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Nutrition regulation and stress in ruminant

Edited by
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Nutrition regulation and stress in ruminant

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Editorial: Nutrition regulation and stress in ruminant

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Editorial on the Research Topic
Nutrition regulation and stress in ruminant

Stress was first introduced by Hans Selye in 1936 and has been defined as the body's systematic nonspecific adaptive response when stimulated by internal and external environmental and physiological factors. Ruminants inevitably face various stresses due to changes in physiological stages, environmental conditions and feeding management during the process of breeding production. Stress negatively impacts the nutrient digestibility and health status of ruminants, which ultimately leads to a decrease in production performance and economic efficiency, especially in young ruminants. The articles within this Research Topic detail the nutritional strategies to mitigate animal stress, and cover a wide-range of alternative feed ingredients or dietary supplements on rumen health, immunity and antioxidant function in ruminants at different physiological stages. It contributes to our current understanding of nutritional regulation to mitigate the negative effects of stress on animals.

There is a growing recognition within the livestock industry and society at large that animal stress is a significant issue that requires attention. With implications for animal health, wellbeing, and productivity, minimizing animal stress through improved animal management procedures and/or selective breeding is becoming a priority. With the development of biotechnology, more and more plant bioactive substances have been isolated and identified, some of which have been found to play a role in mitigating animal stress. On the other hand, Alkaline Mineral Complex (AMC) has been proven to have biological benefits and therapeutic effects on animals. The rumen serves as a vital digestive organ for ruminants and significantly influences their growth, performance, and overall health. Plant bioactive substances and AMC can induce changes in rumen function that impact the health and development of ruminants. To instigate readers' interest in the topic of nutritional regulation of animal stress, we present here the main findings reported by different authors in their nine manuscripts.

Plant bioactive substances are widely found in plants and are digested in the rumen used by ruminants. Starch in hydroponically barley seedlings (HBS) is metabolized into soluble polysaccharides during the growth period. Ma Y. et al. evaluated the effects of replacing different ratios of basal diets with HBS in lactating Hu ewes. The results indicated that HBS instead of 5%−15% of the basal diet were able to improve milk quality and alleviate oxidative stress in the body of ewes. Allium plants have been reported to have a positive effect on rumen fermentation and digestive capacity. Wang et al. found that the

addition of 10 g/day *Allium mongolicum* Regel powder to the diet was beneficial for the stability of the rumen microbiota structure, which had a positive impact on the rumen health of Tibetan sheep lambs. Isatis Leaf (ISL) is a traditional Chinese herbal medicine that contains three main active compounds: indoles, quinazolones, and glucosinolates. The paper by Cao et al. studied the effect of ISL on the growth performance, gastrointestinal tissue morphology and microbiota of fattening sheep. In this study, the addition of 8% ISL to the diet increased rumen ammonia-nitrogen levels, regulated the gastrointestinal microbiota, promoted body fat metabolism, and enhanced immunity and resistance, thereby improving the health of fattening sheep. The improvement of rumen health by dietary composition more or less positively affects the health of sheep. However, supplementation of the plant bioactive substances in the form of feed ingredients requires the addition of larger doses.

Plant extracts have been used for thousands of years due to their high bioactive components, which have a positive effect on certain physiological activities of animals. It is also used as phytogenic feed additives to mitigate the negative effects of stress on ruminants. Feng et al. reported that the addition of 40 mg/kg body weight (BW) each day of *Dioscorea alata* L. anthocyanin (extracted from *Dioscorea alata* L.) to the diet improved antioxidant capacity, immune function and meat quality of Hainan black goats. Zhang et al. demonstrated that the addition of 30 mg/kg BW (sheep/day) ellagic acid (commercial product, purity ≥90%) to the diet of 5-month-old Kazakh sheep could improve dry matter intake and apparent digestibility of neutral detergent fiber and crude fat, increase the content of acetic acid and propionic acid in rumen fluid, regulate rumen microbiota, enhance antioxidant capacity and improve daily weight gain. Xu et al. examined the effects of tea polyphenols on growth performance, cytokine content, intestinal antioxidant status and intestinal barrier function of weaned lambs. The study demonstrated that 4–6 g/kg tea polyphenols (purity = 98.1%) could enhance the immunity and antioxidant capacity of lambs, improve the intestinal barrier function, reduce intestinal damage and protect intestinal health. Moreover, the dietary addition of tea polyphenols had anti-inflammatory and antioxidant effects similar to those of chlortetracycline. Ma X. et al. provide valuable scientific insights for the rational application of *Salvia sclarea* extract in lamb production by adding different levels of *Salvia sclarea* extract to the growth performance, serum immunity, and antioxidant indices of lambs. The addition of 0.12 ml/kg *Salvia sclarea* extract (essential oil, purity = 85%) to the diet improved the growth performance of lambs by increasing feed intake and nutrient digestibility. It also improved the health status of lambs by increasing their serum antioxidant capacity and immune function. Indeed, plant extracts offer the advantage of exerting their bioactive effects in small quantities, and their degradation in the rumen should be taken into account when used on adult ruminants.

Alkaline Mineral Complex is a complex mixture of alkaline ions that helps maintain the acid-base balance of rumen fluid in ruminants. Liu et al. investigated the effects of different concentrations of AMC on fermentation characteristics and bacterial composition *in vitro*. The addition of 2 ml/kg AMC to the substrate stabilized the rumen environment by increasing the fatty acids concentration in fermentation fluid, without altering the rumen microbiota. Ulteriorly, Guo et al. demonstrated that supplementation with AMC water could improve immune function and antioxidant capacity of calves, and reduce diarrhea.

