphates, and further experiments are required to investigate whether inositol binds these two amino acids during metabolism. On day seven of inositol supplementation, phenylacetic acid metabolism was significantly upregulated in the phenylalanine metabolic pathway in the 100 mg/kg DM inositol group compared to the control. Phenylacetic acid is a metabolite of phenylbutyric acid that is further metabolized to phenylacylglutamine and phenylbutyrylglutamine, which are excreted through the urine (40). Phenylacetic acid modulates endoplasmic reticulum chemical stress and plays a protective role in a variety of diseases (41). In rumen microorganisms, phenylacetic acid can

be formed by the conversion of phenylalanine (42) and exerts a stimulatory effect on the fiber digestion of *Ruminococcus* (43). The rumen fluid metabolite results indicated that inositol addition produced some variation in sheep rumen metabolites and may improve animal health.

The addition of inositol only had a small effect on the blood biochemical indices, but there were some clear trends. The 50 mg/kg DM inositol supplementation promoted an increase in total blood cholesterol levels, and an upregulation of blood high-density lipoprotein was observed with increasing treatment time. Cholesterol is an important regulatory molecule in the body, and its excess or deficiency leads to the development of diseases. It can be distributed in various parts of the body as a plasma protein carrier of the fat-soluble vitamins K and E and can be used as a carrier for cellular drugs. Regulation of cholesterol levels is of great importance to animal health. High-density lipoproteins remove excess cholesterol and prevent diseases caused by high cholesterol levels, such as atherosclerosis (44). In previous studies, the addition of inositol reduced cholesterol levels (45), which differs from the increase observed after the addition of inositol in this study, which may be related to the increase in HDL levels. On the fourth day of inositol supplementation, the insulin level was significantly higher than that of the control group. Insulin is the only hormone that can reduce blood glucose concentration, and an increase in its synthesis can reduce the blood glucose concentration in animals after feeding, increase feed intake, and promote nutrient deposition, which is beneficial to the growth and development of animals (46).

Increasing inositol supplementation time increased the levels of IgG, IgM, and IL-4. IL-4 can promote B cell differentiation into antibody-secreting cells and enhance humoral immunity (47), and IgG and IgM can mediate humoral immune responses. An increase in the levels of both can promote specific immune functions, and the continuous addition of inositol may enhance the immune ability of animals. The blood GSH-Px level significantly increased with increasing treatment time, and the MDA levels decreased. GSH-Px is an important cellular antioxidant enzyme that removes free radicals (48). MDA is one of the final products of the peroxidation of polyunsaturated fatty acids in cells, and an increase in free radicals in cells increases the level of MDA (49); therefore, the level of MDA is used as a marker for oxidative stress and antioxidant status in animals. Changes in these levels after inositol supplementation may indicate the potential antioxidant capacity of inositol in ruminant nutrition. Current studies on the antioxidant capacity of inositol, focusing on aquatic animals, have shown that inositol can improve antioxidant and immune capacities (13, 50, 51). However, there are few studies on inositol in ruminants, and the mechanism by which inositol affects their immunity and antioxidant activity requires further investigation.

The addition of inositol promoted the production of lipid or lipid-like molecules, such as palmitoleic acid and the nucleotide metabolite inosine, and inhibited the metabolism of glycohyocholic acid. Palmitoleic acid is capable of regulating different metabolic processes, and studies have shown that phospholipid levels of palmitoleic acid in the blood are associated with elevated high-density lipoproteins (52). Palmitoleic acid inhibits steatosis in the liver and improves insulin sensitivity by modulating GLUT-4 and AMPK phosphorylation (53). Downregulation of SIRT3 gene expression by palmitoleic acid regulates the gluconeogenesis pathway and maintains normal blood sugar levels in the body (54). Palmitoleic acid can also inhibit inflammation and reduce systolic blood pressure to prevent hypertensive diseases (55), thereby playing an important role in maintaining the health of the body.

Inosine is an important secondary metabolite of purine metabolism that participates in a variety of biochemical processes. Intracellularly, it can act as a molecular messenger to participate in physiological activities related to cellular signaling pathways, as well as having immunomodulatory and neuroprotective effects. Extracellularly, inosine can inhibit the production of pro-inflammatory factors, such as TNF-α and IL-1, and slow down the macrophage-mediated endotoxin inflammatory response (56), blocking the production of superoxide induced by formyl peptides (57). The exogenous addition of inosine also mitigates cellular damage and protects body tissues (58), and an increase in the metabolic level of inosine indirectly enhances the immune capacity of the body.

Glycohyocholic acid is the glycine-bound form of primary bile acid and has been implicated in the regulation of intestinal flora activity (59). Bile acids are synthesized from cholesterol by hepatocytes and are involved in many biological functions. Free bile acids in the blood can be removed by transporter proteins located on the basement membrane of hepatocytes (60). Blood concentrations of bile acids are elevated in patients with non-alcoholic fatty liver disease (NAFLD) who exhibit insulin resistance (61), and inositol, with its insulin-mimetic properties, can play a role in diseases associated with insulin resistance and has the potential to modulate NAFLD (62). Glycohyocholic acid metabolism was significantly downregulated in the inositol-supplemented group compared to the control, suggesting that inositol is involved in lipid metabolism and regulates the level of sterols in ruminants.

There are still some shortcomings in this study. The gas composition in total gas production, especially CH_4, was not analyzed in the *in vitro* experiment. Further classification of the gas produced by fermentation can further explore the effects of different inositol dosages on rumen nutrient digestion, microbial diversity, and environmental pollution of sheep. The role of inositol in the growth performance of sheep should be further studied in the future, and the mechanism of inositol's influence on sheep's bodies should be discussed in more detail at the molecular level according to the results obtained in the experiment.

5 Conclusion

Inositol was found to improve rumen fermentation and affect its microbial diversity and metabolism *in vitro*. These results were validated in the feeding experiment, indicating the potential of inositol to reduce inflammation and improve animal health.

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 here: NCBI sequence Read Archive (SRA), accession numbers PRJNA1027573 and PRJNA1027802.

Ethics statement

The animal study was approved by all experimental procedures were performed in accordance with the Guidelines for the Care and Use of Experimental Animals of the Jilin Agricultural University (JLAU-ACUC2022-006). The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

GY: Writing – original draft, Data curation, Software, Validation, Visualization. ZS: Writing – review & editing, Resources. ZW: Writing – review & editing, Investigation. YX: Writing – review & editing, Investigation. LC: Writing – review & editing, Investigation. GQ: Writing – review & editing, Formal analysis, Methodology. NA: Writing – review & editing, Investigation. HL: Writing – review & editing, Supervision. XZ: Writing – review & editing, Formal analysis, Methodology. QW: Writing – review & editing, Investigation. WZhan: Writing – review & editing, Supervision. WZhao: Writing – review & editing, Supervision. TW: Writing – review & editing, Funding acquisition, Supervision. YZ: Writing – review & editing, Formal analysis, Methodology.

Funding

The author(s) declare financial support was received for the research, authorship, and/or publication of this article. This work was supported by the Scientific and Technological Developing Scheme of Jilin Province (grant number 20210202037NC).

Conflict of interest

ZS, WZhan, WZhao, TW, and YZ are employed by Changchun Borui Science and Technology Co., Ltd., China.

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

Publisher's note

All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2024.1359234/full#supplementary-material

References

1. Guyader J, Janzen HH, Kroebel R, Beauchemin KA. Forage use to improve environmental sustainability of ruminant production. *J Anim Sci.* (2016) 94:3147–58. doi: 10.2527/jas.2015-0141

2. McAfee AJ, McSorley EM, Cuskelly GJ, Moss BW, Wallace JMW, Bonham MP, et al. Red meat consumption: an overview of the risks and benefits. *Meat Sci.* (2010) 84:1–13. doi: 10.1016/j.meatsci.2009.08.029

3. Park YW, Nam MS. Bioactive peptides in milk and dairy Products: A Review. *Korean J Food Sci Anim Resour.* (2015) 35:831–40. doi: 10.5851/kosfa.2015.35.6.831

4. Shingfield KJ, Bonnet M, Scollan ND. Recent developments in altering the fatty acid composition of ruminant-derived foods. *Animal.* (2013) 7:132–62. doi: 10.1017/S1751731112001681

5. Xu Q, Qiao Q, Gao Y, Hou J, Hu M, Du Y, et al. Gut microbiota and their role in health and metabolic disease of dairy cow. *Front Nutr.* (2021) 8:701511. doi: 10.3389/fnut.2021.701511

6. Clements RS, Darnell B. Myo-inositol content of common foods: development of a high-myo-inositol diet. *Am J Clin Nutr.* (1980) 33:1954–67. doi: 10.1093/ajcn/33.9.1954

7. Bizzarri M, Carlomagno G. Inositol: history of an effective therapy for Polycystic Ovary Syndrome. *Eur Rev Med Pharmacol Sci.* (2014) 18:1896–903. doi: 10.4314/tjpr.v13i7.24

8. Sun Z, Wang T, Demelash N, Zheng S, Zhao W, Chen X, et al. Effect of yeast culture (Saccharomyces cerevisiae) on broilers: A preliminary study on the effective components of yeast culture. *Animals (Basel).* (2019) 10:68. doi: 10.3390/ani10010068

9. Barker CJ, Berggren P-O. The role of inositol and the principles of labelling, extraction, and analysis of inositides in mammalian cells. *Methods Mol Biol.* (2010) 645:1–19. doi: 10.1007/978-1-60327-175-2_1

10. Deranieh RM, Greenberg ML. Cellular consequences of inositol depletion. *Biochem Soc Trans.* (2009) 37:1099–103. doi: 10.1042/BST0371099

11. Suliman M, Case KC, Schmidtke MW, Lazcano P, Onu CJ, Greenberg ML. Inositol depletion regulates phospholipid metabolism and activates stress signaling in HEK293T cells. *BBA-MOL CELL BIOL L.* (2022) 1867:159137. doi: 10.1016/j.bbalip.2022.159137

12. Bu X, Lin Z, Liu S, Wang C, Wang N, Lei Y, et al. Effects of myo-inositol on growth performance, body composition, antioxidant status, non-specific immunity and lipid metabolism of juvenile Chinese mitten crab (Eriocheir sinensis). *Aquacult Nutr.* (2020) 26:1623–35. doi: 10.1111/anu.13107

13. Chen X, Wang J, Zhao W. Effects of dietary inositol supplementation on growth, digestive performance, antioxidant capacity, and body composition of golden pompano (Trachinotus ovatus). *Front Physiol.* (2022) 13:850470. doi: 10.3389/fphys.2022.850470

14. Menke KH, Steingass H. Estimation of the energetic feed value obtained from chemical analysis and in vitro gas production using rumen fluid. *Anim Res Dev.* (1988) 28:7–55.

15. AOAC. Association of Official Analytical Chemists: Official methods of analysis In: *Association of Official Agricultural Chemists (USA)*. Washington, DC: (1990). 881–2.

16. Callahan BJ, McMurdie PJ, Rosen MJ, Han AW, Johnson AJA, Holmes SP. DADA2: High-resolution sample inference from Illumina amplicon data. *Nat Methods.* (2016) 13:581–3. doi: 10.1038/nmeth.3869

17. Smith CA, Want EJ, O'Maille G, Abagyan R, Siuzdak G. XCMS: processing mass spectrometry data for metabolite profiling using nonlinear peak alignment, matching, and identification. *Anal Chem.* (2006) 78:779–87. doi: 10.1021/ac051437y

18. Dunn WB, Broadhurst D, Begley P, Zelena E, Francis-McIntyre S, Anderson N, et al. Procedures for large-scale metabolic profiling of serum and plasma using gas chromatography and liquid chromatography coupled to mass spectrometry. *Nat Protoc.* (2011) 6:1060–83. doi: 10.1038/nprot.2011.335

19. Li F, Guan LL. Metatranscriptomic profiling reveals linkages between the active rumen microbiome and feed efficiency in beef cattle. *Appl Environ Microbiol.* (2017) 83:e00061–17. doi: 10.1128/AEM.00061-17

20. Bentley OG, Johnson RR, Hershberger TV, Cline JH, Moxon AL. Cellulolytic-factor activity of certain short-chain fatty acids for rumen microorganisms in vitro. *J Nutr.* (1955) 57:389–400. doi: 10.1093/jn/57.3.389

21. Zhang YK, Zhang XX, Li FD, Li C, Li GZ, Zhang DY, et al. Characterization of the rumen microbiota and its relationship with residual feed intake in sheep. *Animal.* (2021) 15:100161. doi: 10.1016/j.animal.2020.100161

22. Morotomi M, Nagai F, Sakon H, Tanaka R. Paraprevotella clara gen. nov., sp. nov. and Paraprevotella xylaniphila sp. nov., members of the family "Prevotellaceae" isolated from human faeces. *Int J Syst Evol Microbiol* (2009) 59:1895–1900. doi: 10.1099/ijs.0.008169-0

23. Xue MY, Sun HZ, Wu XH, Liu JX, Guan LL. Multi-omics reveals that the rumen microbiome and its metabolome together with the host metabolome contribute to individualized dairy cow performance. *Microbiome.* (2020) 8:64. doi: 10.1186/s40168-020-00819-8

24. Tian K, Liu J, Sun Y, Wu Y, Chen J, et al. Effects of dietary supplementation of inulin on rumen fermentation and bacterial microbiota, inflammatory response and growth performance in finishing beef steers fed high or low-concentrate diet. *Anim Feed Sci Tech*. (2019) 258:114299. doi: 10.1016/j.anifeedsci.2019.114299

25. Khafipour E, Krause DO, Plaizier JC. A grain-based subacute ruminal acidosis challenge causes translocation of lipopolysaccharide and triggers inflammation. *J Dairy Sci*. (2009) 92:1060–70. doi: 10.3168/jds.2008-1389

26. Jin D, Zhao S, Wang P, Zheng N, Bu D, Beckers Y, et al. Insights into abundant rumen ureolytic bacterial community using rumen simulation system. *Front Microbiol*. (2016) 7:1006. doi: 10.3389/fmicb.2016.01006

27. de Freitas AS, de David DB, Takagaki BM, Roesch LFW. Microbial patterns in rumen are associated with gain of weight in beef cattle. *Anton Leeuw Int J G*. (2020) 113:1299–312. doi: 10.1007/s10482-020-01440-3

28. Bi Y, Zeng S, Zhang R, Diao Q, Tu Y. Effects of dietary energy levels on rumen bacterial community composition in Holstein heifers under the same forage to concentrate ratio condition. *BMC Microbiol*. (2018) 18:69. doi: 10.1186/s12866-018-1213-9

29. Ziołecki A. Isolation and characterization of large treponemes from the bovine rumen. *Appl Environ Microbiol*. (1979) 37:131–5. doi: 10.1128/aem.37.1.131-135.1979

30. van Gylswyk NO. Succiniclasticum ruminis gen. nov., sp. nov., a ruminal bacterium converting succinate to propionate as the sole energy-yielding mechanism. *Int J Syst Bacteriol*. (1995) 45:297–300. doi: 10.1099/00207713-45-2-297

31. Sijpesteijn AK, Elsden SR. The metabolism of succinic acid in the rumen of the sheep. *Biochem J*. (1952) 52:41–5. doi: 10.1042/bj0520041

32. La Reau AJ, Suen G. The Ruminococci: key symbionts of the gut ecosystem. *J Microbiol*. (2018) 56:199–208. doi: 10.1007/s12275-018-8024-4

33. Sharma S, Gopu V, Sivasankar C, Shetty PH. Hydrocinnamic acid produced by Enterobacter xiangfangensis impairs AHL-based quorum sensing and biofilm formation in Pseudomonas aeruginosa. *RSC Adv*. (2019) 9:28678–87. doi: 10.1039/c9ra05725k

34. Wang GF, Shi LP, Ren YD, Liu QF, Liu HF, Zhang RJ, et al. Anti-hepatitis B virus activity of chlorogenic acid, quinic acid and caffeic acid in vivo and in vitro. *Antiviral Res*. (2009) 83:186–90. doi: 10.1016/j.antiviral.2009.05.002

35. Liao Y, Wu X, Luo W, Chen J, Huang Y, Ma K, et al. Azelaic acid regulates the Renin-Angiotensin System and improves colitis based on network pharmacology and experimentation. *ACS Omega*. (2023) 8:15217–28. doi: 10.1021/acsomega.3c00210

36. Takagi H. Proline as a stress protectant in yeast: physiological functions, metabolic regulations, and biotechnological applications. *Appl Microbiol Biotechnol*. (2008) 81:211–23. doi: 10.1007/s00253-008-1698-5

37. Christgen SL, Becker DF. Role of proline in pathogen and host interactions. *Antioxid Redox Signal*. (2019) 30:683–709. doi: 10.1089/ars.2017.7335

38. Case KC, Schmidtke MW, Greenberg ML. The paradoxical role of inositol in cancer: a consequence of the metabolic state of a tumor. *Cancer Metastasis Rev*. (2022) 41:249–54. doi: 10.1007/s10555-022-10032-8

39. Lockridge O, Schopfer LM. Review of tyrosine and lysine as new motifs for organophosphate binding to proteins that have no active site serine. *Chem Biol Interact*. (2010) 187:344–8. doi: 10.1016/j.cbi.2010.03.002

40. Comte B, Kasumov T, Pierce BA, Puchowicz MA, Scott ME, Dahms W, et al. Identification of phenylbutyrylglutamine, a new metabolite of phenylbutyrate metabolism in humans. *J Mass Spectrom*. (2002) 37:581–90. doi: 10.1002/jms.316

41. Marahatta A, Bhandary B, Lee MR, Kim DS, Lee YC, Kim SR, et al. Determination of phenylbutyric acid and its metabolite phenylacetic acid in different tissues of mouse by liquid chromatography with tandem mass spectrometry and its application in drug tissue distribution. *J Chromatogr B Analyt Technol Biomed Life Sci*. (2012) 903:118–25. doi: 10.1016/j.jchromb.2012.07.004

42. Khan RI, Onodera R, Amin MR, Mohammed N. Production of tyrosine and other aromatic compounds from phenylalanine by rumen microorganisms. *Amino Acids*. (1999) 17:335–46. doi: 10.1007/BF01361659

43. Stack RJ, Hungate RE, Opsahl WP. Phenylacetic acid stimulation of cellulose digestion by Ruminococcus albus 8. *Appl Environ Microbiol*. (1983) 46:539–44. doi: 10.1128/aem.46.3.539-544.1983

44. Kjeldsen EW, Nordestgaard LT, Frikke-Schmidt R. HDL cholesterol and non-cardiovascular disease: a narrative review. *Int J Mol Sci*. (2021) 22:4547. doi: 10.3390/ijms22094547

45. Leança CC, Nunes VS, Nakandakare ER, de Faria EC, Quintao ECR. Does plasma HDL-C concentration interact with whole-body cholesterol metabolism? *Nutr Metab Cardiovasc Dis*. (2013) 23:279–84. doi: 10.1016/j.numecd.2012.12.003

46. Torres N, Noriega L, Tovar AR. Nutrient modulation of insulin secretion. *Vitam Horm*. (2009) 80:217–44. doi: 10.1016/S0083-6729(08)00609-2

47. Matsui S, Matsumoto H, Sonoda Y, Ando K, Aizu-Yokota E, Sato T, et al. Glycyrrhizin and related compounds down-regulate production of inflammatory chemokines IL-8 and eotaxin 1 in a human lung fibroblast cell line. *Int Immunopharmacol*. (2004) 4:1633–44. doi: 10.1016/j.intimp.2004.07.023

48. Handy DE, Loscalzo J. The role of glutathione peroxidase-1 in health and disease. *Free Radic Biol Med*. (2022) 188:146–61. doi: 10.1016/j.freeradbiomed.2022.06.004

49. Gaweł S, Wardas M, Niedworok E, Wardas P. Malondialdehyde (MDA) as a lipid peroxidation marker. *Wiad Lek*. (2004) 57:453–5.

50. Cui W, Ma A, Wang X, Huang Z. Myo-inositol enhances the low-salinity tolerance of turbot (Scophthalmus maximus) by modulating cortisol synthesis. *Biochem Biophys Res Commun*. (2020) 526:913–9. doi: 10.1016/j.bbrc.2020.04.004

51. Jiang WD, Liu Y, Jiang J, Wu P, Feng L, Zhou XQ. Copper exposure induces toxicity to the antioxidant system via the destruction of Nrf2/ARE signaling and caspase-3-regulated DNA damage in fish muscle: amelioration by myo-inositol. *Aquat Toxicol*. (2015) 159:245–55. doi: 10.1016/j.aquatox.2014.12.020

52. Paillard F, Catheline D, Duff FL, Bouriel M, Deugnier Y, Pouchard M, et al. Plasma palmitoleic acid, a product of stearoyl-coA desaturase activity, is an independent marker of triglyceridemia and abdominal adiposity. *Nutr Metab Cardiovasc Dis*. (2008) 18:436–40. doi: 10.1016/j.numecd.2007.02.017

53. Bolsoni-Lopes A, Festuccia WT, Chimin P, Farias TSM, Torres-Leal FL, Cruz MM, et al. Palmitoleic acid (n-7) increases white adipocytes GLUT4 content and glucose uptake in association with AMPK activation. *Lipids Health Dis*. (2014) 13:199. doi: 10.1186/1476-511X-13-199

54. Guo X, Jiang X, Chen K, Liang Q, Zhang S, Zheng J, et al. The role of palmitoleic acid in regulating hepatic gluconeogenesis through SIRT3 in obese mice. *Nutrients*. (2022) 14:1482. doi: 10.3390/nu14071482

55. Tang J, Yang B, Yan Y, Tong W, Zhou R, Zhang J, et al. Palmitoleic acid protects against hypertension by inhibiting NF-κB mediated inflammation. *Mol Nutr Food Res*. (2021) 65:e2001025. doi: 10.1002/mnfr.202001025

56. Haskó G, Kuhel DG, Németh ZH, Mabley JG, Stachlewitz RF, Virág L, et al. Inosine inhibits inflammatory cytokine production by a posttranscriptional mechanism and protects against endotoxin-induced shock. *J Immunol*. (2000) 164:1013–9. doi: 10.4049/jimmunol.164.2.1013

57. Marton A, Pacher P, Murthy KG, Németh ZH, Haskó G, Szabó C. Anti-inflammatory effects of inosine in human monocytes, neutrophils and epithelial cells in vitro. *Int J Mol Med*. (2001) 8:617–21.