This Research Topic of articles describe a variety of nutritional strategies to alleviate animal stress, with plant bioactive substances accounting for the majority of the research. In most of these papers, rumen function and health were positively affected by the dietary treatments, resulting in an improvement of ruminant health. These findings provide theoretical support for the development of nutritional regulation of stress resistance in ruminants.

Author contributions

XD: Supervision, Writing – original draft, Writing – review & editing. JC: Validation, Writing – original draft, Writing – review & editing. ZZ: Supervision, Validation, Writing – review & editing. RH: Supervision, Validation, Writing – review & editing.

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Conflict of interest

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Impact of anthocyanins derived from *Dioscorea alata L.* on growth performance, carcass characteristics, antioxidant capacity, and immune function of Hainan black goats

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Dioscorea alata L. anthocyanins (DAC) are natural compounds found in plants and have shown potential health benefits. The objective of this investigation was to assess the impact of anthocyanins sourced from *Dioscorea alata L.* on the growth, carcass traits, antioxidant potential, and immune response of Hainan black goats. In this study, 30 three-month-old Hainan black goats (with a weight of 11.30±0.82kg) were selected and randomly divided into two groups, with 15 goats in each group. During the 60-day experiment, the control group (CON) and the treatment group (DAC) were, respectively, supplemented with 0 and 40mg/kg BW of DAC in the basal diet. The results showed that DAC had no significant impact on the growth performance and body characteristics of Hainan black goats (p >0.05). However, in terms of meat quality, the addition of DAC significantly increased the pH value and cooking yield 24h post-slaughter (p <0.05), while reducing the shear force of the meat (p <0.05). Compared to the control group, adding DAC to the feed resulted in a significant increase in the total antioxidant capacity (T-AOC) and superoxide dismutase (T-SOD) concentrations in plasma after 30days of feeding (p <0.05). After 60days of feeding, the concentrations of T-AOC, T-SOD, glutathione peroxidase (GSH-Px), and catalase (CAT) in the plasma of the DAC group was higher than that of the control group (p <0.05), while the concentration of malondialdehyde (MDA) was lower than that of the control group (p <0.05). In addition, supplementing DAC significantly increased the content of interleukin-10 (IL-10) and immunoglobulin M (IgM) in the plasma of Hainan black goats after 30days of feeding (p <0.05), while reducing the content of interleukin-6 (IL-6) (p <0.05). After 60days of feeding, the immunoglobulin G (IgG) and IL-10 content in the plasma of the DAC group was significantly increased (p <0.05), while the concentrations of IL-1β, IL-6, and tumor necrosis factor-α (TNF-α) were suppressed (p <0.05). In summary, these results indicate that supplementing DAC can improve the meat quality, enhance the antioxidant capacity, and immune function of Hainan black goats.

KEYWORDS

***Dioscorea alata L.*, antioxidant capacity, immune function, meat quality, Hainan black goats**

1 Introduction

In ruminant production, various factors such as diet, environment, stress, and the psychological and physiological state of animals can influence the occurrence of oxidative stress in the body (1, 2). Failure to effectively and safely eliminate reactive oxygen species (ROS) can directly or indirectly impair the overall health of ruminant animals, resulting in adverse effects on growth, reproduction, and immune function (3). Oxidative stress is also a significant factor contributing to aging, inflammation, and even diseases. Additionally, meat products are prone to lipid oxidation, resulting in color changes and nutrient loss, which lead to a decline in meat quality (4). Anthocyanins, as natural dietary antioxidants, offer defense against the detrimental effects of oxidative stress (5). Extensive scientific evidence also confirms their diverse range of functions, including anti-inflammatory, immune-regulatory, anti-cancer, and antioxidant properties (6). Furthermore, anthocyanins can prevent the oxidation of milk and meat (7). Previous studies have demonstrated the favorable effects of incorporating anthocyanins as feed additives in animal production, benefiting both animal productivity and health (8). According to previous studies, the consumption of anthocyanin-enriched sugarcane silage has been found to enhance plasma's total antioxidant capacity (TAC) while also increasing the activity of superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GSH-Px) (9). Additionally, previous studies have shown that composite extracts containing high levels of polyphenols can enhance growth performance and immunity in growing buffalo, while also reducing excretion of methane, nitrogen, and phosphorus (10).

Dioscorea alata L., a yam species with a global presence, is commonly known as the "greater yam" (11) due to its widespread popularity. It is rich in carbohydrates, proteins, fats, fibers, vitamins, minerals, and other components (12) In addition, *Dioscorea alata L.* is one of the important natural sources of anthocyanins, which have antioxidant properties (13). Due to its location in the tropical region, Hainan Island offers favorable climatic conditions for the growth of *Dioscorea alata L.* The extensive cultivation area and high yield contribute to the advantageous extraction of anthocyanins. Previous research has demonstrated the numerous health advantages of dietary supplementation with *Dioscorea alata L.* anthocyanins (DAC). These benefits include improving intestinal damage in mice afflicted with inflammatory bowel disease (14), as well as diminishing oxidative stress in mice (15). Furthermore, DAC effectively mitigates cellular oxidative stress by impeding the phosphorylation of IκB and p65 proteins (16).

To date, there have been limited reports on the application of *Dioscorea alata L.* anthocyanin (DAC) in ruminant animals. Based on our previous investigation, the incorporation of *Dioscorea alata L.* in the diet by replacing 30% of maize revealed significant potential in enhancing the antioxidant capacity of pregnant goats and newborns (17). This finding serves as a crucial foundation for substantiating the viability of this study. The objective of this study was to assess the impact of dietary DAC on the growth performance, carcass characteristics, antioxidant capacity, and immune function of Hainan Black goats. This study holds significant importance in understanding the nutritional value of *Dioscorea alata L.* anthocyanins and their potential applications in animal husbandry. The findings from this research may offer a novel approach for the feeding and improvement of Hainan Black goats, and could potentially provide insights for other livestock industries.

2 Materials and methods

2.1 Ethics statement

The Ethics Committee of Hainan University (Haikou, China) provided ethical approval for the animal study, under the license number HNUAUCC-2021-00082.

2.2 Relative composition of *Dioscorea alata L.* anthocyanins (DAC)

The required *Dioscorea alata L.* for this experiment were provided by the *Dioscorea alata L.* Base at Hainan University, while *Dioscorea alata L.* extract was prepared by Shaanxi Zhongwei Biological Engineering Co., Ltd. The extraction method for DAC was described in detail by Qiu et al. (15). In brief, fresh *Dioscorea alata L.* were extracted using ethanol to form a solution, which was then filtered, concentrated, and spray-dried to obtain DAC. Table 1 presents the abundance of individual anthocyanins, which were identified using a combined LC–MS technique (18, 19), revealing their relative concentrations.

2.3 Animals, experimental design, and diets

The study involved the selection of thirty 3-month-old castrated male Hainan black goats, with an average weight of 11.30 ± 0.82 kg. A completely randomized block design was used to divide the goats into two groups, each comprising fifteen goats. The goats were divided into two groups: the control (CON) group, which received a basal diet, and the treatment (DAC) group, which received the basal diet supplemented with 40 mg/kg body weight (BW) of *Dioscorea alata L.* anthocyanins. Table 2 displays the composition and nutritional content of the basal diet. The entire experimental period was conducted over 67 days, which included a pre-trial phase lasting 7 days followed by a 60-day trial period. Each animal was housed individually in separate enclosures. During the adaptation period, the lambs were dewormed, ear-tagged, and vaccinated.

To ensure accurate intake of DAC, a small quantity of pelletized feed was pulverized and thoroughly blended with DAC before being individually fed to each goat. Subsequently, the goats were provided with a combination of concentrate feed and king grass. Fresh king grass and concentrated feed were given twice daily, approximately at 8:00 am and 6:00 pm. Water and trace mineral salt blocks were provided *ad libitum* for consumption. The feed intake was periodically adjusted every 4 to 5 days, ensuring a residual feed of 5% to 10% remained. Measurements for dry matter intake (DMI) and feed residue were conducted on a daily basis, while weekly records of body weight were documented prior to each feeding. The calculation of average daily gain (ADG) involves dividing the weight gain by the specific experimental period for analysis. The determination of the

TABLE 1 Anthocyanin profifile of *Dioscorea alata L.* (DAC).

Compound	Rt (min)	MSn (*m*/*z*)	Metabolite name	Relative composition (%)[(a)]
1	9.383	935.24329	Cyanidin3-O-(6‴-caffeoylsophoroside)-5-O-glucoside	68.4408%
2	10.69	787.20471	Petunidin3-O-(6″-p-coumaroylglucoside)-5-O-glucoside	7.4824%
3	7	893.23419	Cyanidin3-O-(6″-p-hydroxybenzoylsophoroside)-5-O-glucoside	5.0421%
4	10.519	303.04956	Delphinidin	4.1572%
5	9.558	963.27875	Peonidin3-O-(6‴-feruloylsophoroside)-5-O-glucoside	4.0333%
6	10.578	1081.28479	Cyanidin 3-O-(6″-caffeoyl-6‴-p-coumarylsophoroside)-5-O-glucoside	3.6506%
7	9.72	773.19092	Delphinidin3-O-(6″-p-coumaroylglucoside)-7-O-glucoside	1.5194%

MSn (*m*/*z*): MS fragmentation model; Rt (min): compound retention time (a) relative composition of anthocyanins calculated from peak areas recorded at 520 nm.

TABLE 2 Composition and nutritional level of the diets of Hainan black goats

Item	Concentrate	King grass
Raw materials %		
Corn	72.50	—
Soybean meal	10.00	—
DDGs	8.20	—
Shell powder	2.00	—
Baking soda	1.00	—
$CaHPO_4$	0.40	—
$Caco_3$	0.40	—
Salt	0.50	—
Premix[1]	5.00	—
Total	100	—
Chemical composition[2]		
DM %	90.53	16.06
DE (MJ/kg)	12.97	—
CP %	12.48	6.40
EE %	3.63	2.17
Ash %	11.77	11.50
NDF %	59.88	68.87
ADF %	23.04	36.15
Ca %	0.53	0.23
P %	0.37	0.27

5% premix[1]: Vitamin A, 200,000 IU/kg; Vitamin D3, 60,000 IU/kg; Vitamin E, 550 IU/kg; D-Biotin, 0.3 mg/kg; Niacin, 350 mg/kg; Iron, 15 g/kg; Copper, 0.5 g/kg; Manganese, 2.4 g/kg; Zinc, 3 g/kg; Iodine, 20 mg/kg; Cobalt, Selenium, 10 mg/kg. All[2] values, except for digestible energy, were measured experimentally.

feed conversion ratio (FCR) was conducted in a professional manner by dividing DMI by ADG.