58. Buckley S, Barsky L, Weinberg K, Warburton D. In vivo inosine protects alveolar epithelial type 2 cells against hyperoxia-induced DNA damage through MAP kinase signaling. *Am J Physiol Lung Cell Mol Physiol*. (2005) 288:L569–75. doi: 10.1152/ajplung.00278.2004

59. Guo L, Milburn MV, Ryals JA, Lonergan SC, Mitchell MW, Wulff JE, et al. Plasma metabolomic profiles enhance precision medicine for volunteers of normal health. *Proc Natl Acad Sci U S A*. (2015) 112:E4901–10. doi: 10.1073/pnas.1508425112

60. Marin JJG, Macias RIR, Briz O, Banales JM, Monte MJ. Bile acids in physiology, pathology and pharmacology. *Curr Drug Metab*. (2015) 17:4–29. doi: 10.2174/1389200216666151103115454

61. Grzych G, Chávez-Talavera O, Descat A, Thuillier D, Verrijken A, Kouach M, et al. NASH-related increases in plasma bile acid levels depend on insulin resistance. *JHEP Rep*. (2021) 3:100222. doi: 10.1016/j.jhepr.2020.100222

62. Pani A, Giossi R, Menichelli D, Fittipaldo VA, Agnelli F, Inglese E, et al. Inositol and non-alcoholic fatty liver disease: a systematic review on deficiencies and supplementation. *Nutrients*. (2020) 12:3379. doi: 10.3390/nu12113379

frontiers | Frontiers in Veterinary Science

TYPE Original Research
PUBLISHED 28 February 2024
DOI 10.3389/fvets.2024.1339940

OPEN ACCESS

EDITED BY
Tao Wang,
Jilin Agricultural University, China

REVIEWED BY
Yujia Tian,
Tianjin Agricultural University, China
Mustafa Shukry,
Kafrelsheikh University, Egypt
Yongxin Yang,
Qingdao Agricultural University, China

*CORRESPONDENCE
Kailun Yang
✉ 172387243@qq.com

RECEIVED 17 November 2023
ACCEPTED 13 February 2024
PUBLISHED 28 February 2024

CITATION
Li X, Ma J, Li H, Li H, Ma Y, Deng H and Yang K (2024) Effect of β-alanine on the athletic performance and blood amino acid metabolism of speed-racing Yili horses. *Front. Vet. Sci.* 11:1339940. doi: 10.3389/fvets.2024.1339940

Effect of β-alanine on the athletic performance and blood amino acid metabolism of speed-racing Yili horses

Xiaobin Li[1,2], Jun Ma[3], Haiying Li[1,2], Hai Li[1,2], Yuhui Ma[1,2], Haifeng Deng[1,2] and Kailun Yang[1,2]*

[1]Xinjiang Key Laboratory of Herbivore Nutrition for Meat and Milk Production, College of Animal Science, Xinjiang Agricultural University, Ürümqi, China, [2]Xinjiang Key Laboratory of Horse Breeding and Exercise Physiology, College of Animal Science, Xinjiang Agricultural University, Ürümqi, China, [3]General Animal Husbandry Station of Ili Kazakh Autonomous Prefecture, Yining, China

The objective of this study was to examine the effects of supplemental β-alanine feeding on the athletic performance of Yili horses involved in speed racing, focusing on alterations in plasma free amino acid patterns pre and post exercise. Additionally, the research aimed to evaluate the effects of carnosine on the plasma acid–base buffering capacity and antioxidant levels in these horses. Twelve Yili horse stallions, averaging 3 years in age and 346.50 ± 21.39 kg in weight, were chosen and randomly divided into two groups: a control group and a test group, each comprising six horses. The control group received a supplementation of 300 mg/kg BW/day of α-alanine, while the test group received 300 mg/kg BW/day of β-alanine. This supplementation regimen was maintained for a 30-day supplementation trial period, under identical feeding and management conditions. Throughout the trial, the horses participated in the 1,000 Speed Race, and three distinct blood samples were gathered for assessing plasma free amino acids, blood gases, biochemical parameters, and antioxidant parameters. The outcomes indicated a considerable enhancement in the 1,000 m exercise performance of the speed racing Yili horses in the test group compared to the control group, showcasing a noteworthy improvement of 12.01%, with the test group completing the race 13.29 s faster. Notably, the α-alanine content in the plasma of the control group Yili horses remained higher than that of the test group, demonstrating a consistent increasing trend. By contrast, the plasma β-alanine content was notably higher in the test group than in the control group. Over the course of the supplementation period, plasma β-alanine exhibited an escalating and then stabilizing trend in the test group, whereas in the control group, although β-alanine content also increased, the trend was less pronounced. The plasma levels of histidine and carnosine showed minimal variance between the two groups. Overall, the test group of Yili horses exhibited slightly higher plasma levels of histidine and carnosine compared to the control group. The addition of β-alanine to their diet for a duration of 30 days notably affected the plasma levels of amino acids both pre- and post-exercise in speed-racing Yili horses. Furthermore, β-alanine demonstrated an inhibitory effect on the catabolism of these horses' bodies during high-intensity exercise. Ten marker amino acids, including valine, leucine, β-alanine, isoleucine, carnosine, 3-methyl-histidine, lysine, ethanolamine, argnine, and taurine, displayed statistically significant changes. β-alanine notably increased the blood glucose levels of Yili horses and played a role in expediting the restoration of blood gas levels post-exercise. Moreover, in the test group of Yili horses, the levels of superoxide dismutase, glutathione peroxidase, and total

antioxidant capacity significantly increased both before and after the race, while the content of malondialdehyde, an oxidation product, exhibited an extremely significant decrease immediately after the race. These outcomes suggest that the addition of β-alanine significantly augmented antioxidant levels during high-intensity exercise in Yili horses. Consequently, it reduced post-exercise injuries and accelerated the recovery process after exercise.

KEYWORDS

β-alanine, carnosine, Yili horse, free amino acids, athletic performance

1 Introduction

Carnosine, an endogenous water-soluble dipeptide, was discovered in 1900 by Guiotto and others. It is widely present in mammalian skeletal muscle, brain, nerves, and various tissues (1–3). The synthesis of carnosine in skeletal muscle is catalyzed by myostatin synthetase, which begins with the pre-activation of β-alanine under ATP-energy conditions to produce the "enzyme-β-alanyl adenosine" complex, followed by the binding of the β-alanyl portion of the enzyme to L-histidine, and the final release of carnosine from the enzyme (4). Carnosine demonstrates strong amphoteric electrolyte characteristics and effective acid–base buffering capacity (5, 6). The molecular structure of carnosine, particularly the imidazole ring and amino residue, grants it robust antioxidant properties (7, 8). In research by Huang et al., β-alanine supplementation was found to increase myostatin content in skeletal muscle. Myostatin, primarily located in myocytes, contributes to maintaining myocytes' internal stability, regulating physiological pH, chelating metal ions, scavenging free radicals, and managing glucose metabolism during intense physical activity. Elevated myostatin levels in skeletal muscle can enhance exercise performance, owing to its exceptional antioxidant properties that shield mitochondria from oxidative damage caused by free radicals, ensuring a continuous energy supply (9). In summary, β-alanine can improve mitochondrial damage, promote mitochondrial integrity, improve energy metabolism and the body's ability to resist oxidative stress during exercise and thus promote exercise performance. Thus, increasing the body's carnosine levels improves the body's exercise capacity (10, 11). Despite its physiological significance, carnosine is inadequately present and utilized in food or feed. Studies indicate that supplementing with β-alanine, which is deficient in common dietary sources, significantly elevates carnosine levels in the body (12–14). The Yili Horse 1,000 m Speed Race is a high-intensity event that requires a high level of energy supply and resistance to exercise stress. Therefore, in the present study, we supplemented β-alanine to 1,000 m speed horses, and investigated whether the level of 1,000 m speed race and the ability of resistance to exercise stress could be improved by increasing the level of myostatin in the body of the horses through detecting the changes in the exercise level of the horses, the concentration of amino acids in the blood, and the indexes of antioxidant and blood gases before and after the race.

Abbreviations: β-ala, β-alanine; Val, Valine; Leu, Leucine; Ile, Isoleucine; Car, Carnosine; 3MHis, 3-methyl-histidine; Lys, Lysine; Eth, Ethanolamine; Arg, Argnine; Tau, Taurine; MDA, Malondialdehyde; T-AOC, Total antioxidant capacity; GSH-px, Glutathione peroxidase; SOD, Superoxide dismutase; CAT, Catalase; Glu, Glucose.

2 Materials and methods

2.1 Ethical considerations

This study protocol received approval from the Animal Care and Utilization Committee of the College of Animal Science, Xinjiang Agricultural University (Urumqi, China). The research was conducted at Zhaosu Horse Farm in Yili Kazakh Autonomous Prefecture, Xinjiang.

2.2 Animal and experimental design

Twelve Yili horse stallions, aged 3 years and weighing an average of (346.50 ± 21.39 kg), with similar 1,000-m speed race performances (135.24 ± 15.75 s) from Zhaosu stable, were selected for this study. They were randomly divided into two groups of six stallions each: the control group and the test group. In accordance with Dunnet (15), the supplemental feeding dose was designed as 300 mg/kg BW/day α-alanine for the control group and 300 mg/kg BW/day β-alanine for the test group. Throughout the test period, each horse received a daily diet comprising 4 kg of high-quality mountain grass and 4 kg of alfalfa hay, along with a concentrate supplement of 3 kg in three divided doses per day, and had free access to water. Table 1 displays the diet composition and nutrient levels. The test horses were individually housed in single stalls, and their bedding was cleaned and replaced every 2 days. Feeding occurred at 07:00, 10:00, 13:00, 16:00, and 19:00 daily. Before feeding concentrate, a small amount of roughage was provided appropriately, and after finishing the roughage, the horse was allowed to move freely in the outdoor activity field. Initially, supplementation was approximately 10 g per horse, mixed with the concentrate supplement to prevent anorexia caused by the additive. The dose was gradually increased to normal levels. Table 2 outlines the training program for Yili horses in speed racing. The test horses underwent speed training once a day, scheduled during 10:00–12:00 or 14:00–16:00. The training involved the rider leading the horse to the training ground, commencing with a warm-up where the jockey slowly walked the test horse in the grass in a circular motion, then transitioned to the sand and gradually increased speed, followed by two laps of warm-up training before starting the formal speed training. Formal training utilized the interval training method, with

intervals lasting 1–2 min. The volume of formal training remained constant throughout the trial period, while the training intensity gradually increased. The training intensity was gauged by the speed horses' 1,000-m performance, with the rider controlling the horses' speed. The intensity was progressively reduced from 1.25 times the time taken to complete the 1,000 m at maximum speed in the initial stages of training to 1.11 times in the later stages.

2.3 Sample collection and analysis

A 1,000-m speed simulation test race took place at the Westfield Racecourse on the day before the commencement of the trial, on the 15th day, and on the 30th day. Professional riders were invited to participate in the race. The event involved four start/finish judges, four timekeepers, and one data recorder. During the race, the timekeepers were responsible for accurately determining the test horses' simulated race score. The average of the valid data was considered as the horses' test race score after validation by the judges, and the scores from the three simulated test race competitions were recorded.

In this trial, three types of blood samples were collected to determine plasma free amino acids, blood gases, biochemical indexes, and antioxidant indexes. To determine plasma free amino acids, blood samples were collected from the horses' jugular veins using EDTA anticoagulant tubes. These samples, measuring 5 mL each, were collected 1 h before, immediately after, and 2 h after the simulated test match on the 30th day of the trial. The collected blood samples were centrifuged at 1,500 × g for 15 min immediately after collection, and the resulting plasma was stored at −80°C.

Blood samples for measuring blood gases and biochemical analyses were obtained from the jugular vein of the horses using regular blood collection tubes at a rate of 3 mL per test. For analysis, 100 μL of whole blood, not exposed to air below the liquid level, was aspirated. Sampling times were 1 h before, immediately after, 20 min after, and 2 h after the simulated test match on the 30th day of the trial period.

Blood gas parameters were measured immediately after the collection of blood samples. For the determination of antioxidant indices, blood samples were collected in sodium heparin anticoagulant tubes from the jugular vein of the horses. These samples, measuring 5 mL each, were collected 1 h before the simulated test match on the 30th day of the trial and immediately after, 20 min after, and 2 h after the match. Similar to the previous samples, these were centrifuged at 1,500 × *g* for 15 min immediately after collection, and the resulting plasma was stored at −80°C.

TABLE 1 Diet composition and nutrient levels (DM basis).

Items	Content (%)
Ingredients %	
Alfalfa hay	72.73
Corn	15.00
Bran	3.27
Soybean meal	7.36
$CaHPO_4$	0.82
NaCl	0.55
Premixes[1]	0.27
Total	100
Nutrient levels[2]	
DM (%)	92.10
OM (%)	92.56
CP (%)	12.92
NDF (%)	20.76
ADF (%)	33.12
Ca (%)	0.86
P (%)	0.09
DM (%)	92.10

[1]The premix provided the following per kg of diets: VA 4.09 mg, VB1 5.80 mg, VB2 2.84 mg, VB6 0.33. Mg, VB12 0.0027 mg, VC 5.68 mg, VD 0.60 mg, VE 218.12 mg. Calcium Pantothenate 1.39 mg, Nicotinamide 3.31 mg, Biotin 2.73 mg, Choline chloride 32.73 mg, Swell soil 1.94 g, Cu (as copper sulfate) 45.09 mg, Fe (as ferrous sulfate) 151.52 mg, Mn (as manganese sulfate) 142.94 mg, Zn (as zinc sulfate) 131.75 mg, I (as potassium iodide) 10.53 mg, Se (as sodium selenite) 25.25 mg and Co (as cobalt chloride) 4.55 mg. [2]The nutrient levels are all measured values.

2.4 Sample determination

2.4.1 Determination of β-alanine-based indicators in plasma

Plasma β-alanines were analyzed by Beijing Purui Huasheng Biological Co., Ltd., using high-performance liquid chromatography–tandem mass spectrometry (HPLC-MS/MS).

2.4.1.1 Sample processing

For the analysis, 50 μL of plasma was mixed with 50 μL of protein precipitant (containing NVL), frozen, and centrifuged at 13,200 rpm for 4 min. Then, 10 μL of the supernatant was mixed with 50 μL of labeling buffer, followed by the addition of 20 μL of derivatization solution. This mixture was subjected to constant temperature derivatization at 55°C for 15 min. After cooling in the refrigerator, the samples were dissociated, and 50 μL of the samples were used for testing in the machine.

TABLE 2 Training program for speed racing Yili horses.

Train time(d)	Warm-up before treatment	Training intensity	Distance	Intermissive time	Repetitional times
1–14 days	The test horses were warmed up first at a slow trot on the grass track and the sand track respectively, and gradually increased their speed, and after two warm-up laps they officially started interval training	1.25 times the time of the 1,000 m speed event	1,000 m	1–2 min	3
15–30 days		1.11 times the time of the 1,000 m speed event	1,000 m	1–2 min	3

2.4.1.2 Instrument model

HPLC-MS/MS (Ultimate3000-API 3200 Q TRAP).

2.4.1.3 Amino acid kit

MSLAB-45 + AA (batch no.: MSLAB451561#; Beijing Mass Spectrometry Medical Research Co.), methanol, and hadronitrile were purchased from Fisher.

The chromatographic conditions were as follows: column, MSLab 45 + AA − C18 (150 × 4.6 mm, 5 μm); mobile phase A, 0.1% formic acid aqueous solution; mobile phase B, acetonitrile; flow rate, 1 mL/min; column temperature, 50°C; injection volume, 5 μL; chromatographic run time, 20 min. The mass spectrometry conditions were as follows: the ion source was an +ESI electrospray ion source; MRM multi-reaction monitoring was used; the curtain gas (CUR) was 20 psi; spray voltage (ESI) was +5,500 V; collision gas (CAD) was medium; collision chamber ejection voltage (CXP) was 2.0; nebulizer (GAS1) was 55 psi; auxiliary gas (GAS2) was 60 psi; nebulization temperature (TEM) is 500°C; and ingress voltage was 10 V. Amino acid standard curves and peak times are shown in Table 3.

2.4.2 Measurement of blood gases and biochemical indicators

Blood gas analysis is a crucial method to rapidly and accurately assess changes and trends in the body's acid–base balance. It involves the determination of O_2 and CO_2 levels in the blood for gas exchange, along with measuring relevant acid–base balance parameters. This analysis aids in understanding the body's ventilation, gas exchange function, and various acid–base imbalance conditions. In domestic animals, blood gas analysis helps comprehend the internal environment's state concerning gas exchange, acid–base balance, and oxygenation. It is closely associated with electrolytes, essential for maintaining osmotic pressure and regulating acid–base balance and metabolism, including cardiac and muscle function regulation. The blood gas and biochemical indices were measured using an i-SATA portable blood gas analyzer. Electrochemical methods were used to determine Na^+ and K^+ levels, galvanometry for Glu and PO_2, voltammetry for pH and PCO_2, selective electrode voltammetry for iCa^{2+}, and electroconductivity for Hct.

2.4.3 Measurement of plasma antioxidant capacity

Plasma levels of malondialdehyde (MDA), total antioxidant capacity (T-AOC), glutathione peroxidase (GSH-px), superoxide dismutase (SOD), and catalase (CAT) activities were assessed using commercially available kits (Nanjing Jianjian Bioengineering Research Institute, Nanjing, China). The antioxidant enzyme activities were determined according to the provided kit manual. Measurements were conducted colorimetrically using a 7230G spectrophotometer for T-AOC, SOD, GSH-Px, CAT, and MDA in plasma.

2.5 Statistical analysis

Data of free amino acids and blood gas/biochemical indices were extracted and normalized using EZinfo2 2.0 software. Free amino acids PCA analyses were performed using the FactoMineR package in R software and the ggplot2 package (version 2.15.3). The PCoA analysis was performed using the WGCNA package, stat packages, and ggplot2 package in R software (Version 2.15.3).

Preliminary analysis of the experimental data was conducted using Excel 2010. The normality (Shapiro–Wilk) test was performed before statistical analysis. The Shapiro–Wilk test determined normality for athletic performance and plasma antioxidant indexes. Further statistical analysis was conducted using SPSS 20.0 software (SPSS Statistics 20, IBM Japan, Ltd., Tokyo, Japan) with independent-samples t-tests. Data were expressed as mean ± standard deviation, with $p < 0.05$ indicating significant differences and $0.05 < p < 0.10$ indicating a significant trend of differences.

3 Results

3.1 Effect of supplemental feeding of β-alanine on the athletic performance of speed-racing Yili horses

Figure 1 depicts the effect of β-alanine supplementation on the athletic performance of Yili horses in speed racing. Initially, the 1,000-m exercise times for the control and test groups of speed racing Yili horses were 135.76 and 134.73 s, respectively. After 15 days, the performance of the test group improved by 2.47%, clocking in 2.97 s faster than the control group, although this difference was not statistically significant ($p > 0.05$). By day 30, the test group's performance significantly improved by 12.01%, completing the 1,000-m race in 13.29 s less than that in the control group ($p < 0.05$).

3.2 Effect of supplemental feeding of β-alanine on β-alanine-like indices in the plasma of speed-racing Yili horses

Figure 2 shows the effect of β-alanine supplementation on β-alanine-related parameters in the plasma of Yili horses during 1,000-m speed races. In Figure 2A, the plasma α-alanine content of the control group exhibited a steady increase, with a slower rising trend after 18:00 (i.e., the 4th supplemental feeding). Conversely, Figure 2B shows higher β-alanine content in the plasma of the test group than in the control group. The plasma β-alanine content remained stable from 9:00 to 15:00 (i.e., 2 h after the initial three supplemental feedings) and displayed a noticeable upward trend from 15:00 onward, with the most significant increase observed from 15:00 to 18:00 (i.e., 2 h after the third and fourth supplemental feedings). The upward trend had slowed down, and the concentration of α-alanine also increased, but the trend was not obvious. The control group also displayed an increase, but the trend was not obvious. Figures 2C,D indicate marginal differences in the plasma levels of histidine and carnosine between the test and control groups. Overall, the test group of Yili horses exhibited slightly higher plasma levels of histidine and carnosine than the control group.

3.3 Effect of supplemental feeding of β-alanine on amino acid metabolism in the plasma of speed-racing Yili horses

Figure 3 illustrates the effect of β-alanine supplementation on amino acid metabolism in plasma both before and after speed racing

TABLE 3 Amino acid standard curve and peak time.

Item	Molecular weight	Peak time (minutes)	Standard curve	R^2
3-Aib	103.12	7.38	y = 0.0126x + 0.00251	0.9994
3-MHis	169.18	3.78	y = 0.000406x − 3.12e − 005	0.9983
5Ava	117.15	7.93	y = 0.0189x + 0.000875	0.9997
5-HT	176.20	10.70	y = 0.0154x − 0.0021	0.9997
5-HTP	220.22	9.31	y = 0.00294x − 0.000907	0.9969
Aad	161.16	6.40	y = 0.00389x + 0.00708	0.9996
Abu	103.12	8.47	y = 0.0145x + 0.000688	0.9986
Ala	89.09	6.51	y = 0.00567x + 0.022	0.9965
Ans	240.26	3.82	y = 0.000176x − 3.58e − 005	0.9941
Arg	174.20	5.31	y = 0.00113x − 0.000548	0.9938
Asa	290.27	3.79	y = 0.00018x + 3.42e − 005	0.9932
Asn	132.12	3.26	y = 0.0101x + 0.00632	0.9974
Asp	133.10	4.63	y = 0.00221x + 0.000114	0.9949
β-Ala	89.09	5.64	y = 0.0108x + 0.023	0.9997
Car	226.23	3.80	y = 0.0108x + 0.023	0.9995
Cit	175.19	4.41	y = 0.000445x + 4.32e − 005	0.9974
Cth	222.26	7.95	y = 0.000603x + 0.000133	0.9997
Cys1	121.16	8.56	y = 0.000178x − 4.94e − 005	0.9974
EtN	61.08	4.56	y = 0.00188x-0.000229	0.9956
GABA	103.12	6.44	y = 0.0107x + 0.00522	0.9966
Gln	146.15	3.55	y = 0.00314x + 0.00217	0.9998
Glu	147.13	4.95	y = 0.00465x + 0.00118	0.9991
Gly	75.07	4.69	y = 0.00285x + 0.000531	0.9969
Harg	188.20	6.26	y = 0.000947x-6.12e − 005	0.9845
Hcit	189.20	5.64	y = 0.00107x + 0.000149	0.9988
His	155.15	3.76	y = 0.000377x − 9.31e − 005	0.9993
Hpro	129.16	10.00	y = 0.00436x − 0.000308	0.9990
Hser	119.10	3.73	y = 0.00276x + 0.00128	0.9917
Hyl	162.20	7.12	y = 0.00106x + 0.000221	0.9993
Hyp	131.13	3.12	y = 0.0274x + 0.00918	0.9927
Ile	131.18	12.70	y = 0.0211x + 0.000765	0.9995
Kyn	208.10	12.60	y = 0.00463x − 0.000324	0.9982
Leu	131.17	13.30	y = 0.0198x + 0.00132	0.9994
Lys	146.19	9.23	y = 0.00126x + 0.000487	0.9984
Met	149.20	10.10	y = 0.00965x + 0.000992	0.9996
Orn	132.16	8.32	y = 0.00133x + 0.000433	0.9991
PEtN	141.06	1.90	y = 0.000234x + 3.93e − 005	0.9973
Phe	165.19	13.9	y = 0.0174x + 0.00579	0.9988
Pro	115.14	7.08	y = 0.0109x + 0.0174	0.9997
Sar	89.09	5.21	y = 0.0118x + 0.00113	0.9997
Ser	105.09	3.74	y = 0.00761x + 0.0235	0.9908
Tau	125.25	2.02	y = 0.000735x-0.000721	0.9938
Thr	119.13	5.44	y = 0.0056x + 0.00018	0.9999
Trp	204.23	14.5	y = 0.0129x + 0.000269	0.9990
Tyr	181.19	9.47	y = 0.00799x + 0.00123	0.9989
Val	117.15	10.30	y = 0.0102x + 0.0145	0.9966

among Yili horses. In Figure 3A and Table 4, the plasma concentration of β-alanine in the Yili horse test group was 10.09 times higher than that of the control group 1 h before the race ($p < 0.01$). Additionally, the concentration of valine was notably 14.67% lower than that of the control group ($p < 0.05$), while the concentration of arginine was significantly higher by 32.10% ($p < 0.01$). No other significant differences in amino acid concentrations were observed between the two groups. The principal coordinate analysis (PCoA) results demonstrated that the horizontal coordinate accounted for 39.11% of sample variation, with the vertical coordinate contributing 30.03%. Notably, there was no substantial similarity observed in amino acid concentrations within the pre-race plasma of control and test Yili horses based on the PCoA and principal component analysis (PCA). The PCA indicated that the horizontal coordinate, contributing 49.18% to sample variance, and the vertical coordinate, contributing 34.02%, did not reveal significant similarities in amino acid concentrations between the pre-race plasmas of control and test Yili horses.

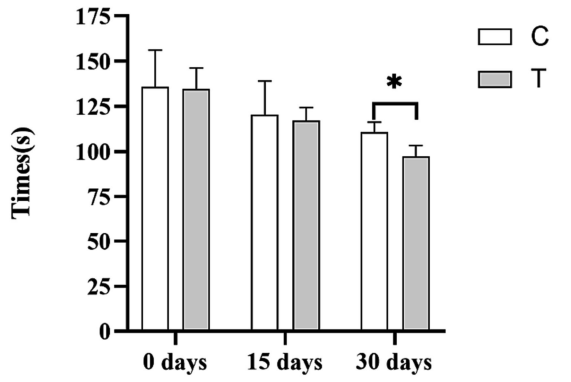


FIGURE 1
Effect of supplemental feeding of β-alanine on the athletic performance of speed-racing Yili horses (C: α-alanine group; T: β-alanine group). Each bar represents the mean of 6 horses ± standard error of the mean (SEM). * indicates a significant difference between the two groups ($p < 0.05$), ** indicates a highly significant difference between the two groups ($p < 0.01$).

As shown in Figure 3B and Table 5, post-competition plasma analysis displayed that the β-alanine concentration in the Yili horse test group was 6.12 times higher than that of the control group ($p < 0.01$). Moreover, concentrations of carnosine and 3-methylhistidine were significantly higher than those of the control group by 72.03 and 70.65% ($p < 0.05$), while valine concentrations were notably lower by 23.71% ($p < 0.01$). Notably, ethanolamine and taurine exhibited substantial changes in plasma concentrations before and after the race. Ethanolamine and taurine concentrations in the test group increased by 3.4 and 1.6 times before the race, respectively. Specifically, ethanolamine concentration in the test group remained higher than that of the

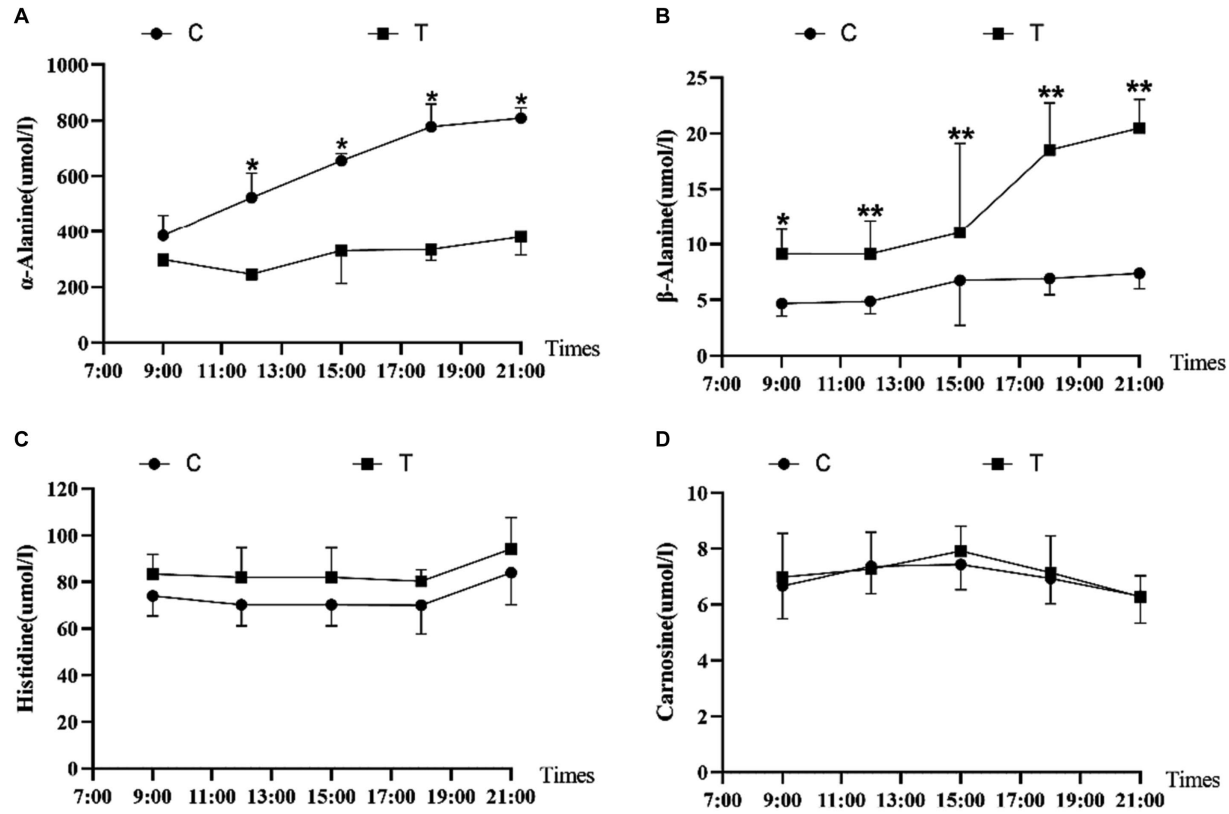


FIGURE 2
Effect of supplemental feeding of β-alanine on beta-alanine-like indices in the plasma of speed-racing Yili horses **(A–D)** (C: α-alanine group; T: β-alanine group). * indicates a significant difference between the two groups ($p < 0.05$), ** indicates a highly significant difference between the two groups ($p < 0.01$).

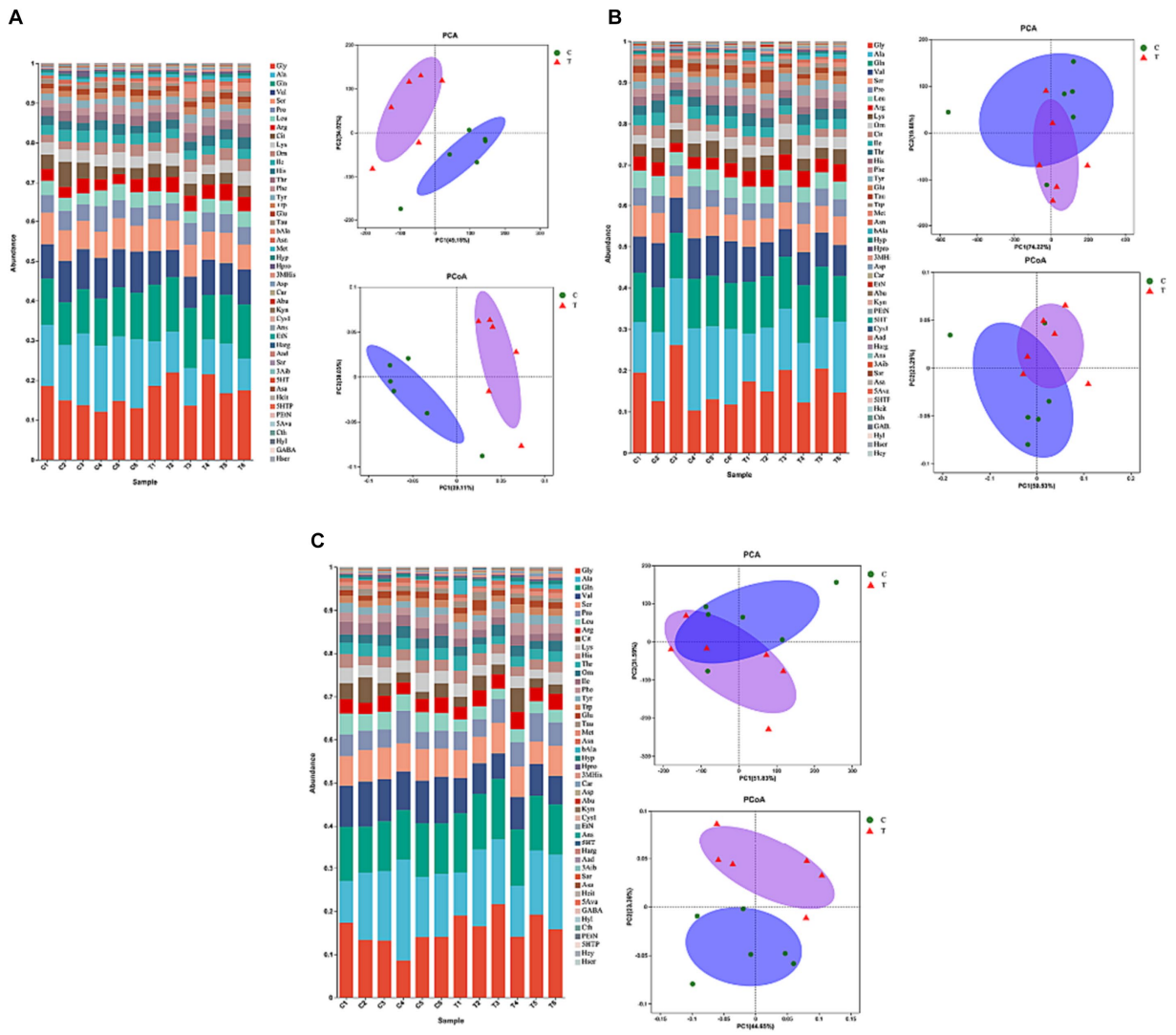


FIGURE 3
Effect of supplemental feeding of beta-alanine on amino acid metabolism in the plasma of speed-racing Yili horses **(A–C)** (C: α-alanine group T: β-alanine group).

control group before and after the race, whereas taurine concentration in the test group was lower than that of the control group immediately after the race. Regarding PCoA analysis, the horizontal coordinate contributed 50.53% to sample variance, while the vertical coordinate contributed 23.29%. Interestingly, there was high amino acid similarity observed in the plasma of control and test Yili horses immediately after the race, as revealed by the PCoA analysis. The PCA analysis reveals that the primary contributor to sample variance is the horizontal coordinate, amounting to 74.22%. Following this, the vertical coordinate represents the second principal component, contributing 19.68% to the sample variance. It exhibited a notable resemblance in the amino acid composition of plasma between control and test Yili horses immediately after the race.

This similarity is evident in Figure 3C and Table 6, where the plasma concentrations of β-alanine, carnosine, and 3-methylhistidine in the Yili test group were significantly higher than those in the control group ($p < 0.05$) at the 2-h mark post-race. Conversely, the plasma concentrations of the major branched-chain amino acids (BCAAs)—valine, leucine, and isoleucine—were 31.88% ($p < 0.01$), 36.75% ($p < 0.01$), and 36.07% ($p < 0.01$) lower, respectively, in the Yili test group than in the control group at the same post-race interval. Plasma lysine levels changed immediately after the race, decreasing in both test and control groups, with the test group exhibiting significantly lower levels than the control group at the 2-h mark post-race ($p < 0.05$). The PCoA analysis results demonstrate that the horizontal coordinate accounts for 44.65% of sample variation, while the vertical coordinate represents other principal components contributing 23.38% to sample variation. There was a lack of significant amino acid similarity in the plasma of control and test Yili horses 2 h after racing. The PCA results showed that the horizontal coordinate is the main component, contributing 51.83% to the sample variance. The vertical indicates the second principal component, contributing

TABLE 4 Effect of β-alanine on the metabolic concentration of amino acids in the plasma of Yili horses 1 h before the race (μmol/L).

Items	Control groups	Test groups
Gly	345.00 ± 95.92	441.67 ± 82.43
Ala	378.50 ± 38.68^{A}	244.67 ± 63.75^{B}
Gln	270.67 ± 30.07	324.00 ± 55.97
Val	229.50 ± 18.45^{a}	195.83 ± 29.03^{b}
Ser	172.00 ± 31.70	179.83 ± 33.89
Pro	114.62 ± 20.08	109.00 ± 11.47
Leu	87.63 ± 10.49	84.60 ± 16.22
Arg	70.88 ± 12.21^{B}	93.63 ± 11.21^{A}
Cit	85.70 ± 41.38	71.28 ± 15.53
Lys	74.33 ± 10.24	74.38 ± 14.71
Orn	54.62 ± 9.89^{b}	70.07 ± 10.49^{a}
Ile	62.93 ± 9.58	56.22 ± 9.30
His	53.32 ± 9.52	63.07 ± 11.97
Thr	54.53 ± 8.01	58.35 ± 12.77
Phe	43.32 ± 3.92^{b}	50.42 ± 4.36^{a}
Tyr	40.12 ± 6.96	43.73 ± 4.89
Trp	34.72 ± 1.53	34.8 ± 4.98
Glu	31.7 ± 3.90	34.67 ± 4.59
Tau	25.98 ± 11.01	25.42 ± 7.13
β-Ala	3.95 ± 1.62^{B}	39.88 ± 22.65^{A}
Asn	19.27 ± 4.82	23.08 ± 5.10
Met	19.2 ± 2.42	20.63 ± 4.07
Hyp	14.17 ± 2.55	15.67 ± 3.06
Hpro	15.73 ± 10.60	13.64 ± 3.05
3-MHis	6.80 ± 1.37	11.26 ± 4.89
Asp	7.86 ± 2.57	9.41 ± 3.33
Car	6.50 ± 2.72	8.73 ± 2.11
Abu	2.91 ± 0.47	2.88 ± 0.51
Kyn	2.20 ± 0.52	2.55 ± 0.33
Cys1	2.58 ± 1.11	2.00 ± 0.47
Ans	2.15 ± 1.15	1.67 ± 0.96
EtN	1.40 ± 0.70	1.48 ± 0.53
Harg	1.31 ± 0.43	1.37 ± 0.22
Aad	1.04 ± 0.42	1.11 ± 0.41
Sar	0.83 ± 0.24	0.96 ± 0.22
3-Aib	0.63 ± 0.20	1.00 ± 0.44
5-HT	0.66 ± 0.15^{A}	0.38 ± 0.09^{B}
Asa	0.62 ± 0.54	0.24 ± 0.28
Hcit	0.30 ± 0.19	0.28 ± 0.23
5-HTP	0.25 ± 0.10	0.29 ± 0.14
PEtN	0.53 ± 0.97	--
5-Ava	0.25 ± 0.32	0.18 ± 0.21
Cth	0.14 ± 0.12	0.22 ± 0.11
Hyl	0.10 ± 0.15	0.15 ± 0.29
GABA	0.05 ± 0.06	0.11 ± 0.14
Hser	0.10 ± 0.07	0.05 ± 0.08

^{a,b}Values within a row without common superscripts differ significantly ($p < 0.05$). ^{A,B}Values in rows with no common superscript, highly significant ($p < 0.01$).

TABLE 5 Effect of β-alanine on the metabolic concentration of amino acids in the plasma of Yili horses in the immediate post-race period (μmol/L).

Items	Control groups	Test groups
Gly	431.67 ± 254.72	412.33 ± 84.91
Ala	446.17 ± 92.74	358 ± 88.14
Gln	305.33 ± 43.27	308.5 ± 21.68
Val	255.17 ± 36.65^{A}	194.67 ± 24.41^{B}
Ser	177.33 ± 12.64	162.17 ± 22.09
Pro	112.07 ± 19.66	107.35 ± 10.61
Leu	113.33 ± 9.07	99.80 ± 17.63
Arg	80.70 ± 7.78	96.77 ± 18.39
Cit	76.33 ± 41.93	53.28 ± 5.83
Lys	90.9 ± 14.73	82.40 ± 18.00
Orn	61.93 ± 10.09	72.52 ± 15.70
Ile	68.52 ± 11.04	58.82 ± 8.56
His	53.67 ± 6.82	54.10 ± 10.43
Thr	61.73 ± 7.68	61.3 ± 10.94
Phe	48.5 ± 4.31	53.4 ± 5.22
Tyr	42.72 ± 4.69	47.83 ± 5.37
Trp	34.15 ± 3.27	29.9 ± 6.44
Glu	41.1 ± 6.53	46.03 ± 15.91
Tau	45.92 ± 14.79	40.5 ± 23.12
β-Ala	4.45 ± 0.81^{B}	27.22 ± 13.51^{A}
Asn	17.65 ± 7.54	17.02 ± 5.84
Met	18.78 ± 1.73	21.57 ± 5.19
Hyp	12.37 ± 2.17	13.43 ± 2.92
Hpro	10.46 ± 5.53	10.63 ± 2.37
3-MHis	7.60 ± 2.14^{b}	12.97 ± 5.34^{a}
Asp	10.86 ± 4.60	8.79 ± 4.71
Car	6.65 ± 2.96^{B}	11.44 ± 1.22^{A}
Abu	2.83 ± 0.88	2.45 ± 0.40
Kyn	2.15 ± 0.45	2.53 ± 0.15
Cys1	1.99 ± 1.73	0.78 ± 0.18
Ans	0.96 ± 0.6	0.83 ± 0.63
EtN	4.54 ± 3.18	5.00 ± 4.53
Harg	1.18 ± 0.4	0.76 ± 0.46
Aad	1.69 ± 2.00	0.76 ± 0.57
Sar	0.69 ± 0.26	0.98 ± 0.29
3-Aib	0.91 ± 0.26	0.84 ± 0.44
5-HT	1.63 ± 1.38	1.60 ± 2.01
Asa	0.18 ± 0.24	0.57 ± 0.41
Hcit	0.19 ± 0.25	0.35 ± 0.35
5-HTP	0.23 ± 0.18	0.36 ± 0.22
PEtN	2.21 ± 3.28	1.11 ± 1.24
5-Ava	0.35 ± 0.40	0.28 ± 0.20
Cth	0.30 ± 0.50	0.13 ± 0.12
Hyl	0.15 ± 0.11	0.09 ± 0.13
GABA	0.25 ± 0.24	0.07 ± 0.11
Hser	0.04 ± 0.06	0.05 ± 0.07

^{a,b}Values within a row without common superscripts differ significantly ($p < 0.05$). ^{A,B}Values in rows with no common superscript, highly significant ($p < 0.01$).

TABLE 6 Effect of β-alanine on the metabolic concentration of amino acids in the plasma of Yili horses 2 h after the race (μmol/L).

Items	Control groups	Test groups
Gly	316.00 ± 72.16	403.17 ± 99.20
Ala	371.67 ± 137.26	335.33 ± 113.53
Gln	283.67 ± 36.97	298.83 ± 49.09
Val	232.67 ± 23.85^{A}	158.50 ± 15.31^{B}
Ser	165.50 ± 24.44	148.00 ± 32.85
Pro	117.28 ± 42.71	118.10 ± 22.59
Leu	99.65 ± 15.01^{A}	63.03 ± 9.94^{B}
Arg	75.68 ± 14.87	79.50 ± 16.83
Cit	78.07 ± 35.72	66.48 ± 26.47
Lys	78.72 ± 16.69a	58.77 ± 10.97b
Orn	56.78 ± 12.45	50.62 ± 13.64
Ile	65.23 ± 11.36^{A}	41.70 ± 6.28^{B}
His	62.17 ± 11.05	56.40 ± 10.81
Thr	63.82 ± 7.22^{a}	53.05 ± 7.57^{b}
Phe	48.62 ± 6.07	49.53 ± 6.16
Tyr	43.95 ± 8.67	41.13 ± 6.02
Trp	39.08 ± 4.43	36.62 ± 4.25
Glu	32.38 ± 6.58	35.8 ± 13.17
Tau	23.66 ± 10.50	26.17 ± 12.96
β-Ala	4.13 ± 1.05^{B}	27.47 ± 17.83^{A}
Asn	22.90 ± 5.38	18.1 ± 5.00
Met	19.97 ± 2.80	21.78 ± 4.80
Hyp	15.73 ± 2.23	15.70 ± 3.89
Hpro	12.02 ± 7.67	10.76 ± 2.33
3MHis	8.43 ± 0.97^{b}	13.52 ± 5.41^{a}
Asp	6.93 ± 2.94	8.74 ± 4.59
Car	7.77 ± 2.06^{b}	11.12 ± 2.79^{a}
Abu	2.76 ± 0.70	2.38 ± 0.41
Kyn	2.09 ± 0.47^{b}	2.62 ± 0.28^{a}
Cys1	1.91 ± 1.24	1.50 ± 0.52
Ans	1.33 ± 0.37	1.59 ± 0.53
EtN	1.59 ± 0.90	1.68 ± 1.09
Harg	1.04 ± 0.19	1.01 ± 0.18
Aad	0.97 ± 0.26	1.00 ± 0.58
Sar	0.75 ± 0.12	0.8 ± 0.32
3-Aib	0.71 ± 0.15	0.90 ± 0.29
5-HT	0.8 ± 0.31	1.66 ± 2.46
Asa	0.25 ± 0.15	0.36 ± 0.46
Hcit	0.28 ± 0.17	0.3 ± 0.31
5-HTP	0.18 ± 0.19	0.06 ± 0.15
PEtN	0.27 ± 0.35	--
5-Ava	0.26 ± 0.22	0.29 ± 0.22
Cth	0.04 ± 0.10^{b}	0.24 ± 0.16^{a}
Hyl	0.20 ± 0.15	0.18 ± 0.19
GABA	0.24 ± 0.09	0.28 ± 0.11
Hser	0.02 ± 0.03	0.04 ± 0.07

^{a,b}Values within a row without common superscripts differ significantly ($p < 0.05$). ^{A,B}Values in rows with no common superscript, highly significant ($p < 0.01$).

31.59% to the sample variance, showing a high similarity of amino acids in the plasma of horses in the control group and Yili horses of the test group 2 h after the race.

3.4 Effect of β-alanine supplementation on blood gas indices before and after a 1,000 m speed race in Yili horses

Figure 4 shows the effect of β-alanine on blood gas indices before and after the 1,000-m speed race in Yili horses. Notably, the VIP blood gas indexes of Glu and Lac in the control group were significantly lower than those in the test group by 24.58% ($p < 0.01$) and 72.48% ($p < 0.01$), respectively, in the immediate post-competition period. Among the non-VIP indicators, TCO_2 and SO_2 were 57.30% ($p < 0.01$) and 16.22% ($p < 0.05$) higher in the control group than in the test group, respectively. The differences in Glu and Lac levels between the two groups persisted at 20 min after the race, while PO_2 was significantly higher in the test group than in the control group. Two hours after the race, the blood indexes had largely recovered, with the Hct of the control group remaining significantly higher than that of the test group. Figure 5 illustrates the effect of supplemental β-alanine feeding on antioxidant levels in Yili horses before and after a 1,000-m speed race.

3.5 Effect of supplemental feeding of β-alanine on plasma antioxidant capacity before and after 1,000 m speed races in Yili horses

In Figure 5A, the CAT activity in the plasma of the Yili horse test group was 11.70% higher than that of control group 1 h before the race ($p < 0.01$). There was no significant difference immediately after and 20 min after the race, but 2 h after the plasma CAT activity in the test group was notably higher than that of the control group 2 h after the race by 9.39% ($p < 0.05$). In Figure 5B, SOD activity in the plasma of Yili horses in the test group was 9.28%, 15.45%, and 8.66% higher than that of the control group 1 h before the race, immediately after, and 20 min after the race, respectively ($p < 0.05$). However, no significant difference was observed between the two groups 2 h after the race. Figure 5C displays no significant difference in GSH-PX activity in the plasma of Yili horses in the test group 1 h before and immediately after the race. Yet, 20 min and 2 h after the race, the test group showed significantly higher levels by 16.39% ($p < 0.01$) and 3.70% ($p < 0.05$), respectively, compared with the control group. In Figure 5D, T-AOC viability in the plasma of Yili horses in the test group showed no significant difference 1 h before and immediately after the race. However, it was significantly higher than that in the control group 20 min after ($p < 0.01$) and 2 h after the race ($p < 0.05$), by 15.40% ($p < 0.01$) and 9.84% ($p < 0.05$), respectively. Figure 5E indicates no significant difference in MDA activity in the plasma of Yili horses in the test group 1 h before, 20 min after, and 2 h after the race. Yet, immediately after the race, the test group exhibited a significant 26.46% reduction in MDA activity compared with the control group ($p < 0.01$).