2.4 Sample collection and processing

On both day 30 and day 60 of the trial period, blood samples were collected from both experimental groups. Prior to the morning feeding, a 10 mL blood collection was conducted from the jugular vein of each goat and transferred into a vacuum blood collection tube. The tubes were then promptly placed in a refrigerated centrifuge to facilitate plasma separation. The obtained plasma, used for further analysis of antioxidant and immune indicators, was stored at a temperature of −20°C in a freezer.

At the end of the feeding trial, a total of five lambs from each group, selected based on closely matching body weights (BW) with the group's average BW, were chosen for slaughter to evaluate both carcass characteristics and meat quality. To determine the slaughter yield percentage, the goats' final body weight (FBW) was recorded before slaughter. On the day of slaughter, the hair, organs, head, forelimb knee joints, and hindlimb toe joints were removed, and the carcass weight was recorded. Fresh meat samples (30 g) from the longissimus dorsi were collected from each lamb within 1 h after slaughter and placed in self-sealing bags. The meat samples were stored in the bags at a temperature of 4°C for further analysis of meat quality following standard protocols.

Following that, the carcasses were subjected to a 24-h chilling period at a temperature of 4°C. Subsequently, the half carcass located on the left side was then transferred to the meat quality laboratory. In order to evaluate the attributes of carcass quality, dissection was performed between the 12th and 13th ribs, which involved measuring the fat thickness at the 12th rib and the area of the longissimus dorsi (LM).

2.5 Analysis of carcass characteristics and meat quality

To assess the cross-sectional area of the latissimus dorsi (LD), we followed the evaluation procedure outlined by Wang et al. (20). Sulfur label paper was used to outline the cross-section in the intercostal region between the 12th and 13th ribs. The backfat thickness was assessed through caliper measurements, which were subsequently adjusted by considering the average backfat thickness between the 12th and 13th ribs. Muscle pH value was determined using a 1 cm-length glass electrode pH meter (HI99161, Hanna Instruments, Italy). The pH measurements were performed over a duration of 45 min and 24 h, and the mean of three readings was documented. Meat color attributes were assessed at three distinct sites using a spectrophotometer (Konica Minolta CR-400, Konica Minolta Camera Inc., Japan). To measure drip loss, the LD sample was suspended parallel to the orientation of the muscle fibers and placed in a sealed environment at 4°C for a period of 24 h. Following the removal of the sample from the bag, it was delicately wiped and subsequently reweighed. Cooking yield was measured by weighing trimmed LD (20–30 g), heating it in an 85°C water bath for 40 min,

absorbing surface moisture with filter paper, and weighing it after cooling to constant temperature. Cooking yield was calculated by determining the proportion of the weight after cooking to the weight before cooking, expressed as a percentage. Shear force was evaluated according to the method of Destefanis (21).

2.6 Nutritional quality analysis of meat

The AOAC method was employed to analyze the routine nutrient quality components of the meat (22). The moisture content of Longissimus dorsi (LD) samples obtained from Hainan black goats was evaluated using a thorough drying process in an oven maintained at a constant temperature of 105°C. The calculation of crude protein content involved the application of the Kjeldahl method, specifically for nitrogen determination. The determination of EE content was conducted using the Soxhlet extraction method, following a similar protocol. The quantification of total ash levels was achieved through calcination of the sample in a crucible at a controlled temperature of 550 ± 25°C for 4 h, followed by calculation of the remaining inorganic matter.

2.7 Analysis of plasma antioxidant capacity and immune function

The quantification of SOD, GSH-Px, CAT, T-AOC, and MDA levels in plasma samples was performed using commercially available test kits from (Jiancheng Bioengineering Institute, Nanjing, China). The analysis was conducted in accordance with the provided guidelines.

Quantification of IgA, IgM, IgG, IL-1β, IL-6, IL-10, and TNF-α in plasma was performed using ELISA kits obtained from (Jiangsu Meibiao Biotechnology Co., Ltd. in Yancheng, China). All measurements were conducted in strict adherence to the recommended protocols.

2.8 Statistical analysis

The quantitative data acquired in this study were analyzed using SPSS software (Version 17.0, SPSS Inc., Chicago, United States). An independent-samples t-test was performed to assess significant differences in average values among the treatments. The outcomes consisted of mean values, corresponding p-values, and the standard error of the mean (SEM). GraphPad Prism 8.0 software (GraphPad Software Inc., La Jolla, United States) was employed to present the statistical findings visually. In this study, statistical significance was defined as $p < 0.05$, while high statistical significance was considered as $p < 0.01$.

3 Results

3.1 Growth performance

The DAC group did not exhibit any statistically significant effects ($p > 0.05$, Table 3) on the final weight, ADG, DMI, and FCR of Hainan black goats at different stages, when compared to the CON group.

TABLE 3 Effect of dietary supplementation of DAC on growth performance of Hainan black goat.