10.3389/fvets.2024.1339940

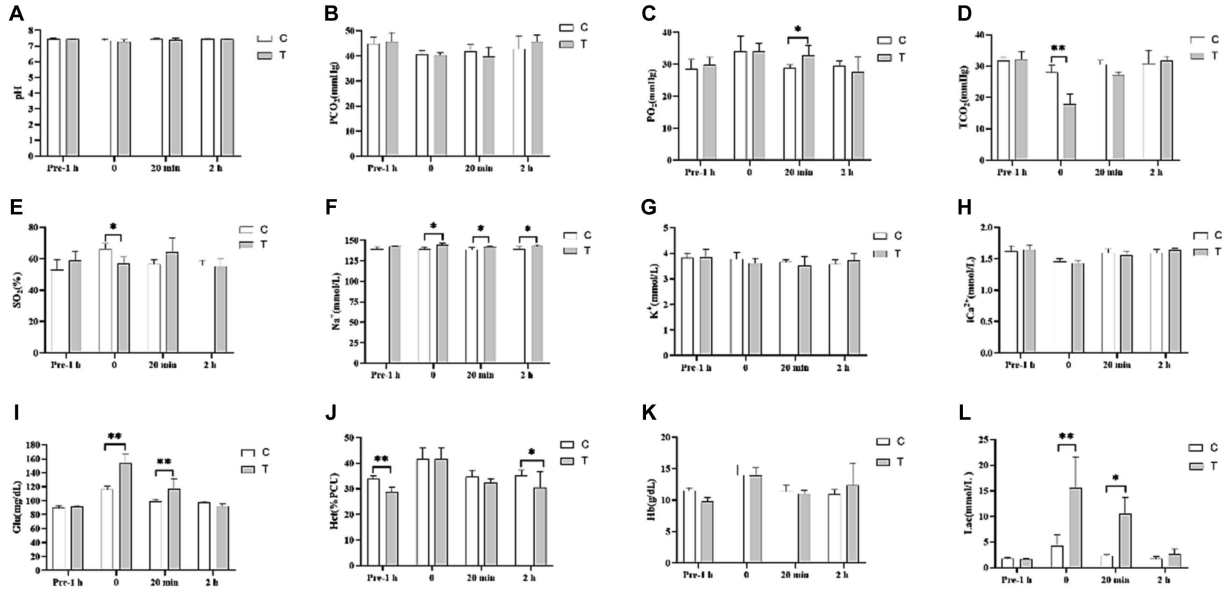


FIGURE 4
Effect of β-alanine supplementation on blood gas indices before and after a 1,000m speed race in Yili horses **(A–L)** (C: α-alanine group T: β-alanine group). Each bar represents the mean of 6 horses ± standard error of the mean (SEM). * indicates a significant difference between the two groups ($p<0.05$), ** indicates a highly significant difference between the two groups ($p<0.01$).

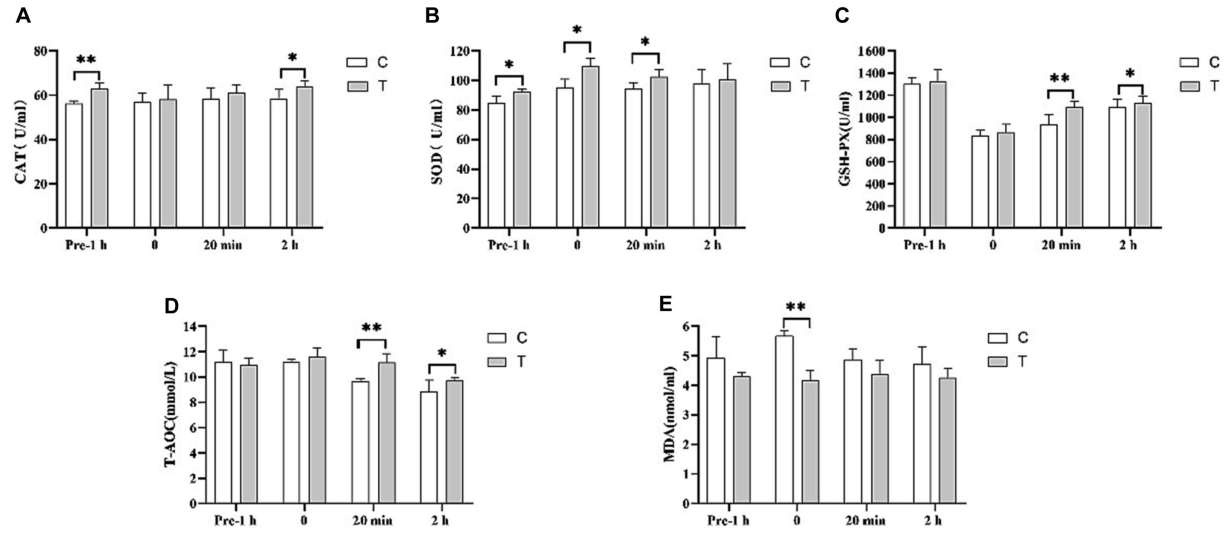


FIGURE 5
Effect of supplemental feeding of β-alanine on plasma antioxidant capacity before and after 1,000 m speed races in Yili horses. **(A–E)** Each bar represents the mean of 6 horses ± standard error of the mean (SEM). * indicates a significant difference between the two groups ($p < 0.05$), ** indicates a highly significant difference between the two groups ($p < 0.01$).

3.6 Pearson's correlation analysis of the top 10 amino acids in the plasma with blood gas indices and antioxidant capacity of 1,000 m speed-racing Yili horses supplemented with β-alanine before and after racing

This study conducted an analysis of the Pearson's correlation between the top 10 amino acids in plasma, blood gas indices, and antioxidant capacity before and after 1,000-m speed races involving Yili horses supplemented with β-alanine. Figure 6A demonstrates that 1 h before the race, T-AOC exhibited a significant positive correlation with 3MHis concerning antioxidant capacity. Moreover, pH was positively correlated with 3MHis, while TCO_2 showed a negative correlation with 3MHis among the blood gas indexes. Additionally, Na^+ exhibited a positive correlation with Leu and Tau, while K^+ showed a positive correlation with β-alanine. Following the race, as shown in Figure 6B, there was a significant positive correlation in antioxidant capacity between MDA and Tau, and a significant negative correlation between GSH-PX and Car. Regarding blood gas indices, pH displayed a

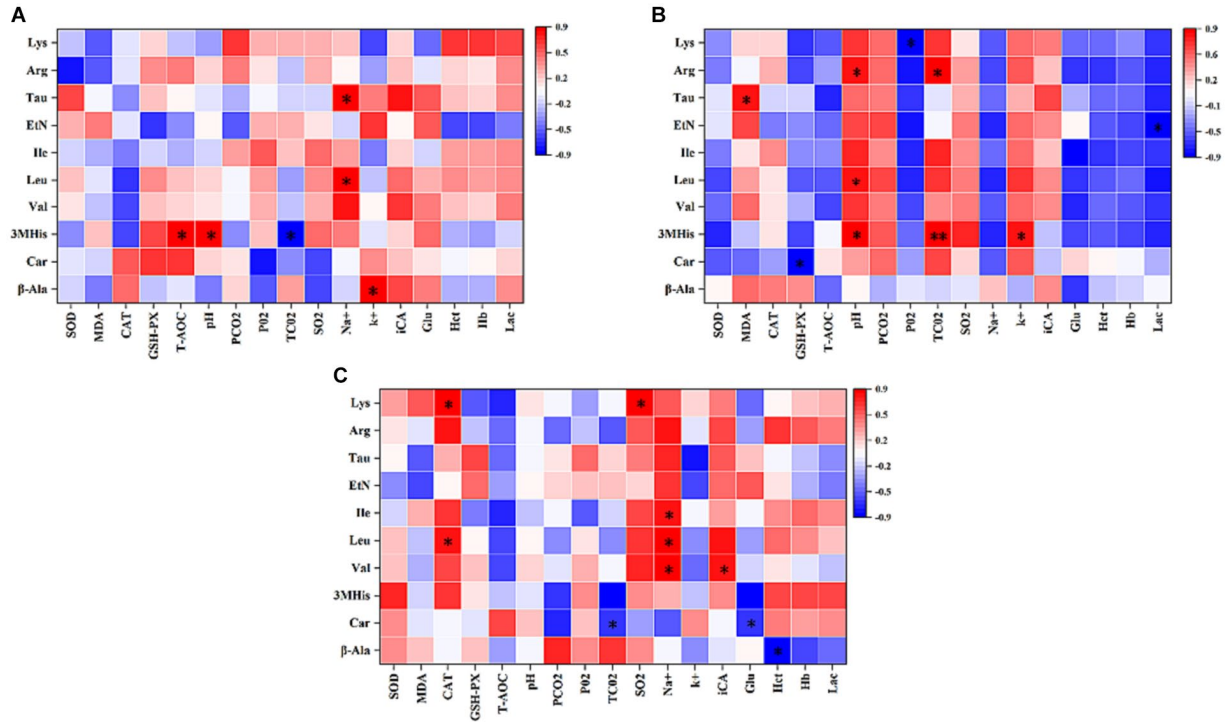


FIGURE 6
Pearson's correlation analysis of the top 10 amino acids in the plasma with blood gas indices and antioxidant capacity of 1,000 m speed-racing Yili horses supplemented with β-alanine before and after racing **(A–C)** (C: α-alanine group T: β-alanine group). The intensity of the colors represents the degree of association. Red represents a significant positive correlation ($p < 0.05$), blue represents significantly negative correlation ($p < 0.05$), and white shows that the correlation was not significant ($p > 0.05$). * indicates a significant difference between the two groups ($p < 0.05$), ** indicates a highly significant difference between the two groups ($p < 0.01$).

significant positive correlation with 3MHis, Leu, and Arg. Conversely, PO_2 exhibited a significant negative correlation with Lys, while TCO_2 showed either a significant or highly significant positive correlation with 3MHis and Arg. Moreover, K^+ demonstrated a significant positive correlation with 3MHis, while Lac exhibited a significant negative correlation with Eth. The relationship between 3-MHis and β-alanine metabolism is direct, with 3MHis being a product of goose carnosine methylation. Elevated plasma levels of β-alanine in speed-racing Erythroxylon horses, subsequent to β-alanine supplementation, stimulate goose carnosine synthesis, leading to increased 3-methylhistidine. *In vivo*, 3-MHis exists in myofibrillar proteins and skeletal muscle myosin, releasing energy during myosin catabolism through oxidation. Notably, plasma 3MHis content displayed significant or highly significant positive correlation with blood gas indexes TCO_2, K^+, and pH immediately after the race. This suggests that Yili horses supplemented with β-alanine experienced heightened anaerobic respiration during the 1,000-m test race, resulting in increased lactate accumulation (Lac) and subsequently elevated blood gas indexes TCO_2, K^+, and pH. Figure 6C depicts that CAT in antioxidant capacity displayed a significant positive correlation with Leu and Lys at the 2-h mark after the race. Among the blood gas indices, TCO_2 exhibited a significant negative correlation with Car, while SO_2 showed a significant positive correlation with Lys. Furthermore, Na^+ exhibited a significant positive correlation with Val, Leu, and Ile, whereas iCa displayed a significant positive correlation with Val. Glu, by contrast, demonstrated a significant negative correlation with Car, and Hct exhibited a significant negative correlation with β-alanine.

4 Discussion

Many studies have suggested the notable increase in skeletal muscle carnosine content through oral administration of β-alanine (12–14). Unlike direct carnosine administration, oral intake of β-alanine is readily absorbed by the intestines, entering the body's circulation and reaching the myocytes to participate in carnosine synthesis within the myocytes (5, 16, 17). Dunnet's study showed that supplemental with β-alanine 300 mg/kg BW/day to untrained pure blood horses gave the peak β-alanine concentration in the blood 6 h after the first dose (15). In our study, Yili horses supplemented with β-alanine showed a notably higher plasma β-alanine level compared with those supplemented with α-alanine 2 h, specifically at 9:00 post-supplementation. This level exhibited an upward trend during 15:00–18:00, gradually declining during 18:00–21:00. Unlike Dunnet's study, our research observed a delayed peak and lacked a plateau (15). Dunnett previously established that blood carnosine predominantly originates from myocyte spillover after injury (15). However, in this study, no strenuous exercise occurred during the period of supplemental feeding, consequently resulting in no significant increase in plasma carnosine levels on the day of supplementation. Nevertheless, the present study conducted a follow-up assessment of β-alanine and carnosine levels in the blood of both groups on the 30th day of the trial. This analysis revealed a significant elevation in plasma β-alanine and carnosine levels among speed-raced Erythroxylon horses that were supplemented with β-alanine for 30 days.

Carnosine's physicochemical properties render it beneficial for enhancing athletic performance. It can mitigate and buffer excessive

acid production by reducing proteolytic metabolism, thereby contributing to muscle toning and strengthening (18–21). Muscle carnosine content plays a pivotal role in high-intensity and long-distance exercise (10). For instance, Wim et al. observed a performance improvement of 0.75 s among 400-m track and field athletes after a 4-week β-alanine supplementation compared with the control group (22). Similarly, Baguet et al. reported a 4.3-s improvement in race performance among 2,000 m professional rowers following a 7-week supplementation of 5 g/day β-alanine compared to the control group, which, prior to the trial, had a 0.3 s faster race time than the supplemented group (4). In our study, at the experiment's outset, the 1,000-m exercise performance for both groups of speed racing Yili horses was recorded at 135.76 s and 134.73 s, respectively. After 30 days, the group of speed-racing Yili horses supplemented with β-alanine exhibited a decrease in their 1,000-m exercise performance by 13.29 s compared with the control group in terms of race time. This marked a significant performance improvement of 12.01%, consistent with the findings of Wim et al. and Baguet et al. This suggests that β-alanine supplementation can increase skeletal muscle carnosine content, mitigate physiological declines and energy depletion due to cellular acidification, and enhance the post-race recovery capability of speed-racing Yili horses, thereby improving their overall performance.

During exercise, the body undergoes changes in energy and substance metabolism, reflected in metabolite concentrations. Amino acids, integral to body metabolites, not only signify protein metabolism but also monitor pre- and post-exercise bodily changes. Studying amino acid variations pre- and post-exercise can offer insights into internal metabolism. In our research, speed-racing Yili horses supplemented with β-alanine exhibited notably higher plasma levels of β-alanine compared with those supplemented with α-alanine. Additionally, β-alanine supplementation resulted in significantly elevated plasma levels of carnosine in these horses. Dunnett et al. inferred that carnosine in plasma primarily stemmed from myocyte overflow post-injury (23). The plasma carnosine content in Yili horses supplemented with β-alanine notably increased immediately after and 2 h post-race. This suggests a substantial release of carnosine due to muscle injury during the race, indirectly supporting the notion that β-alanine supplementation aids carnosine synthesis in the skeletal muscle. Valine, leucine, and isoleucine, collectively known as BCAAs, are essential amino acids. BCAAs exhibit a rapid transamination and full oxidation capability, generating ATP at a higher rate compared to other amino acids (24). Consequently, they stand out as a crucial energy source during exercise, contributing to 14% of the total oxidative energy supply in skeletal muscle, with leucine displaying the highest activity in this regard (25). Blomstrand showed that increased utilization of BCAAs during high-intensity exercise leads to decreased plasma concentrations of BCAAs (26). In the present study, concentrations of leucine and isoleucine in the plasma of speed-racing horses were notably reduced both 1 h before and immediately after the race. This reduction was particularly significant in horses supplemented with β-alanine 2 h after the race, aligning with the results of Blomstrand's study. This suggests that the utilization of BCAAs in the skeletal muscles of horses supplemented with β-alanine was higher than that of the speed-racing horses supplemented with α-alanine during the 1,000-m test event, resulting in substantial depletion of these amino acids. The utilization rate was notably higher in β-alanine supplemented speed-racing horses than in α-alanine supplemented. Thus, a large amount of BCAAs were consumed, resulting in lower plasma levels of BCAAs in β-alanine-supplemented speed racing horses 2 h after the race.

During moderate- to high-intensity exercise, the accumulation of hydrogen ions causes a decrease in intramuscular pH, influencing the muscle's oxidative energy supply. Increased reliance on glycolysis for energy production leads to higher lactate (Lac) and hydrogen ion concentrations in the resulting products. Davery and Lily demonstrated that carnosine functions as an H^+ buffer within the body's metabolism, contributing to nearly 10% of the buffering capacity (24, 27). The hypothesis proposed by Blancquaert suggests that carnosine regulates the acid–base environment within myocytes by readily binding to Ca^{2+} and H^+ within the cell, facilitating the transport of these ions inside the cell (10). During anaerobic respiration, myocytes within the sarcolemma metabolize glucose to generate ATP, resulting in a significant accumulation of H^+ ions. These H^+ ions competitively bind to the carnosine-Ca^{2+} complex, releasing Ca^{2+}. The released Ca^{2+} near the sarcolemma immediately participates in muscle contraction. Subsequently, the carnosine-H^+ complex moves toward the sarcoplasmic reticulum near the myocyte membrane. Simultaneously, it releases a substantial amount of H^+ into the bloodstream to regulate the internal stability of myocytes. In our study, no significant differences in pH, TCO_2, PCO_2, Na^+, K^+, and iCa were observed between the two groups 1 h before the race. However, immediately after the race, β-alanine-supplemented speed-racing Yili horses exhibited notably higher blood levels of Glu, Lac, and Na^+ compared to α-alanine-supplemented speed-racing Yili horses. Additionally, the pH was lower in the β-alanine-supplemented group. These differences could be attributed to their performance during the 1,000-m exercise. The anaerobic respiratory work performed by speed-racing Yili horses supplemented with β-alanine during the 1,000-m test race was greater than that of horses supplemented with α-alanine. Consequently, this led to increased production of Lac and H^+, resulting in substantial Lac accumulation post-race. However, after two subsequent speed races, the Lac content in the whole blood of speed-racing Yili horses decreased from elevated levels to within the normal range. Additionally, other blood gas levels, such as pH, TCO_2, PCO_2, Na^+, K^+, and iCa, were restored to normal levels. This suggests that carnosine potentially enhances the myocytes' acid–base environment by interacting with H^+ and Ca^{2+} within the myocyte, leading to the normalization of Lac and pH levels. Hematocrit (Hct) demonstrates a positive correlation with blood viscosity during exercise, while blood viscosity shows a negative correlation with blood mobility. Higher blood viscosity adversely affects the perfusion of tissues and organs, leading to more pronounced tissue hypoxia. Conversely, lower blood viscosity enhances oxygen transport through the blood, augmenting oxygen supply to the tissues. This facilitates improved exercise levels and accelerates post-exercise recovery (28). In our study, pre- and post-race Hct levels, measured 2 h after racing, were notably lower in speed-racing Yili horses supplemented with β-alanine than in those supplemented with α-alanine. This suggests that β-alanine supplementation in speed-racing Yili horses enhances their resting blood oxygen supply capacity.

Several recent studies have illustrated carnosine's extensive antioxidant properties, showcasing its ability to interact with various free radicals. Carnosine regulates the content of reactive oxygen

species, effectively scavenges oxygen radicals, and serves as an antioxidant (2, 29). Additionally, carnosine directly interacts with peroxidation products of lipids and superoxide anion radicals, thereby improving the oxidative stability of skeletal muscle (30). High-intensity exercise generates substantial free radicals within skeletal muscle cells. Carnosine's antioxidant mechanism involves neutralizing and binding to carbonyl groups on cell membrane proteins. This process produces relatively stable carbonylated proteins, preventing damaging cross-linking with other proteins and their expulsion from the cell. Guo's research demonstrated that carnosine can effectively inhibit lipid oxidation in liver homogenates, reduce peroxidation-associated MDA, and decrease the accumulation of lipid oxidation products and then concluded that carnosine not only scavenges free radicals but also inhibits lipid peroxidation by reacting with lipid oxidation products and eliminating the accumulation of lipid oxidation products (31). Huang's study revealed that administering β-alanine to SD rats for 4 weeks increased SOD and GSH-px activity in the gastrocnemius muscle in the high-dose group. Additionally, MDA content decreased significantly in the medium-dose group, substantially enhancing the skeletal muscle's antioxidant capacity in SD rats (32). Wang's findings showed that supplementing fattening pigs' diet with 600 mg/kg β-alanine increased the activities of glutathione peroxidase and catalase while decreasing malondialdehyde concentration (33). In our study, plasma SOD activity in speed-racing horses supplemented with β-alanine was significantly higher than that of speed-racing horses supplemented with α-alanine, observed from 1 h before the race to 20 min after. GSH-px and T-AOC were notably higher in the β-alanine group at 20 min after the race, and MDA levels immediately post-race were significantly lower compared to horses supplemented with α-alanine. The enhanced antioxidant capacity in the plasma of speed-racing Yili horses corresponded to an increase in plasma taurine content. Taurine aids in neutralizing free radicals produced during exercise, minimizing oxidative stress damage to myocytes. This enables sustained energy supply to the myocytes and contributes to the improvement observed in the 1,000-m speed races of the β-alanine supplementation group (34).

5 Conclusion

In conclusion, supplementing with β-alanine enhances post-race acid–base recovery ability by elevating plasma levels of β-alanine and carnosine. This supplementation also improves the antioxidant status of speed-racing Yili horses, reducing post-exercise injuries and bolstering their post-race recovery ability. Consequently, it enhances the performance of Yili horses in 1,000-m races.

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 procedures in this study were approved by the Animal Experiment Ethics Committee of Xinjiang Agricultural University (permit number: 2018012). The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

XL: Writing – original draft. JM: Writing – original draft. HaiyL: Writing – review & editing. HaiL: Writing – review & editing. YM: Writing – original draft. HD: Writing – original draft. KY: Writing – review & editing.

Funding

The author(s) declare financial support was received for the research, authorship, and/or publication of this article. This study was supported by the Major Science and Technology Special Project of Xinjiang Uygur Autonomous Region (2022A02013-2 and 2022A02013-2-1).

Conflict of interest

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

Publisher's note

All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

References

1. Guiotto A, Calderan A, Ruzza P, Borin G. Carnosine and carnosine-related antioxidants: a review. *Curr Med Chem*. (2005) 12:2293–315. doi: 10.2174/0929867054864796

2. Aldini G, de Courten B, Regazzoni L, Gilardoni E, Ferrario G, Baron G, et al. Understanding the antioxidant and carbonyl sequestering activity of carnosine: direct and indirect mechanisms. *Free Radic Res*. (2021) 55:321–30. doi: 10.1080/10715762.2020.1856830

3. Solana-Manrique C, Sanz FJ, Martínez-Carrión G, Paricio N. Antioxidant and neuroprotective effects of carnosine: therapeutic implications in neurodegenerative diseases. *Antioxidants*. (2022) 11:848–8. doi: 10.3390/antiox11050848

4. Baguet A, Bourgois J, Vanhee L, Achten E, Derave W. Important role of muscle carnosine in rowing performance. *J Appl Physiol*. (2010) 109:1096–101. doi: 10.1152/japplphysiol.00141.2010

5. Boldyrev AA, Aldini G, Derave W. Physiology and pathophysiology of carnosine. *Physiol Rev*. (2013) 93:1803–45. doi: 10.1152/physrev.00039.2012

6. Damon BM, Hsu AC, Stark HJ, Dawson MJ. The carnosine C-2 proton's chemical shift reports intracellular pH in oxidative and glycolytic muscle fibers. *Magn Reson Med*. (2003) 49:233–40. doi: 10.1002/mrm.10384

7. Tamba M, Torreggiani A. Hydroxyl radical scavenging by carnosine and cu(II)-carnosine complexes: a pulse-radiolysis and spectroscopic study. *Int J Radiat Biol*. (1999) 75:1177–88. doi: 10.1080/095530099139656

8. Prokopieva VD, Yarygina EG, Bokhan NA, Ivanova SA. Use of carnosine for oxidative stress reduction in different pathologies. *Oxidative Med Cell Longev*. (2016) 2016:1–8. doi: 10.1155/2016/2939087

9. Huang Y, Duan J, Chen H, Chen M, Chen G. Separation and determination of carnosine-related peptides using capillary electrophoresis with laser-induced fluorescence detection. *Electrophoresis*. (2005) 26:593–9. doi: 10.1002/elps.200406130

10. Blancquaert L, Everaert I, Derave W. Beta-alanine supplementation, muscle carnosine and exercise performance. *Curr Opin Clin Nutr Metab Care*. (2015) 18:63–70. doi: 10.1097/mco.0000000000000127

11. Maughan RJ, Greenhaff PL, Hespel P. Dietary supplements for athletes: emerging trends and recurring themes. *J Sports Sci*. (2013) 29:65–74. doi: 10.4324/9781315873268-14

12. Gaunitz F, Hipkiss AR. Carnosine and cancer:a perspective. *Amino Acids*. (2012) 43:135–42. doi: 10.1007/s00726-012-1271-5

13. Derave W, Everaert I, Beeckman S, Baguet A. Muscle carnosine metabolism and β-alanine supplementation in relation to exercise and training. *Sports Med*. (2010) 40:247–63. doi: 10.2165/11530310-000000000-00000

14. Perim P, Marticorena F, Riberio F, Barreto G, Gobbi N, Kerksick C, et al. Can the skeletal muscle carnosine response to Beta-alanine supplementation be optimized? *Front Nutr*. (2019) 6:135. doi: 10.3389/fnut.2019.00135

15. Dunnett M, Harris RC. Influence of oral ß-alanine and L-histidine supplementation on the carnosine content of the gluteus medius. *Equine Vet J*. (1999) 31:499–504. doi: 10.1111/j.2042-3306.1999.tb05273.x

16. Hoffman JR, Landau G, Stout JR, Hoffman MW, Shavit N, Rosen P, et al. β-Alanine ingestion increases muscle carnosine content and combat specific performance in soldiers. *Amino Acids*. (2015) 47:627–36. doi: 10.1007/s00726-014-1896-7

17. Gonzalez D, McAllister MJ, Waldman HS, Ferrando AA, Joyce J, Barringer ND, et al. International society of sports nutrition position stand: tactical athlete nutrition. *J Int Soc Sports Nutr*. (2022) 19:267–315. doi: 10.1080/15502783.2022.2086017

18. Matthews JJ, Artioli GG, Turner MD, Sale C. The physiological roles of carnosine and β-alanine in exercising human skeletal muscle. *Med Sci Sports Exerc*. (2019) 51:2098–108. doi: 10.1249/mss.0000000000002033

19. Creighton J, de Souza Gonçalves L, Artioli GG, Tan D, Elliott-Sale KJ, Turner MD, et al. Physiological roles of carnosine in myocardial function and health. *Adv Nutr*. (2022) 13:1914–29. doi: 10.1093/advances/nmac059

20. Peters V, Yard B, Schmitt CP. Carnosine and diabetic nephropathy. *Curr Med Chem*. (2020) 27:1801–12. doi: 10.2174/0929867326666190326111851

21. Kilis-Pstrusinska K. Carnosine and kidney diseases: what we currently know? *Curr Med Chem*. (2020) 27:1764–81. doi: 10.2174/0929867326666190730130024

22. Derave W, Özdemir MS, Harris RC, Pottier A, Reyngoudt H, Koppo K, et al. β-Alanine supplementation augments muscle carnosine content and attenuates fatigue during repeated isokinetic contraction bouts in trained sprinters. *J Appl Physiol*. (2007) 103:1736–43. doi: 10.1152/japplphysiol.00397.2007

23. Dunnett M, Harris RC, Dunnett CE, Harris PA. Plasma carnosine concentration: diurnal variation and effects of age, exercise and muscle damage. *Equine Vet J*. (2010) 34:283–7. doi: 10.1111/j.2042-3306.2002.tb05434.x

24. Rennie MJ. Influence of exercise on protein and amino acid metabolism. *Compr Physiol*. (1996):995–1035. doi: 10.1002/cphy.cp120122

25. Ji L, Miller RH, Nagle FJ, Lardy HA, Stratman FW. Amino acid metabolism during exercise in trained rats: the potential role of carnitine in the metabolic fate of branched-chain amino acids. *Metab Clin Exp*. (1987) 36:748–52. doi: 10.1016/0026-0495(87)90111-9

26. Blomstrand E, Celsing F, Newsholme EA. Changes in plasma concentrations of aromatic and branched-chain amino acids during sustained exercise in man and their possible role in fatigue. *Acta Physiol Scand*. (1988) 133:115–21. doi: 10.1111/j.1748-1716.1988.tb08388.x

27. Lily E, Papandreou D. The role and effects of carnosine and β-alanine on exercise: an updated mini review. *Nutr Food Sci*. (2014). doi: 10.1108/nfs-04-2013-0044

28. Zelenkova I, Zotkin S, Korneev P, Koprov S, Grushin A. Comprehensive overview of hemoglobin mass and blood volume in elite athletes across a wide range of different sporting disciplines. *J Sports Med Phys Fitness*. (2019) 59:179–86. doi: 10.23736/s0022-4707.18.08018-0

29. Caruso G, Fresta CG, Musso N, Giambirtone M, Grasso M, Spampinato S, et al. Carnosine prevents Aβ-induced oxidative stress and inflammation in microglial cells: a key role of TGF-β1. *Cell*. (2019) 8:64–4. doi: 10.3390/cells8010064

30. Zhao J, Posa DK, Kumar V, Hoetker D, Kumar A, Ganesan S, et al. Carnosine protects cardiac myocytes against lipid peroxidation products. *Amino Acids*. (2019) 51:123–38. doi: 10.1007/s00726-018-2676-6

31. Guo S, Yang GY, Han LQ. Inhibitory effect of myostatin on CCl4-induced lipid peroxidation in liver homogenates. *Livestock Vet Med*. (2005) 37:25–6. doi: 10.3969/j.issn.0529-5130.2005.01.010

32. Huang C. Effect of beta-alanine supplementation on myostatin content and antioxidant capacity of skeletal muscle in exercising rats. (2015). Ph.D. Thesis. Beijing Sports University30-31.

33. Wang F, Yin Y, Wang Q, Xie J, Fu C, Guo H, et al. Effects of dietary β -alanine supplementation on growth performance, meat quality, carnosine content, amino acid composition and muscular antioxidant capacity in Chinese indigenous Ningxiang pig. *J Anim Physiol Anim Nutr*. (2023) 107:878–86. doi: 10.1111/jpn.13797

34. Ohta H, Azuma J, Awata N, Hamaguchi T, Tanaka Y, Sawamura A, et al. Mechanism of the protective action of taurine against isoprenaline induced myocardial damage. *Cardiovasc Res*. (1988) 22:407–13. doi: 10.1093/cvr/22.6.407

frontiers | Frontiers in Veterinary Science

TYPE Original Research
PUBLISHED 01 March 2024
DOI 10.3389/fvets.2024.1361908

OPEN ACCESS

EDITED BY
Tao Wang,
Jilin Agricultural University, China

REVIEWED BY
Yan Liu,
Zhejiang Academy of Agricultural Sciences, China
Daniel Hernandez-Patlan,
National Autonomous University of Mexico, Mexico

*CORRESPONDENCE
Yajuan Liu
✉ lyj8258@126.com
Baojiang Chen
✉ chenbaojiang@vip.sina.com

†These authors have contributed equally to this work

RECEIVED 27 December 2023
ACCEPTED 21 February 2024
PUBLISHED 01 March 2024

CITATION
Xia M, LI C, Wu D, Wu F, Kong L, Jia Z, Han W, Chen S, Fang W, Liu Y and Chen B (2024) Benefits of heat-killed *Lactobacillus acidophilus* on growth performance, nutrient digestibility, antioxidant status, immunity, and cecal microbiota of rabbits. *Front. Vet. Sci.* 11:1361908. doi: 10.3389/fvets.2024.1361908

Benefits of heat-killed *Lactobacillus acidophilus* on growth performance, nutrient digestibility, antioxidant status, immunity, and cecal microbiota of rabbits

Miao Xia[1,2†], Chong LI[1†], Diange Wu[1], Fengyang Wu[1], Lingchang Kong[1,2], Zifan Jia[1,2], Wenxiao Han[1,2], Saijuan Chen[2,3], Wei Fang[4], Yajuan Liu[2]* and Baojiang Chen[1]*

[1]College of Animal Science and Technology, Hebei Agricultural University, Baoding, China, [2]Mountainous Area Research Institute of Hebei Province, Hebei Agricultural University, Baoding, China, [3]Agricultural Technology Innovation Center in Mountainous Areas of Hebei Province, Baoding, China, [4]Biosource Biotechnology Co., Ltd., Shenzhen, China

Introduction: Heat-killed probiotics, as a type of inactivated beneficial microorganisms, possess an extended shelf life and broader adaptability compared to their live counterparts. This study aimed to investigate the impact of heat-killed *Lactobacillus acidophilus* (*L. acidophilus*, LA) - a deactivated probiotic on the growth performance, digestibility, antioxidant status, immunity and cecal microbiota of rabbits.

Methods: Two hundred weaned Hyla rabbits were randomly allocated into five equal groups (CON, L_{200}, L_{400}, L_{600}, and L_{800}). Over a 28-day period, the rabbits were fed basal diets supplemented with 0, 200, 400, 600, and 800 mg/kg of heat-killed LA, respectively.

Results: Results revealed a significant reduction in the feed-to-gain ratio (F/G) in the L_{600} and L_{800} groups ($p < 0.05$). Additionally, the L_{800} group exhibited significantly higher apparent digestibility of crude fiber (CF) and crude protein (CP) ($p < 0.05$). Regarding digestive enzyme activities, enhanced trypsin and fibrinase activities were observed in the L_{600} and L_{800} groups ($p < 0.05$). Concerning the regulation of the body's antioxidant status, the L_{800} group demonstrated elevated levels of superoxide dismutase (SOD) and total antioxidant capacity (T-AOC) in both serum and ileal tissue ($p < 0.05$). In terms of immune capacity modulation, serum tumor necrosis factor-α (TNF-α) levels were significantly lower in the L_{600} and L_{800} groups ($p < 0.05$), while immunoglobulin A (IgA) and immunoglobulin M (IgM) levels were higher ($p < 0.05$). Additionally, the L_{800} group exhibited a substantial increase in secretory immunoglobulin A (SIgA) levels in the intestinal mucosa ($p < 0.05$). In comparison to the CON group, the L_{800} group exhibited a significant increase in the relative abundance of *Phascolarctobacterium* and *Alistipes* in the cecum ($p < 0.05$). *Phascolarctobacterium* demonstrated a positive correlation with SIgA ($p < 0.05$), IgM ($p < 0.01$), and Glutathione peroxidase (GSH-Px) ($p < 0.05$), while displaying a negative correlation with TNF-α levels ($p < 0.05$). Concurrently, Alistipes exhibited positive correlations with IgA ($p < 0.05$), IgM ($p < 0.05$), SIgA ($p < 0.01$), GSH-Px ($p < 0.05$), SOD ($p < 0.05$), and T-AOC ($p < 0.01$), and a negative correlation with TNF-α ($p < 0.05$).

Discussion: In conclusion, the dietary incorporation of 600 mg/kg and 800 mg/kg of heat-killed LA positively influenced the growth performance, nutrient digestibility, antioxidant status, immune capacity and cecal microbiota of rabbits. This highlights the potential benefits of utilizing heat-killed probiotics in animal nutrition.

KEYWORDS

heat-killed *Lactobacillus acidophilus*, rabbit, growth performance, antioxidant status, immune capacity, cecum microbiology

1 Introduction

Probiotics, defined as living microorganisms with proven health benefits upon proper administration, have been extensively studied for their capacity to enhance host immunity, optimize gut health, and improve overall animal growth performance (1, 2). Commonly employed probiotic strains encompass *Lactobacillus acidophilus*, *Lactobacillus plantarum*, *Bacillus subtilis*, and *Saccharomyces cerevisiae* (3–5). However, the widespread utilization of live probiotics has unveiled associated risks, including variable colonization patterns, instability of live bacteria, and potential strain transfer across the intestinal barrier (6). Consequently, concerns regarding these risks have led to an increased adoption of paraprobiotics, specifically inactivated probiotics (7). Various methods, including heat, high pressure, and ultraviolet light, have been explored for probiotic deactivation, with heat treatment often preferred. This choice is due to its effectiveness, cost-efficiency, and ability to preserve probiotic integrity, ensuring prolonged functionality and stability. Heat treatment stands as a widely adopted approach for preserving probiotic attributes across different contexts (8). Recent studies have substantiated that non-viable bacteria, notably inactivated probiotics, can confer health benefits akin to their living counterparts. These benefits encompass the modulation of immunity, pathogen suppression, and the enhancement of gut microbes to uphold overall animal health. Notably, these functional attributes of inactivated probiotics are attributed to their release of lipophosphatidic acid (LTA), peptidoglycan, and extracellular polysaccharide (EPS) components (9, 10). Additionally, the ease of storage and transportation of inactivated probiotics, compared to live bacteria, further underscores their practical advantages (7, 11, 12).

The weaning period poses a critical juncture for rabbits, characterized by an underdeveloped digestive tract and exposure to multiple stressors, including dietary transitions and relocation to new enclosures, potentially impacting their overall growth and developmental trajectories (13). Post-weaning, the abrupt cessation of breast milk antigen supply results in a rapid decline in intestinal immunity (14). Consequently, rabbits become more susceptible to environmental variations, eliciting stress responses that can manifest as diarrhea and, in severe cases, culminate in death (15).

Prior research has demonstrated the positive impact of dietary supplementation with alive LA on enhancing the dietary conversion rate, promoting growth performance, and augmenting beneficial intestinal flora in rabbits (4). Moreover, LA has been shown to fortify immunity, improve the integrity of the intestinal barrier, diminish the occurrence of diarrhea, and reduce mortality rates (4, 5, 16). Despite these established benefits, there remains a scarcity of literature addressing the utilization of heat-killed LA in rabbit production. This study aims to bridge this knowledge gap by investigating the repercussions of incorporating heat-killed LA into rabbit diets. The investigation also encompasses an in-depth analysis of its effects on growth performance, digestibility, digestive enzymes, antioxidant status, and immune capacity in rabbits. The results of this research will provide valuable scientific insights and provide a powerful reference for the rational application of heat-killed LA in rabbit production.

2 Materials and methods

2.1 Animals and management

A total of 200 healthy Hyla meat rabbits, uniform in weight at 35 days of age, were selected and randomly allocated into 5 groups, each comprising 8 replicates, with 5 rabbits in each replicate. Rabbits in the control group (CON) were exclusively fed a basal diet without any supplementation. In contrast, rabbits in the 4 treated groups, namely L_{200}, L_{400}, L_{600}, and L_{800}, received basal diets enriched with 200, 400, 600, and 800 mg/kg of heat-killed LA, respectively, for a duration of 28 days. The heat-killed LA was sourced from Biosource Biotechnology Co., Ltd. (Shenzhen, China) and contained 1×10^9 cfu/g of LA in a dried product stored at room temperature (the main ingredients in this product include inactivated *Lactobacillus acidophilus* and silica). The formulation of the base diet adhered to the nutritional recommendations for rabbits as outlined by De Blas (17). The composition and nutritional levels of the base diet are detailed in Table 1. Both feeding and digestion experiments were carried out at the rabbit farm located within the Animal Husbandry Teaching Base of Hebei Agricultural University. Prior to the commencement of the experiments, meticulous cleaning and disinfection procedures were implemented for all rabbit cages. The feeding trial spanned a duration of 35 days, encompassing a pre-feeding phase of 7 days and a subsequent standard trial period of 28 days. Throughout the trial, all rabbits were afforded *ad libitum* access to both feed and water.

2.2 Growth performance

The composition and nutritional levels of the base diet are detailed in Table 1. Initial body weight (IBW) and final body weight (FBW) were recorded at the beginning and at the end of the experiment (70 days of age) by weighing on an empty stomach. Record the feeding amount and remaining feed amount of each group of rabbits, calculate the average daily feed intake (ADFI), average daily weight gain (ADG) and feed-to-weight ratio (F/G). ADFI, ADG and F/G were calculated as follows: ADFI = total feed intake/experimental days; ADG = (FBW − IBW)/experimental days; F/G = ADFI/ADG.

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

Ingredients	Content	Nutrient level[b]	Content
Yellow maize grain	12.00	Dry matter	89.23
Wheat bran	15.00	Digestible energy (MJ/kg)	9.83
Chaff	5.00	Crude protein	15.09
Soybean meal	13.00	Ether extract	2.34
Peanut vine	20.00	Crude fiber	16.02
Peanut shell	10.00	Neutral detergent fiber	31.77
Artemisia argyi powder	5.00	Acid detergent fiber	18.91
Wheat middlings	6.00	Calcium	1.29
Corn germ meal	10.00	Total phosphorus	0.63
Sodium chloride	0.30		
DL-methionine	0.10		
L-lysine	0.10		
Premix[a]	3.50		
Total	100.00		

[a]The premix provided the following per kg of diets: Cu (as copper sulfate) 20 mg, Fe (as ferric sulfate) 70 mg, Zn (as zinc sulfate) 70 mg, Se (as sodium sulphate) 0.25 mg, Mn (as manganese sulfate) 10 mg, Co 0.15 mg, I 0.2 mg, VB1 2 mg, VB2 6 mg, VB12 0.02 mg, Pyridoxine 2 mg, Pantothenic acid 50 mg, Nicotinic acid 50 mg, Choline 1,000 mg, Biotin 0.2 mg, VA 101000 IU, VD 900 IU, VE 50 mg, VK 2 mg.
[b]Digestible energy was a calculated value, while the others were measured values.

2.3 Nutrient digestibility

At the end of the experiment, a 7 days digestibility test was conducted. During the trial, feces from each replicate were collected, weighed, and frozen at −20°C to be prepared for chemical analysis. Crude protein (CP), crude fat (EE), crude fiber (CF), acid detergent fiber (ADF), and neutral detergent fiber (NDF) and other nutrients in feed and feces were determined using the Association of Official Analytical Chemists (AOAC) method (18). Calculation of the digestibility of rabbits according to the total manure collection method. The digestibility of nutrients was calculated according to the following formula:

$$\text{Nutrient digestibility}\% = \left[\left(t - f\right)/t\right] \times 100$$

where t is the nutrient intake [g] during collection and f is the amount of nutrients excreted in the feces [g].

2.4 Samples collection

Upon completion of the experimental period, rabbits underwent a 12 h fasting period. Subsequently, one rabbit was randomly chosen from each replicate, and a blood volume of 10 mL was collected from the marginal ear vein. Blood samples were collected in vacuum containers and subjected to centrifugation at 3,000 × g for 10 min at 4°C to isolate the serum. The serum samples were preserved at −20°C until further analysis. Following blood collection, rabbits were humanely euthanized using the pentobarbital method. Tissue samples from the jejunum, ileum, and cecum were obtained using meticulously sterilized surgical instruments. The jejunum, ileum, and cecal chyme were carefully stored in sterile lyophilized test tubes. The 5 cm jejunum and ileum segments were placed on an ice tray and the mucosa was gently scraped off with a slide (the mucosa volume was 3–3.5 mL). The scraped mucous membranes were stored in sterile lyophilized test tubes at −80°C for subsequent analysis.

2.5 Immune capacity

The quantification of IgA, IgG, IgM, IFN-γ, TNF-α, IL-4, and IL-6 levels was conducted using enzyme-linked immunosorbent assay (ELISA). Similarly, intestinal mucosal Secretory SIgA and MUC2 were also assessed via ELISA kits. All ELISA kits utilized in the study were procured from Beijing Borealis Technology Co (Beijing, China), and specific procedural guidelines outlined in the respective kit instructions were followed for accurate implementation.

2.6 Digestive enzyme activity and antioxidant status

The α-amylase, trypsin, lipase activity, and cellulase activity of intestinal contents, as well as the total antioxidant capacity (T-AOC), glutathione peroxidase (GSH-Px), superoxide dismutase (SOD) activities and malondialdehyde (MDA) content in serum, jejunal and ileal tissues were measured using ELISA kits (Beijing Borui Long Term Technology Co (Beijing, China)).

2.7 16S rRNA gene sequencing and analysis

The cecum contents, approximately 5 mL, were collected in sterile cryopreservation tubes and stored at −80°C. Total genomic DNA was extracted from cecal digesta using the OMEGA Soil DNA Kit (M5635-02; Omega Bio-Tek, Norcross, GA, United States) following the manufacturer's instructions. The quantity and quality of the extracted DNA were assessed using a NanoDrop NC2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, United States) and agarose gel electrophoresis, respectively. PCR amplification of the V3–V4 region of the bacterial 16S rRNA gene was performed using forward primer 338F (5′-ACTCCTACGGGAGGCAGCA-3′) and reverse primer 806R (5′-GGACTACHVGGGTWTCTAAT-3′). The amplification procedure consisted of: 98°C for 5 min, followed by 25 cycles of 98°C for 30 s, 53°C for 30 s, and 72°C for 45 s, with a final extension at 72°C for 5 min. The amplicons were purified with Vazyme VAHTSTM DNA Clean Beads (Vazyme, Nanjing, China) and quantified using the Quant-iT PicoGreen dsDNA Assay Kit (Invitrogen, Carlsbad, CA, United States). After individual quantification, the amplicons were pooled in equal amounts, and paired-end sequenced (2 × 250 bp) at Shanghai Personal Biotechnology Co., Ltd. (Shanghai, China) on the Illumina NovaSeq Platform using the NovaSeq 6,000 SP Reagent Kit (500 cycles). To estimate microbial diversity in individual samples, alpha diversity indices, such as the Chao1 richness estimator, Shannon diversity index, Simpson index, and Goods_coverage. Observed_species at the amplicon sequence variant (ASV) level were calculated using the ASV table in QIIME2.

Principal coordinate analysis (PCOA) was performed based on Bray–Curtis and UniFrac distance metrics.

2.8 Date analysis

Statistical data analyses were performed using Excel 2020 and SPSS 26.0 software. One-way analysis of variance (ANOVA) was used to test for significant differences between groups of data, while Duncan's method was used for multiple comparisons. p-value <0.05 indicated significant differences between groups.

3 Results

3.1 Growth performance

The supplementation of heat-killed LA in the diet did not yield statistically significant differences ($p > 0.05$) in FBW, ADF, and ADG among rabbits, as indicated in Table 2. Nevertheless, noteworthy reductions were observed in the Feed-to-Gain ratio (F/G) within the L_{600} and L_{800} groups, and these reductions were statistically significant ($p < 0.05$).

3.2 Nutrient digestibility

As presented in Table 3, the digestibility of CP exhibited a notable increase in the L_{800} group, achieving statistical significance ($p < 0.05$). Moreover, the digestibility of CF and NDF in the L_{600} and L_{800} groups demonstrated a significant improvement when compared to the CON group ($p < 0.05$). Conversely, the digestibility of GE and other nutrients, including DM, ADF, EE, Ash, Ca, and P, did not manifest significant differences in rabbits when compared to the CON group ($p > 0.05$).

3.3 Digestive enzyme activity

The effects of the different treatments on intestinal digestive enzyme levels are presented in Figure 1. Compared with CON group, the activities of jejunum and ileum trypsin and cecum cellulase in L_{600} and L_{800} groups were significantly increased ($p < 0.05$) (Figures 1B,E,G), and the jejunum lipase activity in L_{800} group was also significantly higher ($p < 0.05$) (Figure 1C). Compared with CON group, α-amylase activity in jejunum and ileum and lipase in ileum of rabbits in experimental groups were not significantly different ($p > 0.05$).