Items	Treatments		SEM	p-value
	CON	DAC		
0-30d				
IBW	11.29	11.32	0.20	0.94
BW	13.3	12.98	0.31	0.61
DMI[1] (g/d)	414.42	405.28	5.31	0.45
DMI[2] (g/d)	112.24	102.43	12.83	0.75
ADG (g/d)	66.76	57.22	4.88	0.39
FCR	7.89	8.37	0.65	0.62
31-60d				
FBW	15.43	15.46	0.47	0.97
DMI[1] (g/d)	528.51	539.19	27.55	0.87
DMI[2] (g/d)	109.45	112.32	8.71	0.89
ADG (g/d)	78.89	84.26	10.91	0.84
FCR	8.04	7.72	0.53	0.87
0-60d				
DMI[1] (g/d)	471.47	472.23	13.19	0.98
DMI[2] (g/d)	110.85	107.34	8.72	0.87
ADG (g/d)	69.12	70.74	3.23	0.84
FCR	8.42	8.20	0.13	0.45

SEM = standard error; DMI[1] = (concentrated feed) dry matter intake; DMI[2] = (King grass) dry matter intake; IBW = initial body weight; FBW = final body weight; ADG = average daily gain; FCR= feed conversion ratio.

3.2 Carcass characteristics and meat quality

No statistically significant differences ($p > 0.05$, Table 4) were observed between the various treatments in terms of backfat thickness, carcass weight, slaughter yield, and the longest muscle area of the back. No significant differences were found between the two groups in terms of the L*, a*, and b* values in the LM ($p > 0.05$, Table 5). The addition of DAC to the diet significantly improved the pH value and cooking yield of the LM after 24 h, as compared to the CON group ($p < 0.05$, Table 5). Furthermore, the DAC group showed a noteworthy reduction in shear force of the LM compared to the CON group ($p < 0.05$, Table 5).

3.3 Nutritional quality of meat

No significant disparities were found in the moisture and ash content of the longissimus muscle (LM) between the Hainan black goats in the DAC and CON groups ($p > 0.05$, Table 6). However, it should be noted that the fat and protein content of the DAC group was slightly higher compared to the CON group, although the difference was not statistically significant ($p > 0.05$, as shown in Table 6).

3.4 Plasma antioxidant index

Figure 1 illustrates the impact of DAC dietary supplementation on antioxidant indicators within the plasma of Hainan black goats. After

TABLE 4 Effect of dietary supplementation of DAC on carcass characteristics of Hainan black goat

Items	Treatments		SEM	*p*-value
	CON	DAC		
Carcass weight (kg)	7.67	7.52	0.14	0.62
Dressing percentage %	46.62	47.75	0.98	0.59
Back fat thickness (mm)	6.86	6.47	0.72	0.81
LM area (cm^2)	12.43	13.07	1.04	0.77

SEM = standard error; LM = longissimus dorsi muscle.

TABLE 5 Effect of dietary supplementation of DAC on meat quality of Hainan black goat.

Items	Treatments		SEM	*p*-value
	CON	DAC		
L*	36.68	36.64	0.28	0.95
a*	13.64	14.21	0.19	0.13
b*	8.68	8.58	0.23	0.84
PH_{45min}	6.84	6.78	0.04	0.49
PH_{24h}	6.08^a	6.21^b	0.03	0.04
Drip loss %	3.96	3.48	0.29	0.06
Cooking yield %	55.71^a	58.49^b	0.69	0.04
Shear force N	85.86^a	76.02^b	2.22	0.02

SEM = standard error; color measurements: L* = lightness; a* = red; b* = yellow; a, b Means followed by different superscripts are significantly different ($p < 0.05$).

30 days, the addition of DAC to the diet led to a noteworthy enhancement in both plasma T-AOC and T-SOD concentrations, with statistical significance ($p < 0.05$). After 60 days of incorporating DAC into the diet, the group supplemented with DAC displayed substantial improvements in plasma T-AOC, T-SOD, and GSH-Px concentrations compared to the CON group, with statistical significance ($p < 0.05$). Moreover, the DAC group also experienced a noteworthy elevation in plasma CAT concentration ($p < 0.01$). Additionally, there was a significant decrease in MDA content observed in the DAC group when compared to the CON group, with statistical significance ($p < 0.01$).

3.5 Plasma immune parameters

The immune parameters among different treatment groups are presented in Figure 2. After a 30-day intervention of DAC dietary supplementation, the DAC group demonstrated a significant decrease in IL-6 levels in comparison to the CON group, with statistical significance ($p < 0.05$). Additionally, DAC dietary supplementation led to noteworthy increases in plasma IL-10 and IgM levels ($p < 0.05$). In a comprehensive 60-day experimental trial on Hainan black goats, the incorporation of DAC into their diet resulted in a remarkable reduction in plasma levels of IL-1β ($p < 0.01$), IL-6 ($p < 0.01$), and TNF-α ($p < 0.05$). Additionally, the dietary supplementation of DAC in Hainan black goats significantly elevated plasma levels of IgG ($p < 0.05$) and IL-10 ($p < 0.01$).

TABLE 6 Effect of dietary supplementation of DAC on the nutritional quality of the longissimus dorsi muscle of Hainan black goat.

Items	Treatments		SEM	*p*-value
	CON	DAC		
Moisture (%)	75.60	75.48	0.18	0.75
Protein (%)	22.81	22.89	0.15	0.80
Fat (%)	2.37	2.60	0.15	0.45
Ash (%)	1.16	1.03	0.02	0.31

SEM = standard error.

4 Discussion

The growth performance of animals is easily influenced by factors such as breed, feed composition, and environmental conditions (23–25). Anthocyanins, belonging to the flavonoid group, are commonly applied to animal production as feed additives. Plant-derived flavonoids, which are categorized as secondary metabolites, have been previously shown to play a vital role in the growth of ruminant animals (26). However, the reports on the effects of anthocyanin-rich black sugarcane and red corn on goat growth performance and carcass characteristics did not show any significant changes (9, 27). Consistent with previous research findings, the inclusion of DAC in the diet did not have a significant impact on the growth performance and carcass characteristics of Hainan black goats in this study. Nevertheless, other studies have reported favorable outcomes (28). The potential factors contributing to the observed differences in outcomes may include variations in the formulation of anthocyanin extract, slaughter age, and differences in the bioavailability of phenolic compounds in ruminant and monogastric animals (29). Based on the findings of this study, it can be deduced that the inclusion of DAC in the animal diet does not adversely impact animal performance. These findings can provide valuable insights for future applications in goat production.