3.4 Antioxidant indexes

In comparison to the CON group, the SOD activity in the serum and intestine tissue of the L_{600} and L_{800} groups exhibited a significant increase ($p < 0.05$) (Figures 2B,F,J). Additionally, the serum T-AOC activity was significantly elevated in the L_{800} group ($p < 0.05$, Figure 2D). Furthermore, the T-AOC activity in the ileum of rabbits in the L_{600} and L_{800} groups demonstrated a noteworthy increase in comparison to the CON group ($p < 0.05$) (Figure 2L). However, there were no statistically significant differences observed in MDA content and GSH-Px activity within the serum, jejunum, and ileum between the experimental groups and the CON group ($p > 0.05$).

3.5 Serum immune capacity

In Figure 3, it was shown that serum TNF-α levels were significantly reduced in the L_{600} and L_{800} groups compared with the CON group ($p < 0.05$) (Figure 3D). The serum IgA levels in the L_{800} group were significantly increased ($p < 0.05$) (Figure 3E). In addition, serum IgM levels were significantly higher in the L_{600} and L_{800} groups compared with the CON group ($p < 0.05$) (Figure 3G). And IFN-γ, IL-4, IL-6, and IgG levels were not significantly different compared with the CON group ($p > 0.05$).

3.6 Intestinal mucosal immune capacity

The findings presented in Figure 4 indicate a substantial increase in SigA content in the jejunal mucosa of rabbits within the L_{800} group compared to the CON group ($p < 0.05$) (Figure 4A). Similarly, ileum mucosa was significantly increased in the L_{600} and L_{800} groups (Figure 4C). However, there were no significant differences observed in mucosal MUC2 content in the jejunum and ileum between the experimental groups and the CON group ($p > 0.05$).

3.7 Cecum microflora

The operational taxonomic unit (OTU) analysis revealed varying numbers of identified OTUs across the experimental groups, with

TABLE 2 Effect of heat-killed *Lactobacillus acidophilus* on the growth performance of rabbits.

Item	Groups					SEM	p-value
	CON	L_{200}	L_{400}	L_{600}	L_{800}		
IBW, g	1257.71	1247.10	1248.39	1249.52	1233.99	8.426	0.943
FBW, g	2274.07	2270.25	2295.53	2312.49	2287.48	9.917	0.689
ADG, g/d	36.30	36.54	37.40	37.96	37.62	0.264	0.209
ADFI, g/d	158.17	155.00	158.85	156.67	155.46	0.535	0.215
F/G	4.38[a]	4.30[a]	4.26[a]	4.15[b]	4.15[b]	0.028	0.027

IBW, initial body weight; FBW, final body weight; ADFI, average daily feed intake; ADG, average daily gain; F/G, ratio of feed and gain. Data are means for 8 replicates of 5 rabbits per replicate. SEM, total standard error of the means ($n = 8$). [a,b]Means with different superscripts in the same row are significantly different ($p < 0.05$).

4,605, 4,085, 4,421, 3,890, and 4,446 OTUs in the CON, L_{200}, L_{400}, L_{600}, and L_{800} groups, respectively. Notably, 1,116 OTUs were shared among the four groups, as illustrated in Figure 5. The microbial diversity within an individual sample was assessed by the Chao1, Goods_coverage, Shannon, Simpson indices, and Observed_species but no significant differences between all groups were observed (Figure 6). Principal component analysis of OTUs was performed to evaluate the similarities and differences between the control group and the experimental group (Figure 7), and the results showed that the cecal microbiota of CON group was significantly separated from that of L_{400}, L_{600}, and L_{800} groups. Taxonomic profiling indicated that *Firmicutes* and *Bacteroidetes* accounted for most of the intestinal bacteria of rabbits (Figure 8). At the phylum level, there were no significant differences in the relative bacterial abundance among all groups ($p > 0.05$) (Table 4). The relative abundance of the 20 dominant genera in each group at the genus level was also analyzed. *Ruminococcus* and *Oscillospira* were found to be the main genera in the 4 treatment groups (Figure 9). As shown in Table 5, compared with the CON group, the relative abundance of *Phascolarctobacterium* in L_{600} and L_{800} groups was significantly increased, *Subdoligranulum* in L_{200} group was significantly increased, and *Dehalobacterium* in L_{400}, L_{600}, and L_{800} groups was significantly decreased. The relative abundance of *Alistipes* in group L_{800} was significantly increased ($p < 0.05$).

TABLE 3 Effect of heat-killed *Lactobacillus acidophilus* on apparent digestibility (%) of energy and nutrients in rabbits.

Item	Groups					SEM	*p*-value
	CON	L_{200}	L_{400}	L_{600}	L_{800}		
DM	52.12	51.39	52.71	50.14	51.03	0.550	0.646
GE	57.26	57.46	58.05	54.16	57.36	0.543	0.166
CP	76.70^{b}	76.60^{b}	76.67^{b}	77.27^{b}	79.99^{a}	0.373	0.010
CF	19.43^{c}	18.70^{c}	20.90^{bc}	26.04^{ab}	29.91^{a}	1.059	0.001
NDF	29.56^{c}	30.80^{bc}	29.34^{c}	34.84^{ab}	36.40^{a}	0.781	0.004
ADF	20.41	20.96	25.00	26.68	25.59	1.256	0.403
EE	87.07	87.11	87.94	88.03	88.33	0.194	0.095
Ash	35.49	29.13	40.67	34.12	33.92	1.246	0.057
Ca	56.25	65.43	60.88	55.38	61.21	1.266	0.070
P	16.55	13.65	17.62	18.24	17.74	0.918	0.539

DM, dry matter; GE, gross energy; CP, crude protein; EE, ether extract; CF, crude fiber; NDF, neutral detergent fiber; ADF, acid detergent fiber; Ca, calcium; P, total phosphorus. SEM, total standard error of the means ($n = 8$). ^{a,b,c}Means with different superscripts in the same row are significantly different ($p < 0.05$).

3.8 Association of cecum microflora with immunity and antioxidant capacity

In order to further understand the potential relationship between gut microbes and immunity and antioxidant capacity, Spearman correlation analysis was used to analyze the relationship. As shown in Figure 10, *Phascolarctobacterium* was positively correlated with jejumum SIgA ($p < 0.05$) and serum IgM ($p < 0.01$), and negatively correlated with serum TNF-α level ($p < 0.05$). Serum IgA ($p < 0.05$), serum IgM ($p < 0.05$), and jejunum SIgA ($p < 0.01$) were positively correlated with *Alistipes*, and serum TNF-α ($p < 0.05$) was negatively correlated with *Alistipes*. *Parabacteroides* was negatively correlated with serum IgG ($p < 0.01$). *Clostridiaceae_Clostridium* was positively

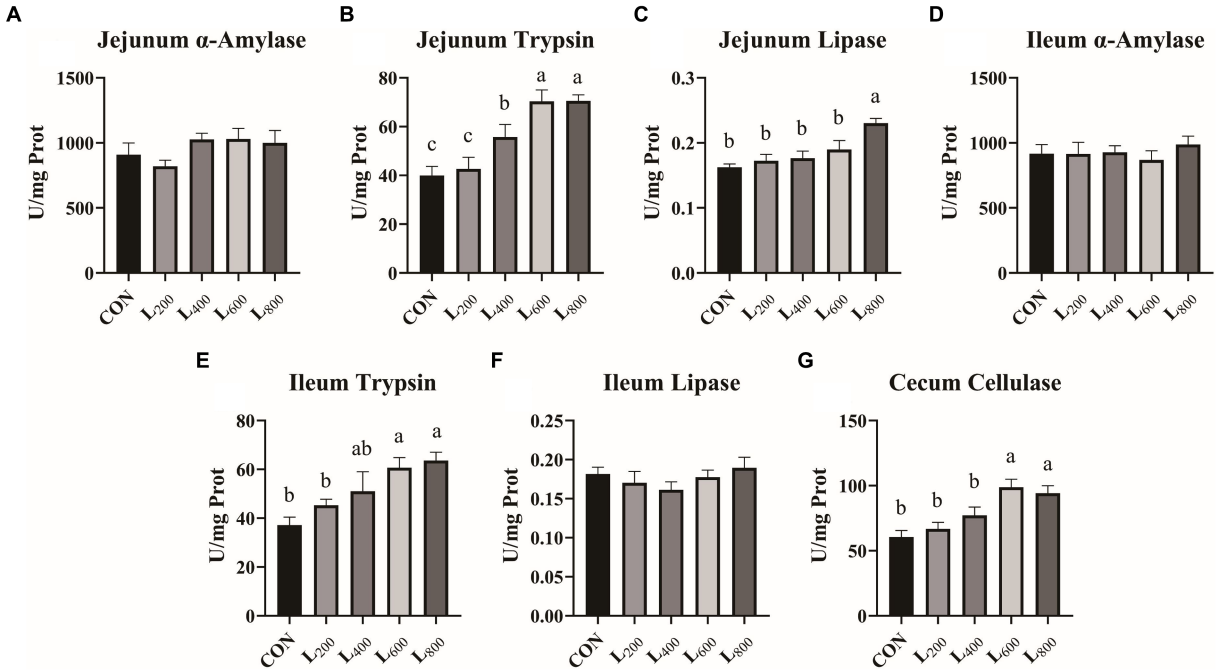


FIGURE 1
Effect of heat-killed *Lactobacillus acidophilus* on intestinal digestive enzymes in rabbits. Values are means ± standard error ($n = 8$). **(A–C)** α-Amylase, trypsin, lipase in jejunum; **(D–F)** α-Amylase, trypsin, lipase in ileum; **(G)** Cellulase in cecum. ^{a,b,c}Means among the experimental groups with different letters are significantly different ($p < 0.05$).

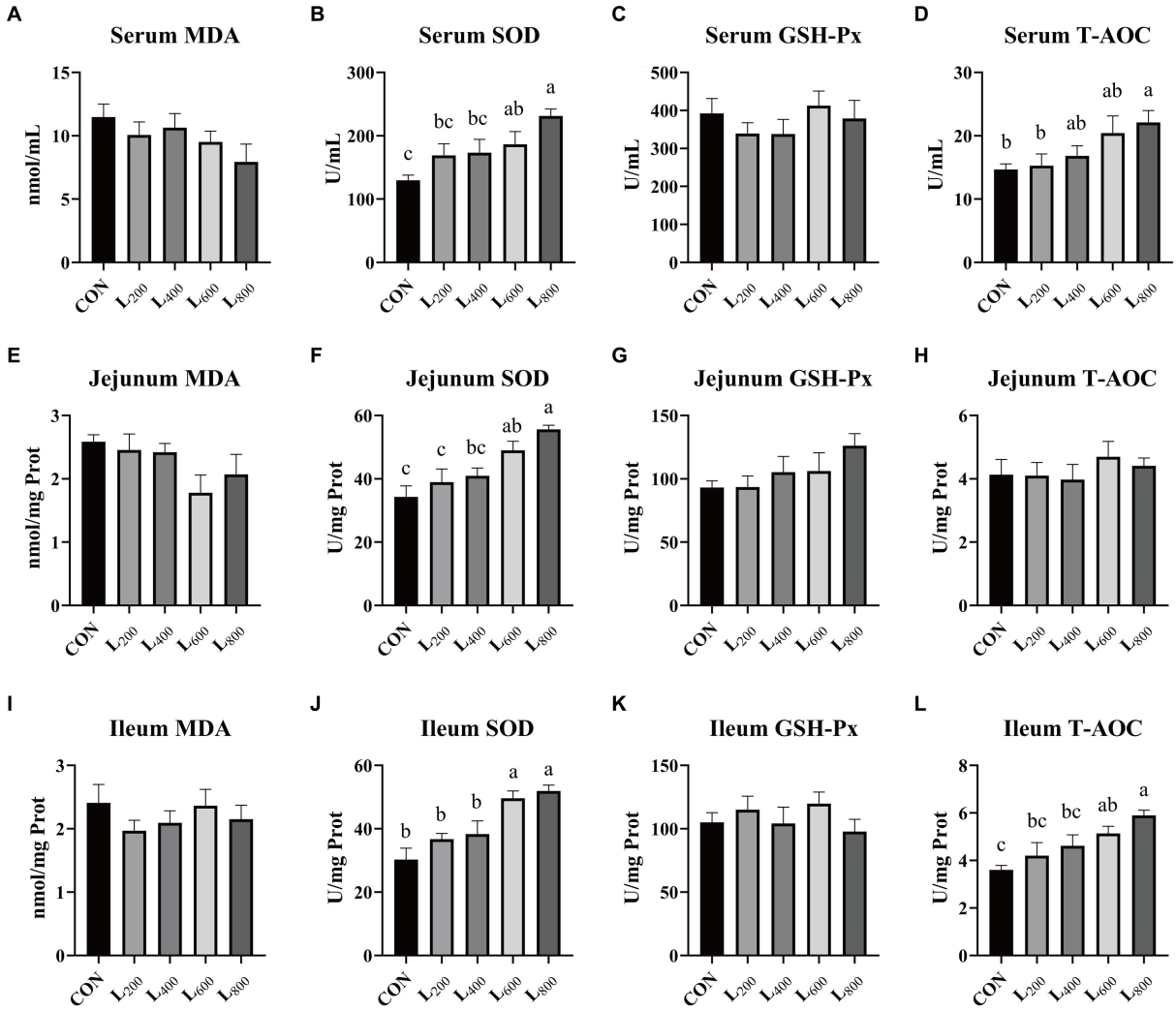


FIGURE 2
Effect of heat-killed *Lactobacillus acidophilus* on antioxidant status of rabbits. Values are means ± standard error ($n = 8$). **(A–D)** MDA, SOD, GSH-Px, TAOC in serum; **(E–H)** MDA, SOD, GSH-Px, TAOC in jejunum; **(I–L)** MDA, SOD, GSH-Px, TAOC in ileum. MDA, malondialdehyde; SOD, superoxide dismutase; GSH-Px, glutathione peroxidase; T-AOC, total antioxidant capacity. [a,b,c]Means among the experimental groups with different letters are significantly different ($p < 0.05$).

correlated with serum TNF-α ($p < 0.05$), and negatively correlated with serum IgG ($p < 0.05$), serum IgM ($p < 0.01$), and ileum SIgA ($p < 0.01$). *Dehalobacterium* was positively correlated with serum TNF-α ($p < 0.05$) and negatively correlated with serum IL-6 ($p < 0.05$), and jejunum SIgA ($p < 0.05$). *Subdoligranulum* was negatively correlated with serum IgA ($p < 0.01$). *Bilophila* was positively correlated with serum IgG ($p < 0.05$). *Akkermansia* was negatively correlated with serum TNF-α ($p < 0.01$), and positively correlated with serum IgM ($p < 0.05$), and ileum SIgA ($p < 0.01$). *Bacteroides* was negatively correlated with serum TNF-α ($p < 0.05$) and positively correlated with jejunum SIgA ($p < 0.01$).

Microorganisms associated with antioxidant results as shown in Figure 11, *Phascolarctobacterium* was positively correlated with ileum GSH-Px ($p < 0.05$). *Alistipes* was positively correlated with jejunum GSH-Px ($p < 0.05$), ileum SOD ($p < 0.05$), and serum T-AOC ($p < 0.01$). *Rikenella* was positively correlated with jejunum GSH-Px ($p < 0.01$). *Bacteroides* was positively correlated with jejunum SOD ($p < 0.05$) and ileum GSH-Px ($p < 0.05$). *Akkermansia* was positively correlated with ileum SOD ($p < 0.05$), serum T-AOC ($p < 0.05$), and jejunum T-AOC ($p < 0.01$). *Clostridiaceae_Clostridium* was positively correlated with serum SOD ($p < 0.05$) and jejunum GSH-Px ($p < 0.05$), and negatively correlated with ileum MDA ($p < 0.05$). *Subdoligranulum* was positively correlated with jejunum MDA ($p < 0.01$) and negatively correlated with ileum GSH-Px ($p < 0.05$). *Campylobacter* was negatively correlated with serum SOD ($p < 0.05$).

4 Discussion

The weaning stage poses a critical period for rabbits, susceptible to external stimuli such as dietary changes and environmental shifts, potentially leading to appetite loss, and even an increase in mortality and diarrhea rates (19). Zhu et al. (20) observed that the supplementation of 500 mg/kg of inactivated complex probiotics in the diet positively influenced cecum microbiota, consequently improving diet conversion and growth performance in yellow-finned

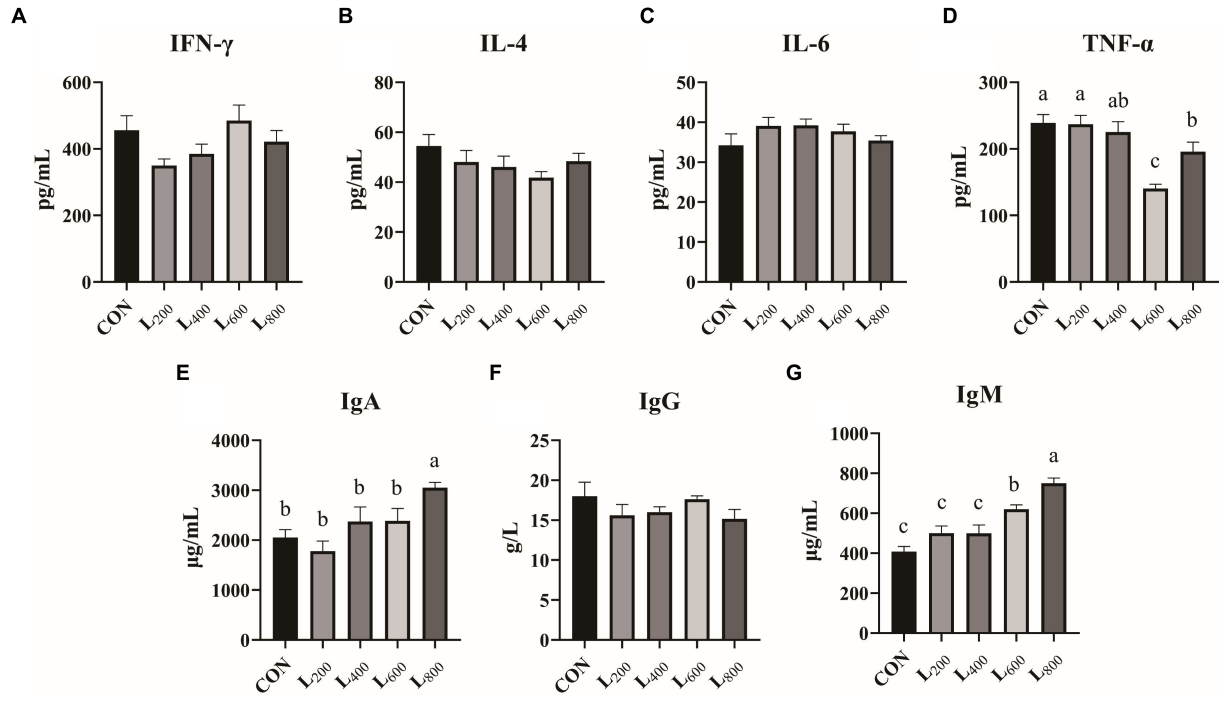


FIGURE 3
Effect of heat-killed *Lactobacillus acidophilus* on serum immunity in rabbits. Values are means ± standard error ($n = 8$). **(A–G)** serum immunological index. IFN-γ, interferon-γ; IL-4, interleukin-4; IL-6, interleukin-6; TNF-α, tumor necrosis factor-α; IgA, immunoglobulin A; IgG, immunoglobulin G; IgM, immunoglobulin M. [a,b,c]Means among the experimental groups with different letters are significantly different ($p < 0.05$).

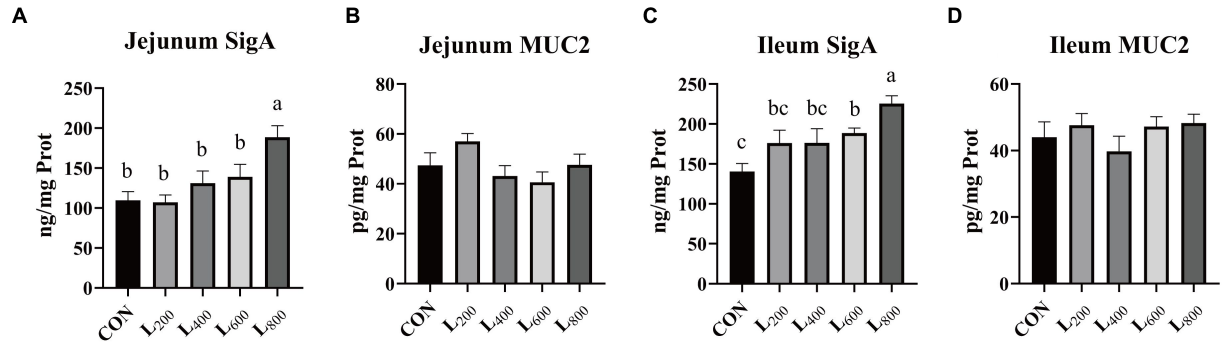


FIGURE 4
Effect of heat-killed *Lactobacillus acidophilus* on intestinal mucosal immunity in rabbits. Values are means ± standard error ($n = 8$). **(A,B)** SigA, Muc2 in jejunum; **(C,D)** SigA, Muc2 in ileum. SigA, Secretory immunoglobulin A; Muc2, Mucin 2. [a,b,c]Means among the experimental groups with different letters are significantly different ($p < 0.05$).

broilers. Noteworthy findings by Tartrakoon et al. (21) demonstrated enhanced feed efficiency and ADG in pigs through the utilization of 20 mg/kg of heat-killed *L. plantarum*. Consistent with prior studies, the current research underscores that the addition of 600 mg/kg and 800 mg/kg of heat-killed LA significantly reduces F/G and enhances the growth performance of rabbits. This improvement in feed efficiency correlates with the observed elevation in the activity of digestive enzymes (lipase, protease, cellulase) within the intestinal tract of rabbits (22). Notably, the potential role of heat-killed LA in regulating gut microflora and diminishing the incidence of diarrhea aligns with the findings of Moal et al., who observed a reduction in harmful bacterial adhesion in the intestine through the use of inactivated probiotics (23), resulting in a decreased frequency of diarrhea in piglets (24). In summary, we hypothesize that the improved growth performance may also be related to heat-killed LA by modulating gut microflora and mitigating the occurrence of diarrhea.

Apparent digestibility serves as a key indicator of an animal's proficiency in nutrient digestion within the diet, and this is intricately linked to digestive enzyme activity, contributing positively to growth performance (25, 26). Mammalian food nutrients primarily undergo digestion orchestrated by various digestive enzymes, with a minor portion being subject to digestion by intestinal bacteria (27, 28). Palamidi et al. (29) reported an elevation in lipase activity and

FIGURE 5
Venn diagram showing common and unique operational taxonomic units (OTUs) in the cecal digest of rabbits. According to the feature abundance table, the number of features in each group was calculated, and the number of common and unique features in each group was visualized by Venn diagram, if the feature existed in only one group, it was unique to that group.

improved digestibility in broilers when fed heat-killed complex probiotics. Similarly, Dawood et al. (30) observed a significant increase in amylase, protease, and lipase activities following the inactivation of *L. plantarum*, leading to enhanced nutrient digestion. In concordance with these findings, the current study demonstrated a substantial increase in trypsin, lipase, and cellulase activities upon supplementing diets with 600 mg/kg and 800 mg/kg of heat-killed LA. This trend aligned with the concurrent increase in the apparent digestibility of CP and CF in rabbits. Furthermore, the inactivation of LA was found to enhance the intestinal microbial composition, favoring an increased relative abundance of beneficial bacteria such as Lactobacillus and Bacillus in the intestine (31, 32). This augmentation in beneficial microbial communities within the gut can potentiate their ability to induce the production of endogenous enzymes (33, 34). Consequently, the inactivation of LA appears to contribute to the enhancement of digestive enzyme activity, ultimately improving digestibility and, consequently, the growth performance of rabbits.

Oxidation reactions, yielding ROS, are constant processes within organisms. Under normal physiological conditions, animal cells maintain a delicate equilibrium between ROS production and clearance (35). Excessive accumulation of free radicals poses a significant threat to tissues, leading to intestinal damage, nutrient malabsorption, and severe diarrhea (36, 37). Antioxidant defense enzymes, including SOD and GSH-Px, play pivotal roles in

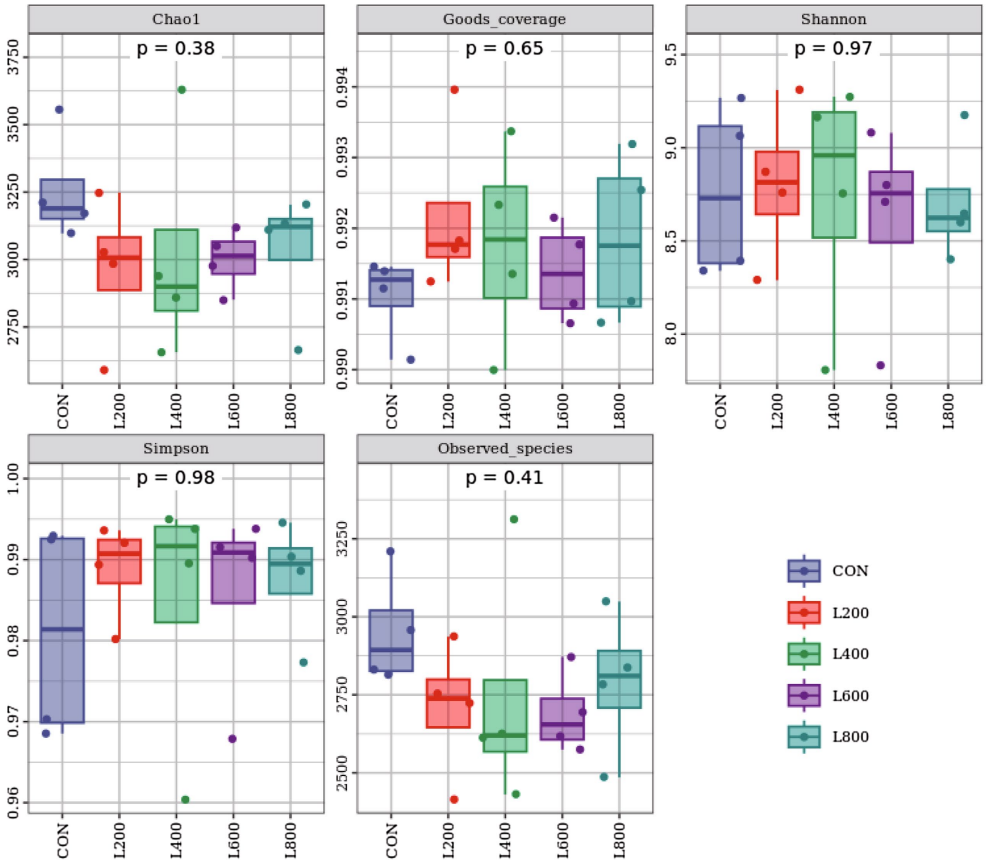


FIGURE 6
Alpha diversity indices of cecum microbiota. Chao 1 and Observed_otus indices mainly reflect the richness of the species in the sample; Goods_coverage reflects the low abundance feature coverage of the sample; Simpson and Shannon reflect the richness and evenness of the species together.

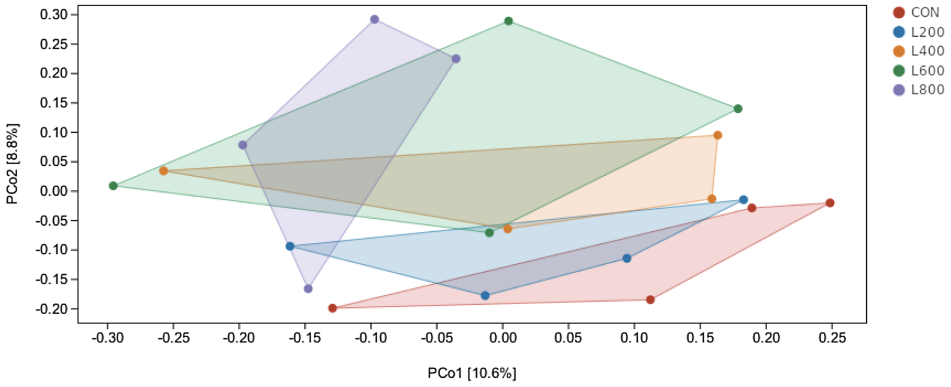


FIGURE 7
Principal co-ordinates analysis diagram of cecum microflora composition. Principal Coordinate Analysis (PCoA) is based on a sample distance matrix for ranking. The PCoA analysis allows the difference among the groups to be observed. The different colours in the results represent the different groupings and the closer the distance, the more similar the structure of the microbial composition and the less difference.

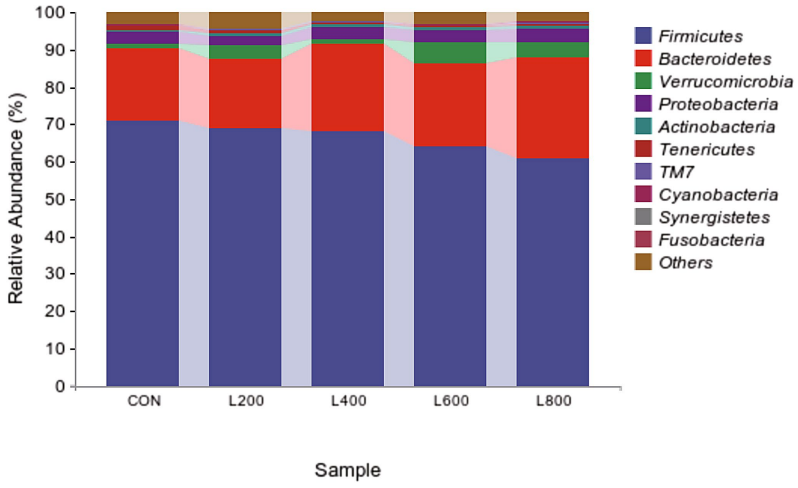


FIGURE 8
Relative abundance of cecum microbiota at phylum level.

TABLE 4 Relative abundance of caecal microbiota at phylum level.

Item	Groups					SEM	*p*-value
	CON	L_{200}	L_{400}	L_{600}	L_{800}		
Firmicutes	70.81	68.79	68.25	64.08	60.97	1.668	0.360
Bacteroidetes	19.30	18.66	23.23	22.19	26.75	1.714	0.621
Verrucomicrobia	1.48	3.63	1.30	5.69	4.12	0.651	0.153
Proteobacteria	3.06	2.42	3.19	3.00	3.68	0.174	0.265
Actinobacteria	0.65	0.80	0.71	0.90	0.77	0.061	0.790
Tenericutes	0.88	0.82	0.57	0.51	0.65	0.078	0.540
TM7	0.24	0.27	0.16	0.19	0.30	0.032	0.647
Cyanobacteria	0.32	0.13	0.26	0.16	0.13	0.034	0.321
Firmicutes	0.00	0.13	0.04	0.02	0.06	0.029	0.710
Bacteroidetes	0.00	0.00	0.00	0.00	0.01	0.001	0.324

SEM, total standard error of the means ($n = 4$). [a,b,c]Means with different superscripts in the same row are significantly different ($p < 0.05$).

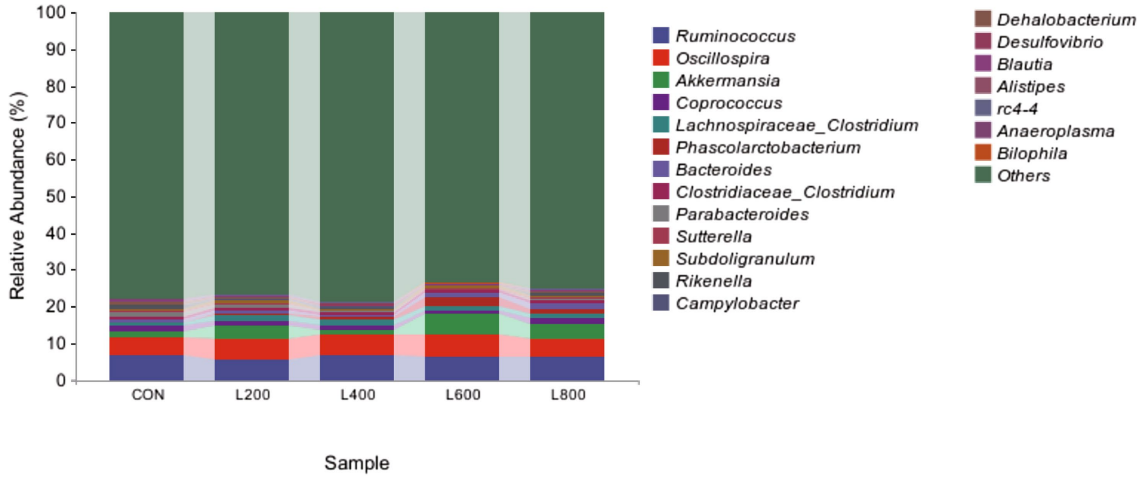


FIGURE 9
Relative abundance of cecum microbiota at genus level.

TABLE 5 Relative abundance of caecal microbiota at genus level.

Item	Groups					SEM	*p*-value
	CON	L_{200}	L_{400}	L_{600}	L_{800}		
Ruminococcus	6.78	5.51	6.76	6.38	6.39	0.316	0.756
Oscillospira	4.90	5.42	5.55	5.90	4.67	0.319	0.788
Akkermansia	1.48	3.63	1.30	5.68	4.12	0.650	0.154
Coprococcus	1.43	1.47	1.28	0.72	1.68	0.200	0.668
Lachnospiraceae-Clostridium	1.16	1.50	1.33	1.22	1.10	0.103	0.798
Phascolarctobacterium	0.00^{c}	0.32^{c}	0.88^{bc}	2.47^{a}	1.43^{b}	0.235	<0.001
Bacteroides	0.44	0.79	0.50	1.24	1.55	0.177	0.204
Clostridiaceae-Clostridium	1.10	0.78	0.88	0.66	0.66	0.057	0.066
Parabacteroides	0.99	0.95	0.23	0.24	0.36	0.206	0.640
Sutterella	0.61	0.59	0.23	0.49	0.55	0.080	0.618
Subdoligranulum	0.45^{bc}	0.71^{a}	0.25^{c}	0.52^{ab}	0.32^{bc}	0.048	0.004
Rikenella	0.97	0.07	0.52	0.03	0.62	0.160	0.315
Campylobacter	0.22	0.18	0.42	0.06	0.32	0.061	0.442
Dehalobacterium	0.54^{a}	0.40^{ab}	0.05^{c}	0.01^{c}	0.18^{bc}	0.061	0.008
Desulfovibrio	0.24	0.29	0.45	0.09	0.10	0.074	0.567
Blautia	0.19	0.24	0.18	0.16	0.23	0.015	0.437
Alistipes	0.06^{b}	0.10^{b}	0.12^{b}	0.11^{b}	0.32^{a}	0.023	<0.001
rc4-4	0.11	0.16	0.12	0.10	0.10	0.011	0.610
Anaeroplasma	0.18	0.14	0.05	0.09	0.14	0.039	0.901
Bilophila	0.10	0.10	0.12	0.12	0.08	0.020	0.966

SEM, total standard error of the means ($n = 4$). ^{a,b,c}Means with different superscripts in the same row are significantly different ($p < 0.05$).

counteracting oxidative stress (38). SOD, functioning as the primary defense against free radicals, not only exhibits superb antioxidant effects but also inhibits the synthesis and release of inflammatory factors (39). T-AOC, encompassing both enzymatic and non-enzymatic antioxidant activities, provides a comprehensive assessment of the organism's antioxidant status (40). Previous research, such as Liu et al. (41) and Dawood et al. (30) has highlighted the antioxidant properties of heat-killed LA and heat-killed *L. plantarum*, respectively. In this experiment, supplementation with heat-killed LA demonstrated a notable increase in SOD activity and T-AOC capacity in rabbits, with the most pronounced effects observed at supplementation doses of 600 mg/kg and 800 mg/kg. This

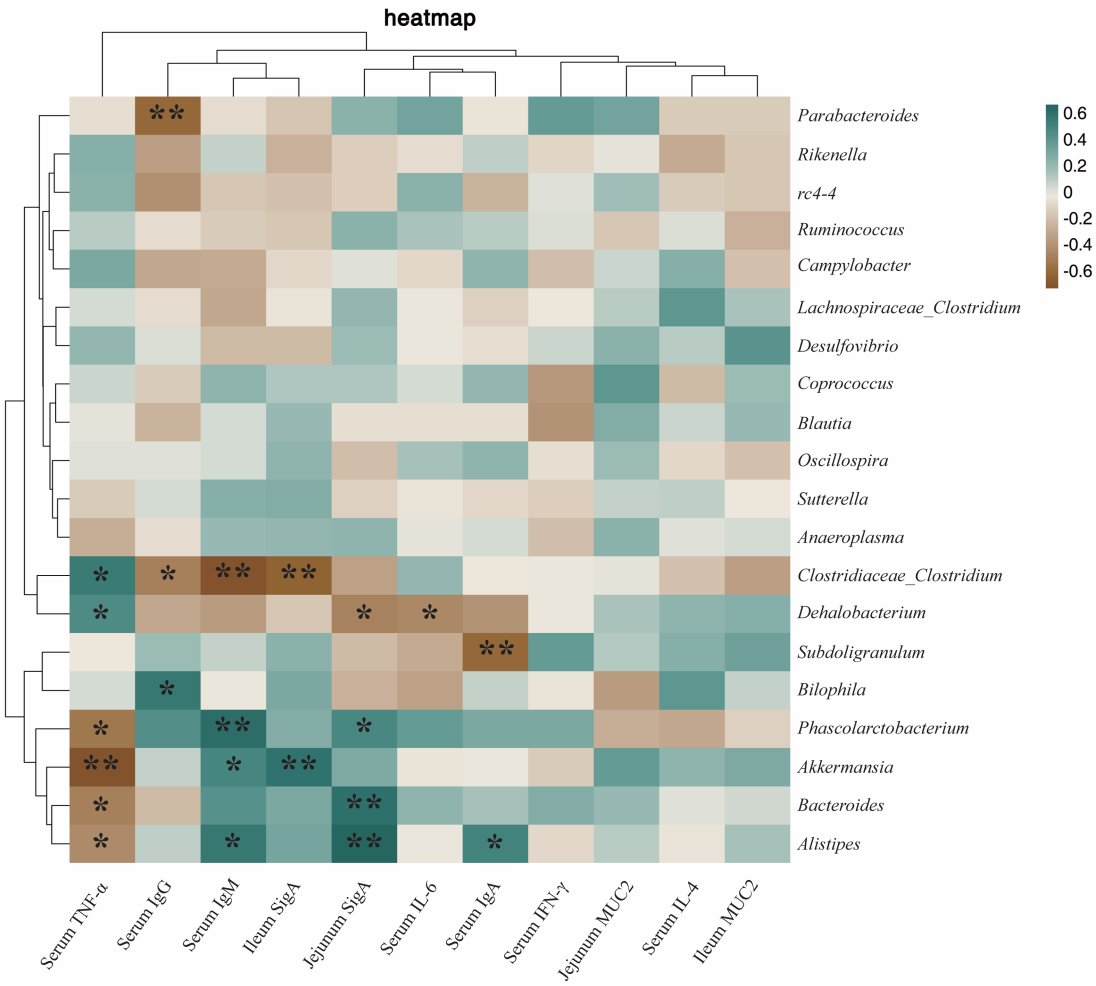


FIGURE 10
Heatmap of the correlation between cecum microbiota and immunity. The correlations were significant (*$p < 0.05$ and **$p < 0.01$), positive in green and negative in brown.

augmentation in antioxidant-related enzyme activity may be attributed to the ability of heat-killed Lactobacillus to activate the Nrf2 signaling pathway (42, 43). Activation of the Nrf2 signaling pathway triggers the transcription and translation of downstream antioxidant genes (such as SOD and T-AOC), bolstering antioxidant status in response to environmental stress and sustaining normal intestinal function (44).

Immunoglobulin (IgA, IgM, and SigA etc.) is one of the components of animal immune system and an important parameter reflecting the immune capacity of animal body (45). IgA plays a crucial role in protecting the mucosal surface by preventing the entry, binding, and colonization of toxins and pathogens (46). IgM is the immunoglobulin with the strongest initial immune effect, and is an important barrier against pathogenic invasion (47). In this study, supplementation with heat-killed LA significantly increased the levels of IgA and IgM in serum, which reflected that heat-killed LA could improve the immune capacity of rabbits. As the main immune defense line of intestinal mucosa, SigA has the function of neutralizing pathogens and bacterial exotoxins in intestinal mucosa and maintaining the stability of intestinal flora (48). Danladi et al. found that supplementation of feed with inactivated *Lactobacillus plantarum* significantly increased the intestinal SigA content of broiler chickens and improved immunity (49). Consistent with the results of previous studies, we found that feed supplementation with 600 mg/kg and 800 mg/kg of heat-killed LA significantly enhanced the secretion of intestinal SigA, protected the intestinal immune barrier, and reduced the occurrence of intestinal diseases. TNF-α is a pro-inflammatory cytokine produced mainly by macrophages and monocytes, which is involved in the inflammatory response, and when its level is too high, it increases intestinal permeability and causes an enteritis response (50). It was found that LTA isolated from Lactobacillus can block the phosphorylation of NF-κB and the phosphorylation degradation of IkB, thereby inhibiting the expression of TNF-α (51). In this experiment, we found that supplementation with heat-killed LA reduced the serum levels of TNF-α. This may be related to the blocking of NF-κB phosphorylation by heat-killed LA. It was also shown that heat inactivation of LA does not cause an inflammatory response in the meat rabbit organism and has no adverse effect on its health.

There are a large number of microorganisms in the intestinal tract of animals, and their species composition and activity play an important role in the immune and inflammatory responses of animals (52, 53). Therefore, the balance of intestinal flora is related to the health of animals. Under normal conditions, the composition of the

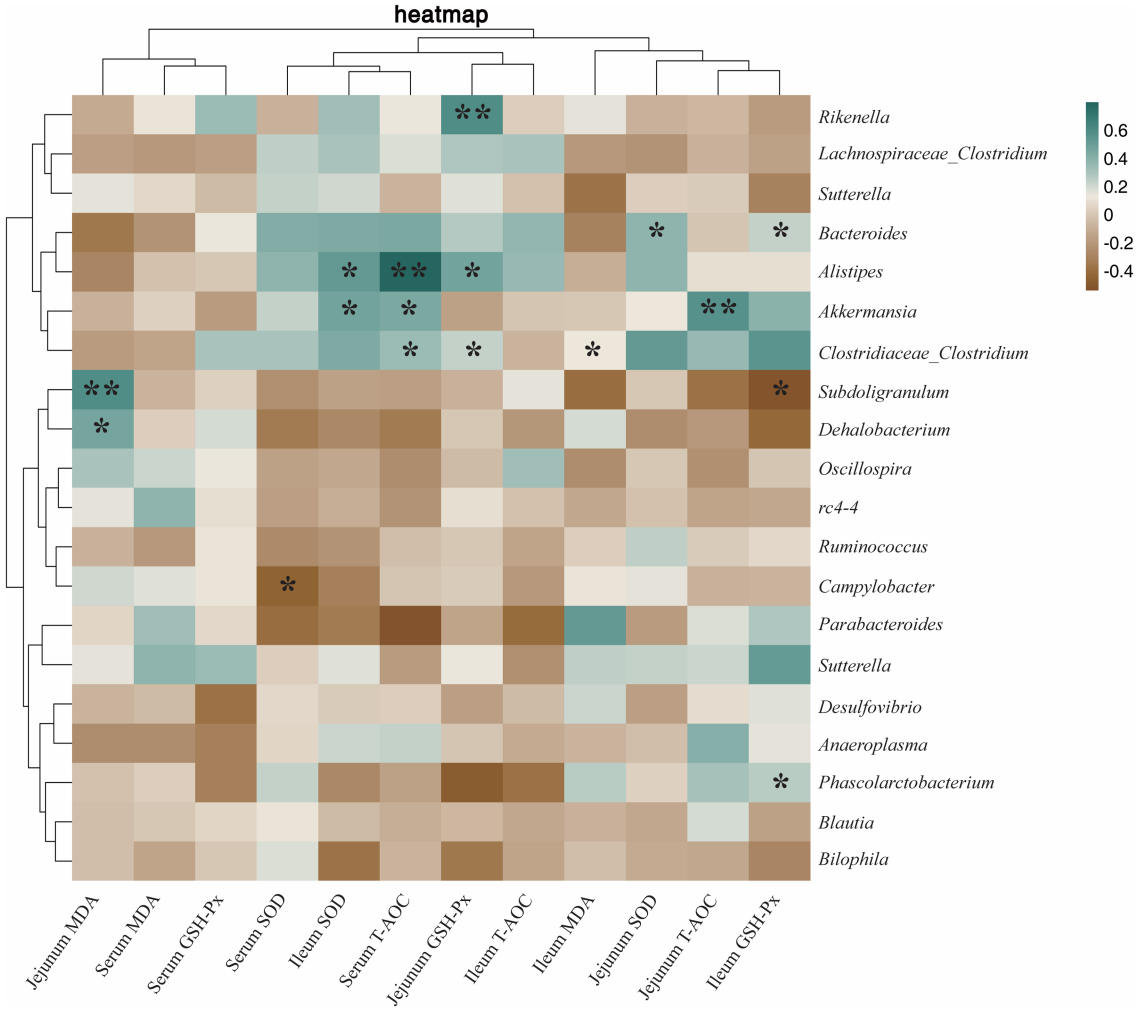


FIGURE 11
Heatmap of the correlation between cecum microbiota and antioxidant capacity. The correlations were significant (*$p < 0.05$ and **$p < 0.01$), positive in green and negative in brown.

intestinal flora is relatively stable, but at the same time it can be disturbed by external factors (54). In the current study, probiotic supplements or probiotic products were found to be one of the effective ways to maintain a healthy balance of gut microbiota (55–58). In addition, species-rich communities enhance intestinal microecological stability and reduce susceptibility to bacterial invasion and intestinal inflammation (59). In this study, dietary heat-killed LA can regulate the cecal microflora structure of rabbits and increase the relative abundance of *Phascolarctobacterium* and *Alistipes*. *Phascolarctobacterium* and *Alistipes* are probiotics that promote intestinal health. Their main function is to produce short chain fatty acid (SCFAs), which play a vital role in maintaining nutrient metabolism and microbial homeostasis (60, 61). Acetic acid is not only the most abundant SCFAs in the gut, but also inhibits the NF-κB signaling pathway by down-regulating the expression of TLR gene, thereby reducing the content of TNF-α in the body and reducing the inflammatory response (62). In addition, SCFAs can also accelerate the activation, differentiation and antibody production of B cells by promoting the synthesis of acetyl-CoA, 5'-adenosine triphosphate and fatty acids in B cells, thus promoting the secretion of SigA, IgA, and IgM (63, 64). SCFAs can also act as an activator of Keap1-Nrf2 signaling pathway (65, 66), and the activation of Nrf2 is positively correlated with SOD, GSH-Px, and T-AOC (44, 67). The above results showed that heat-killed LA can promote the proliferation of *Phascolarctobacterium* and *Alistipes*, thereby reducing serum TNF-α level, and increasing jejunum SigA, serum IgM, serum IgA, ileum GSH-Px, jejunum GSH-Px, ileum SOD and serum T-AOC levels, which may be due to the regulation of SCFAs by *Phascolarctobacterium* and *Alistipes*. These results provide a theoretical basis for understanding heat-killed LA-mediated microbial promotion of immunity and antioxidant capacity in rabbits. However, further microbiome-based studies are needed to elucidate the mechanisms by which *Phascolarctobacterium* and *Alistipes* promote immunity and antioxidant capacity in rabbits.