Meat quality can be effectively assessed by considering key indicators such as pH, cooking yield, and shear force measurements. The degree of decrease in muscle pH may be associated with the glycogen content in skeletal muscles (30). Glycogen is converted to lactic acid through anaerobic or glycolytic pathways, resulting in the production of H+ and subsequently decreasing muscle pH (31). However, the concentration of glycogen is influenced by various factors, including pre-slaughter stress (32). Jiao et al. reported that the addition of grape seeds rich in anthocyanins to sweet sorghum could increase the pH value of lamb meat 24 h after slaughter (33). In this study, it was found that the addition of DAC to the diet can increase the pH value of Hainan Black Goat muscles 24 h after slaughter. The observed phenomenon is likely attributed to the relief of pre-slaughter stress in lambs, which subsequently affects muscle glycogen storage and inhibits muscle glycolysis. Previous research has reported that meat with higher cooking yields tends to lose less moisture during the heating process (34). Our research findings demonstrate that the DAC group exhibits higher cooking yield, indicating the potential of anthocyanins in enhancing muscle water-holding capacity and juiciness. Shear force serves as the most direct and indicative measure

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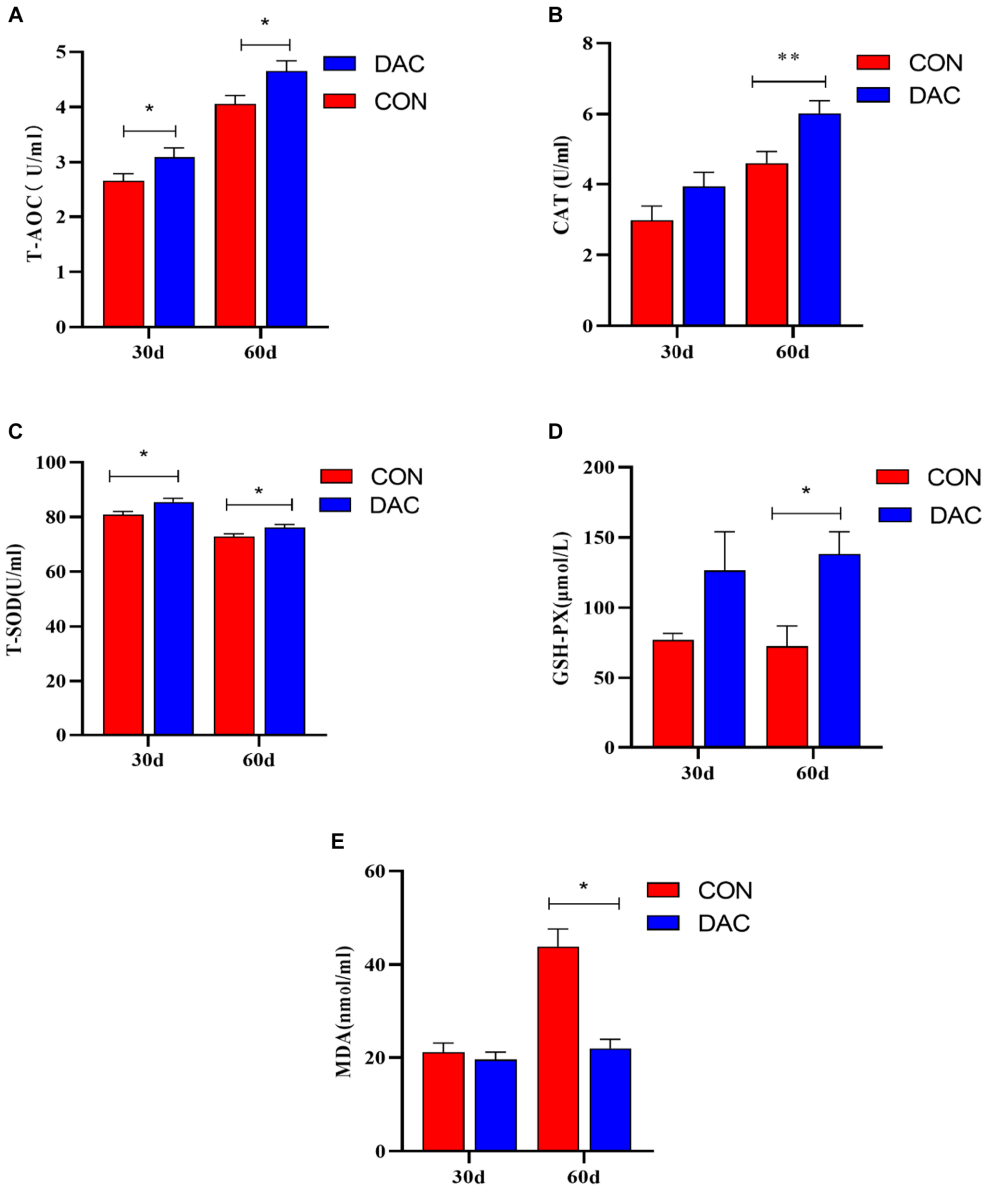