5 Conclusion

In conclusion, the incorporation of heat-killed LA into the diet demonstrates a capacity to enhance intestinal digestive enzyme

activity, bolster antioxidant status, modulate the immune capacity, increase the relative abundance of beneficial bacteria in the cecum and foster overall organismal health in rabbits. This ultimately translates into improved growth performance. Upon comprehensive analysis, the recommended additive levels of heat-killed LA in rabbit diets appear to be 600 and 800 mg/kg.

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 Animal Care and Use Committee of Hebei Agriculture University (Baoding, China). The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

MX: Writing – original draft, Conceptualization, Funding acquisition, Project administration, Resources, Supervision, Validation, Writing – review & editing. CL: Writing – original draft, Conceptualization, Data curation, Formal analysis, Methodology, Writing – review & editing. DW: Writing – original draft, Data curation, Formal analysis, Investigation, Methodology, Resources, Software. FW: Methodology, Project administration, Software, Writing – review & editing. LK: Writing – original draft, Data curation, Formal analysis, Investigation, Methodology. ZJ: Writing – original draft, Data curation, Formal analysis, Investigation, Methodology. WH: Writing – original draft, Data curation, Formal analysis, Investigation, Methodology. SC: Writing – original draft, Data curation, Formal analysis, Investigation, Methodology. WF: Writing – original draft, Data curation, Formal analysis, Investigation, Methodology. YL: Writing – original draft, Conceptualization, Project administration, Resources, Supervision, Validation, Visualization, Writing – review & editing. BC: Writing – original draft, Funding acquisition, Supervision.

Funding

The author(s) declare that financial support was received for the research, authorship, and/or publication of this article. This study was supported by a special fund for "Modern Agriculture Industry Technology System of Rabbit, China (CARS-43-B-2)".

Conflict of interest

WF was employed by Biosource 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.

Publisher's note

References

1. Hill C, Guarner F, Reid G, Gibson GR, Merenstein DJ, Pot B, et al. Expert consensus document. The international scientific Association for Probiotics and Prebiotics consensus statement on the scope and appropriate use of the term probiotic. *Nat Rev Gastroenterol Hepatol.* (2014) 11:506–14. doi: 10.1038/nrgastro.2014.66

2. Grant A, Gay CG, Lillehoj HS. Bacillus spp. as direct-fed microbial antibiotic alternatives to enhance growth, immunity, and gut health in poultry. *Avian Pathol.* (2018) 47:339–51. doi: 10.1080/03079457.2018.1464117

3. Liu Y, Gu W, Liu X, Zou Y, Wu Y, Xu Y, et al. Joint application of Lactobacillus plantarum and Bacillus subtilis improves growth performance, immune function and intestinal integrity in weaned piglets. *Vet Sci.* (2022) 9:20668. doi: 10.3390/vetsci9120668

4. Phuoc TL, Jamikorn U. Effects of probiotic supplement (Bacillus subtilis and Lactobacillus acidophilus) on feed efficiency, growth performance, and microbial population of weaning rabbits. *Asian Australas J Anim Sci.* (2017) 30:198–205. doi: 10.5713/ajas.15.0823

5. Wu Z, Yang K, Zhang A, Chang W, Zheng A, Chen Z, et al. Effects of Lactobacillus acidophilus on the growth performance, immune response, and intestinal barrier function of broiler chickens challenged with Escherichia coli O157. *Poult Sci.* (2021) 100:101323. doi: 10.1016/j.psj.2021.101323

6. Alayande KA, Aiyegoro OA, Ateba CN. Probiotics in animal husbandry: applicability and associated risk factors. *Sustain For.* (2020) 12:12. doi: 10.3390/su12031087

7. Akter S, Park JH, Jung HK. Potential health-promoting benefits of Paraprobiotics, inactivated probiotic cells. *J Microbiol Biotechnol.* (2020) 30:477–81. doi: 10.4014/jmb.1911.11019

8. Cuevas-González PF, Liceaga AM, Aguilar-Toalá JE. Postbiotics and paraprobiotics: from concepts to applications. *Food Res Int.* (2020) 136:109502. doi: 10.1016/j.foodres.2020.109502

9. Hong YF, Lee H, Jung BJ, Jang S, Chung DK, Kim H. Lipoteichoic acid isolated from Lactobacillus plantarum down-regulates UV-induced MMP-1 expression and up-regulates type I procollagen through the inhibition of reactive oxygen species generation. *Mol Immunol.* (2015) 67:248–55. doi: 10.1016/j.molimm.2015.05.019

10. Piqué N, Berlanga M, Miñana-Galbis D. Health benefits of heat-killed (Tyndallized) probiotics: an overview. *Int J Mol Sci.* (2019) 20:20. doi: 10.3390/ijms20102534

11. Almada CN, Almada CN, Martinez RCR, Sant'Ana ASP. Evidences on their ability to modify biological responses, inactivation methods and perspectives on their application in foods. *Trends Food Sci Technol.* (2016) 58:96. doi: 10.1016/j.tifs.2016.09.011

12. Ou CC, Lin SL, Tsai JJ, Lin MY. Heat-killed lactic acid bacteria enhance immunomodulatory potential by skewing the immune response toward Th1 polarization. *J Food Sci.* (2011) 76:M260–7. doi: 10.1111/j.1750-3841.2011.02161.x

13. Velasco-Galilea M, Guivernau M, Piles M, Viñas M, Rafel O, Sánchez A, et al. Breeding farm, level of feeding and presence of antibiotics in the feed influence rabbit cecal microbiota. *Anim Microbiome.* (2020) 2:40. doi: 10.1186/s42523-020-00059-z

14. Bivolarski BL, Vachkova EG. Morphological and functional events associated to weaning in rabbits. *J Anim Physiol Anim Nutr.* (2014) 98:9–18. doi: 10.1111/jpn.12058

15. Gharib HSA, Abdel-Fattah AF, Mohammed HA, Abdel-Fattah DM. Weaning induces changes in behavior and stress indicators in young New Zealand rabbits. *J Adv Vet Animal Res.* (2018) 5:5. doi: 10.5455/javar.2018.e262

16. María Remes Troche J, Coss Adame E, Ángel Valdovinos Díaz M, Gómez Escudero O, Eugenia Icaza Chávez M, Antonio Chávez-Barrera J, et al. Lactobacillus acidophilus LB: a useful pharmabiotic for the treatment of digestive disorders. *Ther Adv Gastroenterol.* (2020) 13:1756284820971201. doi: 10.1177/1756284820971201

17. De Blas C, Wiseman J. *Nutrition of the rabbit* CAB International (2020).

18. AOAC. *Official methods of analysis. 18th* ed. Arlington, VA: AOAC Int (2006).

19. Liang ZL, Chen F, Park S, Balasubramanian B, Liu WC. Impacts of heat stress on rabbit immune function, endocrine, blood biochemical changes, antioxidant capacity and production performance, and the potential mitigation strategies of nutritional intervention. *Front Vet Sci*. (2022) 9:906084. doi: 10.3389/fvets.2022.906084

20. Zhu C, Gong L, Huang K, Li F, Tong D, Zhang H. Effect of heat-inactivated compound probiotics on growth performance, plasma biochemical indices, and Cecal microbiome in yellow-feathered broilers. *Front Microbiol*. (2020) 11:585623. doi: 10.3389/fmicb.2020.585623

21. Tartrakoon W, Charoensook R, Incharoen T, Numthuam S, Pechrkong T, Onoda S, et al. Effects of heat-killed Lactobacillus plantarum L-137 supplementation on growth performance, blood profiles, intestinal morphology, and immune gene expression in pigs. *Vet Sci*. (2023) 10:20087. doi: 10.3390/vetsci10020087

22. Yeganeh S, Adel M, Nosratimovafagh A, Dawood MAO. The effect of Lactococcus lactis subsp. lactis PTCC 1403 on the growth performance, digestive enzymes activity, Antioxidative status, immune response, and disease resistance of rainbow trout (Oncorhynchus mykiss). *Probiotics Antimicrob Proteins*. (2021) 13:1723–33. doi: 10.1007/s12602-021-09787-3

23. Liévin-Le Moal V. A gastrointestinal anti-infectious biotherapeutic agent: the heat-treated Lactobacillus LB. *Ther Adv Gastroenterol*. (2016) 9:57–75. doi: 10.1177/1756283x15602831

24. Kang J, Lee JJ, Cho JH, Choe J, Kyoung H, Kim SH, et al. Effects of dietary inactivated probiotics on growth performance and immune responses of weaned pigs. *J Anim Sci Technol*. (2021) 63:520–30. doi: 10.5187/jast.2021.e44.doi:10.5187/jast.2021.e44

25. Huang P, Cui X, Wang Z, Xiao C, Ji Q, Wei Q, et al. Effects of clostridium butyricum and a bacteriophage cocktail on growth performance, serum biochemistry, digestive enzyme activities, intestinal morphology, immune responses, and the intestinal microbiota in rabbits. *Antibiotics*. (2021) 10:10. doi: 10.3390/antibiotics10111347

26. Jackson AD, McLaughlin J. Digestion and absorption. *Surgery*. (2009) 27:231. doi: 10.1016/j.mpsur.2009.03.003

27. Drozdowski LA, Thomson AB. Intestinal sugar transport. *World J Gastroenterol*. (2006) 12:1657–70. doi: 10.3748/wjg.v12.i11.1657

28. Switzar L, Giera M, Niessen WM. Protein digestion: an overview of the available techniques and recent developments. *J Proteome Res*. (2013) 12:1067–77. doi: 10.1021/pr301201x

29. Palamidi I, Fegeros K, Mohnl M, Abdelrahman WHA, Schatzmayr G, Theodoropoulos G, et al. Probiotic form effects on growth performance, digestive function, and immune related biomarkers in broilers. *Poult Sci*. (2016) 95:1598–608. doi: 10.3382/ps/pew052

30. Dawood MAO, Magouz FI, Salem MFI, Abdel-Daim HA. Modulation of digestive enzyme activity, blood health, oxidative responses and growth-related gene expression in GIFT by heat-killed Lactobacillus plantarum (L-137). *Aquaculture*. (2019) 505:127. doi: 10.1016/j.aquaculture.2019.02.053

31. Danladi Y, Loh TC, Foo HL, Akit H, Md Tamrin NA, Mohammad Naeem A. Impact of feeding Postbiotics and Paraprobiotics produced from Lactiplantibacillus plantarum on Colon mucosa microbiota in broiler chickens. *Front Vet Sci*. (2022) 9:859284. doi: 10.3389/fvets.2022.859284

32. Delong M, Qiang H, Qingshuang Z, Zhe Y, Shubin L, Yalin Y, et al. A compound of paraprobiotic and postbiotic derived from autochthonous microorganisms improved growth performance, epidermal mucus, liver and gut health and gut microbiota of common carp (Cyprinus carpio). *Aquaculture*. (2023) 570:739378. doi: 10.1016/j.aquaculture.2023.739378

33. Liu H, Wang S, Cai Y, Guo X, Cao Z, Zhang Y, et al. Dietary administration of Bacillus subtilis HAINUP40 enhances growth, digestive enzyme activities, innate immune responses and disease resistance of tilapia, Oreochromis niloticus. *Fish Shellfish Immunol*. (2017) 60:326–33. doi: 10.1016/j.fsi.2016.12.003

34. Wu ZX, Feng X, Xie LL, Peng XY, Yuan J, Chen XX. Effect of probiotic Bacillus subtilis Ch9 for grass carp, Ctenopharyngodon idella (Valenciennes, 1844), on growth performance, digestive enzyme activities and intestinal microflora. *J Appl Ichthyol*. (2012) 28:721. doi: 10.1111/j.14390426.2012.01968.x

35. Yang S, Lian G. ROS and diseases: role in metabolism and energy supply. *Mol Cell Biochem*. (2020) 467:1–12. doi: 10.1007/s11010-019-03667-9

36. Lauridsen C. From oxidative stress to inflammation: redox balance and immune system. *Poult Sci*. (2019) 98:4240–6. doi: 10.3382/ps/pey407

37. Thomson A, Hemphill D, Jeejeebhoy KN. Oxidative stress and antioxidants in intestinal disease. *Dig Dis*. (1998) 16:152–8. doi: 10.1159/000016859

38. Mishra V, Shah C, Mokashe N, Chavan R, Yadav H, Prajapati J. Probiotics as potential antioxidants: a systematic review. *J Agric Food Chem*. (2015) 63:3615–26. doi: 10.1021/jf506326t

39. Yasui K, Baba A. Therapeutic potential of superoxide dismutase (SOD) for resolution of inflammation. *Inflamm Res*. (2006) 55:359–63. doi: 10.1007/s00011-006-5195-y

40. Ferrari CK, Souto PC, França EL, Honorio-França AC. Oxidative and nitrosative stress on phagocytes' function: from effective defense to immunity evasion mechanisms. *Arch Immunol Ther Exp*. (2011) 59:441–8. doi: 10.1007/s00005-011-0144-z

41. Liu CF, Hu CL, Chiang SS, Tseng KC, Yu RC, Pan TM. Beneficial preventive effects of gastric mucosal lesion for soy-skim milk fermented by lactic acid bacteria. *J Agric Food Chem*. (2009) 57:4433–8. doi: 10.1021/jf900465c

42. Xu J, Zhang X, Song Y, Zheng B, Wen Z, Gong M, et al. Heat-killed Lacticaseibacillus paracasei ameliorated UVB-induced oxidative damage and Photoaging and its underlying mechanisms. *Antioxidants*. (2022) 11:1875. doi: 10.3390/antiox11101875

43. Zhang X, Xu J, Ma M, Zhao Y, Song Y, Zheng B, et al. Heat-killed Lactobacillus rhamnosus ATCC 7469 improved UVB-induced Photoaging via Antiwrinkle and Antimelanogenesis impacts. *Photochem Photobiol*. (2023) 99:1318–31. doi: 10.1111/php.13775

44. Fu K, Chen M, Zheng H, Li C, Yang F, Niu Q. Pelargonidin ameliorates MCAO-induced cerebral ischemia/reperfusion injury in rats by the action on the Nrf2/HO-1 pathway. *Transl Neurosci*. (2021) 12:20–31. doi: 10.1515/tnsci-2021-0006

45. Wang K, Cao G, Zhang H, Li Q, Yang C. Effects of Clostridium butyricum and Enterococcus faecalis on growth performance, immune function, intestinal morphology, volatile fatty acids, and intestinal flora in a piglet model. *Food Funct*. (2019) 10:7844–54. doi: 10.1039/c9fo01650c

46. Carlier FM, Sibille Y, Pilette C. The epithelial barrier and immunoglobulin a system in allergy. *Clin Exp Allergy*. (2016) 46:1372–88. doi: 10.1111/cea.12830

47. Ehrenstein MR, Cook HT, Neuberger MS. Deficiency in serum immunoglobulin (Ig) M predisposes to development of IgG autoantibodies. *J Exp Med*. (2000) 191:1253–8. doi: 10.1084/jem.191.7.1253

48. Pietrzak B, Tomela K, Olejnik-Schmidt A, Mackiewicz A, Schmidt M. Secretory IgA in intestinal mucosal secretions as an adaptive barrier against microbial cells. *Int J Mol Sci*. (2020) 21:e254. doi: 10.3390/ijms21239254

49. Danladi Y, Loh TC, Foo HL, Akit H, Md Tamrin NA, Naeem Azizi M. Effects of Postbiotics and Paraprobiotics as replacements for antibiotics on growth performance, carcass characteristics, small intestine Histomorphology, immune status and hepatic growth gene expression in broiler chickens. *Animals*. (2022) 12:12. doi: 10.3390/ani12070917

50. Kim KY, Oh TW, Do HJ, Yang JH, Yang IJ, Jeon YH, et al. Acer palmatum thumb. Ethanol extract alleviates Interleukin-6-induced barrier dysfunction and dextran sodium sulfate-induced colitis by improving intestinal barrier function and reducing inflammation. *J Immunol Res*. (2018) 2018:8396. doi: 10.1155/2018/5718396

51. Gao Q, Gao Q, Min M, Zhang C, Peng S, Shi Z. Ability of Lactobacillus plantarum lipoteichoic acid to inhibit Vibrio anguillarum-induced inflammation and apoptosis in silvery pomfret (Pampus argenteus) intestinal epithelial cells. *Fish Shellfish Immunol*. (2016) 54:573–9. doi: 10.1016/j.fsi.2016.05.013

52. de Almada CN, Nunes de Almada C, Martinez RC, Sant'Ana Ade S. Characterization of the intestinal microbiota and its interaction with probiotics and health impacts. *Appl Microbiol Biotechnol*. (2015) 99:4175–99. doi: 10.1007/s00253-015-6582-5

53. Dong H, Rowland I, Thomas LV, Yaqoob P. Immunomodulatory effects of a probiotic drink containing Lactobacillus casei Shirota in healthy older volunteers. *Eur J Nutr*. (2013) 52:1853–63. doi: 10.1007/s00394-012-0487-1

54. Generoso SV, Viana ML, Santos RG, Arantes RM, Martins FS, Nicoli JR, et al. Protection against increased intestinal permeability and bacterial translocation induced by intestinal obstruction in mice treated with viable and heat-killed Saccharomyces boulardii. *Eur J Nutr*. (2011) 50:261–9. doi: 10.1007/s00394-010-0134-7

55. Adams CA. The probiotic paradox: live and dead cells are biological response modifiers. *Nutr Res Rev*. (2010) 23:37–46. doi: 10.1017/s0954422410000090

56. Biswas G, Korenaga H, Nagamine R, Takayama H, Kawahara S, Takeda S, et al. Cytokine responses in the Japanese pufferfish (Takifugu rubripes) head kidney cells induced with heat-killed probiotics isolated from the Mongolian dairy products. *Fish Shellfish Immunol*. (2013) 34:1170–7. doi: 10.1016/j.fsi.2013.01.024

57. Dantas AB, Jesus VF, Silva R, Almada CN, Esmerino EA, Cappato LP, et al. Manufacture of probiotic Minas Frescal cheese with Lactobacillus casei Zhang. *J Dairy Sci*. (2016) 99:18–30. doi: 10.3168/jds.2015-9880

58. Zhang J, Wang L, Guo Z, Sun Z, Gesudu Q, Kwok L, et al. 454 pyrosequencing reveals changes in the faecal microbiota of adults consuming Lactobacillus casei Zhang. *FEMS Microbiol Ecol*. (2014) 88:612–22. doi: 10.1111/1574-6941.12328

59. Ott SJ, Musfeldt M, Wenderoth DF, Hampe J, Brant O, Fölsch UR, et al. Reduction in diversity of the colonic mucosa associated bacterial microflora in patients with active inflammatory bowel disease. *Gut*. (2004) 53:685–93. doi: 10.1136/gut.2003.025403

60. Parker BJ, Wearsch PA, Veloo ACM, Rodriguez-Palacios A. The genus Alistipes: gut Bacteria with emerging implications to inflammation, Cancer, and mental health. *Front Immunol*. (2020) 11:906. doi: 10.3389/fimmu.2020.00906

61. Wu F, Guo X, Zhang J, Zhang M, Ou Z, Peng Y. Phascolarctobacterium faecium abundant colonization in human gastrointestinal tract. *Exp Ther Med*. (2017) 14:3122–6. doi: 10.3892/etm.2017.4878

62. Wu D, Xia M, Yan A, Jiang H, Fan J, Zhou S, et al. Carvacrol attenuated lipopolysaccharide-induced intestinal injury by down-regulating TLRs gene expression and regulating the gut microbiota in rabbit. *Sci Rep*. (2023) 13:11447. doi: 10.1038/s41598-023-38577-w

63. Amu S, Gjertsson I, Brisslert M. Functional characterization of murine CD25 expressing B cells. *Scand J Immunol*. (2010) 71:275–82. doi: 10.1111/j.1365-3083.2010.02380.x

64. Kim M, Qie Y, Park J, Kim CH. Gut microbial metabolites fuel host antibody responses. *Cell Host Microbe*. (2016) 20:202–14. doi: 10.1016/j.chom.2016.07.001

65. Guo W, Liu J, Sun J, Gong Q, Ma H, Kan X, et al. Butyrate alleviates oxidative stress by regulating NRF2 nuclear accumulation and H3K9/14 acetylation via GPR109A in bovine mammary epithelial cells and mammary glands. *Free Radic Biol Med*. (2020) 152:728–42. doi: 10.1016/j.freeradbiomed.2020.01.016

66. Hoyles L, Snelling T, Umlai UK, Nicholson JK, Carding SR, Glen RC, et al. Microbiome-host systems interactions: protective effects of propionate upon the blood-brain barrier. *Microbiome*. (2018) 6:55. doi: 10.1186/s40168-018-0439-y

67. Nakamura BN, Lawson G, Chan JY, Banuelos J, Cortés MM, Hoang YD, et al. Knockout of the transcription factor NRF2 disrupts spermatogenesis in an age-dependent manner. *Free Radic Biol Med*. (2010) 49:1368–79. doi: 10.1016/j.freeradbiomed.2010.07.019

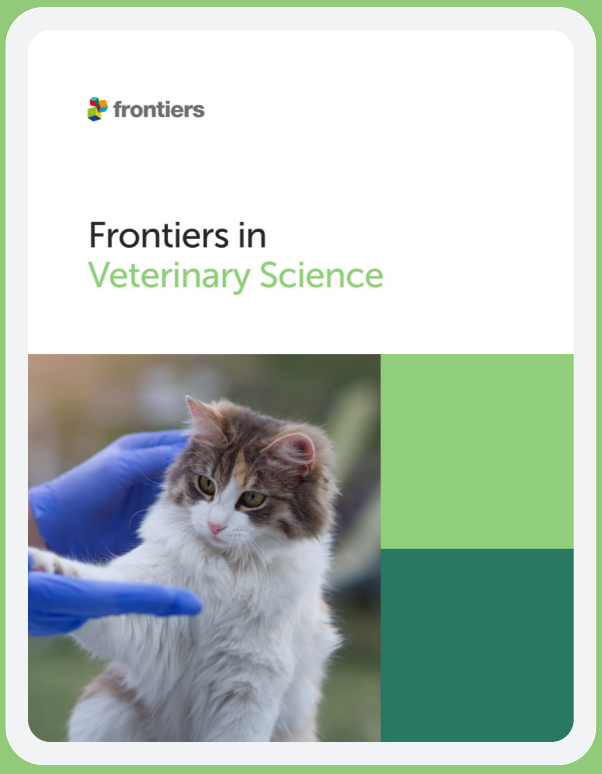
Frontiers in Veterinary Science

Transforms how we investigate and improve animal health

The third most-cited veterinary science journal, bridging animal and human health with a comparative approach to medical challenges. It explores innovative biotechnology and therapy for improved health outcomes.

Discover the latest Research Topics

See more →



Frontiers

Avenue du Tribunal-Fédéral 34
1005 Lausanne, Switzerland
frontiersin.org

Contact us

+41 (0)21 510 17 00
frontiersin.org/about/contact