FIGURE 1
Effect of dietary supplementation with DAC on plasma antioxidant indexes in Hainan black goats. The primary indicators reflecting antioxidant capacity include: **(A)** T-AOC = total antioxidant capacity; **(B)** CAT = catalase; **(C)** T-SOD = total superoxide dismutase; **(D)** GSH-Px = glutathione peroxidase; and **(E)** MDA = malondialdehyde; results are expressed as means ± SEM. * indicated significant difference ($p < 0.05$), and ** indicated extremely significant difference ($p < 0.01$).

of meat tenderness (35). Conversely, shear force is influenced by factors such as collagen content, characteristics of collagen proteins, and muscle fiber structure in connective tissues (36). Moreover, the tenderness of meat is also associated with the speed of glycolysis and the ultimate pH level (37). Scientific evidence supports the claim that polyphenols can enhance tenderness in meat by suppressing the expression of fiber-related genes involved in glycolysis, thereby reducing shear force (38). In this study, the incorporation of DAC in the diet resulted in a reduction in shear force measurement. Furthermore, in combination with our pH value results, this suggests that DAC may influence glycolysis, thereby impacting muscle fiber structure, leading to a decrease in shear force and an improvement in tenderness. Similar results have been observed in ruminant animals with the dietary inclusion of plant extracts rich in anthocyanins (39, 40).

Meat consists of water, proteins, lipids, minerals, and a small amount of carbohydrates. No significant variations were detected in the nutritional composition across the groups investigated, suggesting that the inclusion of DAC did not impact the regulation of nutritional components in goats. Our findings are consistent with the results reported by Salzano et al. (41), which demonstrated that feeding goats with anthocyanin-rich extracts from red oranges and lemons did not result in any differences in the general nutrition facts of the meat. The limited absorption of anthocyanins in animals may explain this phenomenon, although further research is necessary to confirm this hypothesis.

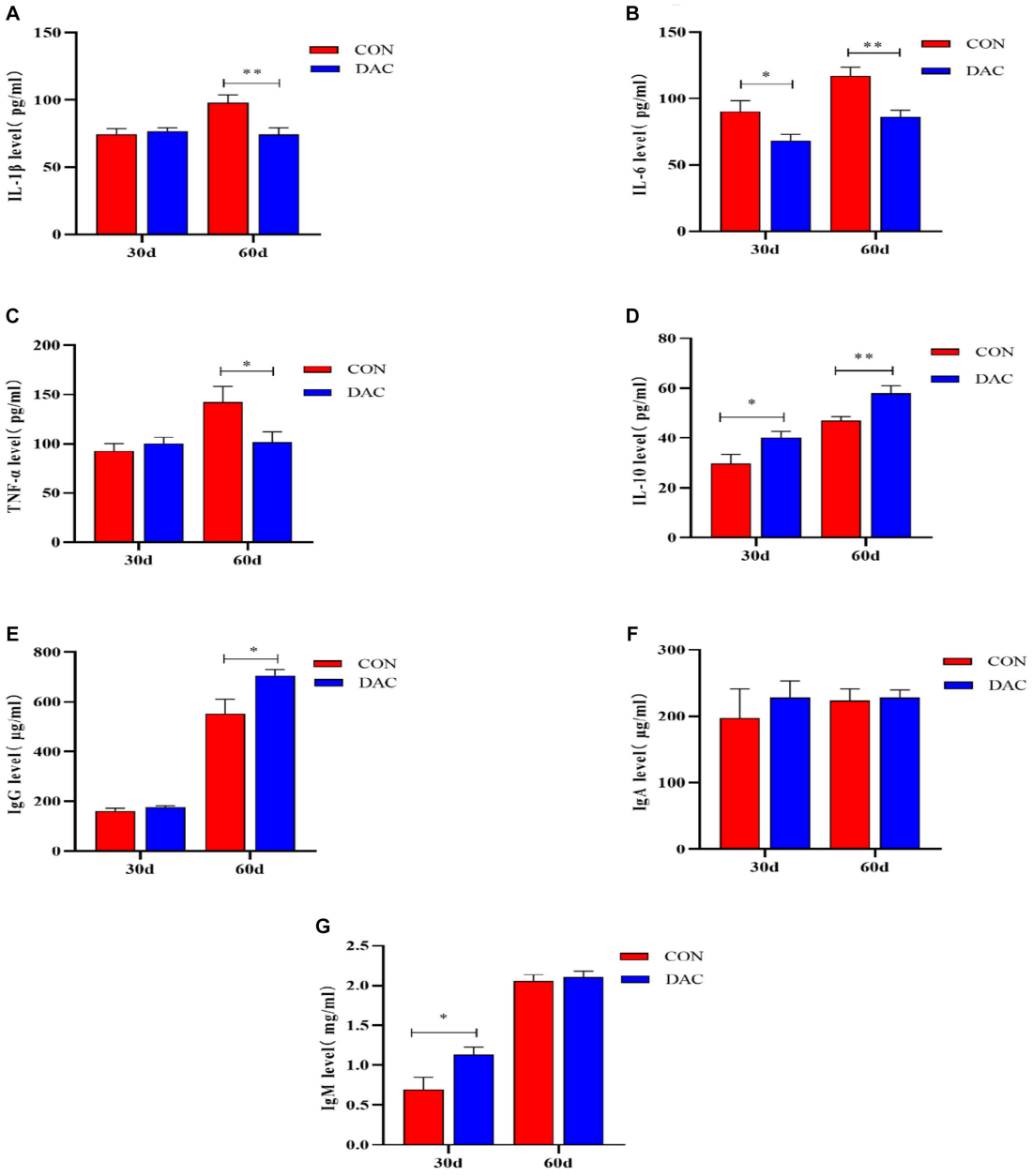

FIGURE 2
Effect of dietary supplementation with DAC on Plasma Immune Parameters in Hainan black goats. The primary indicators reflecting immune function include: **(A)** IL-1β = Interleukin-1β; **(B)** IL-6 = Interleukin-6; **(C)** TNF-α = tumor necrosis factor-α; **(D)** IL-10 = Interleukin-10; **(E)** IgG = Immunoglobulin G; **(F)** IgA = Immunoglobulin A; and **(G)** IgM = Immunoglobulin M; Results are expressed as means ± SEM. * indicated significant difference ($p < 0.05$), and ** indicated extremely significant difference ($p < 0.01$).

The proper functioning of the body and its organs depends on the presence of free radicals (FR), ROS, and reactive nitrogen species (RNS), as well as a well-balanced correlation between these free radicals and the antioxidant system in healthy animals (42). Under normal circumstances, the body can improve animal health by eliminating excessive free radicals through endogenous antioxidant enzymes such as SOD, GPX, and CAT (43). However, the body may occasionally experience oxidative stress (OS) due to an imbalance in this state. It is typically necessary to supplement natural antioxidants in livestock diets to alleviate oxidative stress in animals. Anthocyanins, as natural antioxidants, possess the ability to inhibit or prevent compound oxidation by efficiently scavenging FR and reducing OS (44). It has been confirmed that anthocyanins are absorbed into the bloodstream upon consumption (45). In our study, feeding with DAC for 30 and 60 days significantly increased the activity of SOD and T-AOC in plasma. This outcome can possibly be attributed to the absorption of anthocyanins into the bloodstream, which alleviates OS due to the hydrogenating (electron-donating) ability of flavonoid molecules. Furthermore, feeding with DAC for 60 days resulted in higher activity of CAT and GPX, as well as significantly lower activity of malondialdehyde (MDA). Our findings corroborate with the results obtained by Taethaisong et al. (46) who fed goats with diets containing

purple taro leaves rich in anthocyanins. They observed an increase in plasma levels of T-AOC, SOD, GPX, and CAT, along with a significant decrease in MDA concentration. It has been reported that the expression levels of SOD, GPX, and CAT are primarily regulated by nuclear factor-E2-related factor 2 (Nrf2), which is a key factor in protecting against oxidative stress (47). In addition, the consumption of silage feed made from purple corn stalks rich in anthocyanins was found to increase the levels of SOD in plasma and the expression levels of SOD2, GPX1, and GPX2 mRNA in the mammary glands (48). Therefore, it is hypothesized that dietary supplementation of DAC to increase antioxidant enzyme activity may be associated with the enhancement of the body's antioxidant capacity through the modulation of the Nrf2 signaling pathway by anthocyanins (49). However, further research is needed to elucidate the exact reasons and mechanisms underlying this hypothesis.

The immune function of ruminant animals is crucial for maintaining their overall health status. Immunoglobulins play a significant role in host-mediated humoral immunity, where IgG activates the complement system and resists the invasion of various bacteria and toxins (50, 51). IgM binds to complement and dissolves pathogens (52). Current research indicates that the DAC group exhibited increased concentrations of IgM and IgG in the plasma at 30 days and 60 days, respectively. A study reported that supplementing the diet with hibiscus anthocyanins enhanced the immunity level of IgG in the spleens of chickens (53). The enhanced immune globulin (IgM and IgG) activities demonstrated by the DAC group may be attributed to the antioxidant and antibacterial capacities of the extract, primarily driven by its flavonoid content (mainly anthocyanins) (54, 55). Oxidative stress can induce the activation of nuclear factor-kappa B (NF-κB) (56), which in turn triggers inflammatory responses (57). The activation of NF-κB has been shown to promote gene expression and the synthesis of various pro-inflammatory cytokines (58). Cytokines serve as crucial indicators of the inflammatory status within organisms, which can be classified into anti-inflammatory interleukin-10 (IL-10) and pro-inflammatory cytokines including interleukin-1β (IL-1β), interleukin-6 (IL-6), and tumor necrosis factor-α (TNF-α), among others (59). Based on a study, it has been verified that anthocyanin supplements efficiently inhibit the transactivation of NF-κB, which leads to a reduction in the plasma concentrations of pro-inflammatory chemokines, cytokines, and mediators (60). Our research findings demonstrate that dietary supplementation with DAC can reduce the levels of pro-inflammatory cytokines (IL-1β, IL-6, TNF-α) in plasma, while promoting an increase in the concentration of anti-inflammatory cytokine IL-10. This effect becomes more pronounced after feeding with DAC for 60 days. The supplementary inclusion of Dioscorea in the diet of goats exhibited a similar trend to our findings in terms of plasma pro-inflammatory factors (61). The study findings suggest that DAC supplementation in the diet of weaned Hainan black goat lambs effectively mitigates serum inflammatory response. This process involves the stimulation of anti-inflammatory factors and suppression of pro-inflammatory factors, thereby boosting their immune function.

5 Conclusion

Based on the present results, it can be concluded that the inclusion of DAC in the diet does not affect the growth performance and carcass characteristics of Hainan black goats. However, it does improve meat quality to some extent. The addition of 40 mg/kg BW of DAC to the diet leads to an improved immune response and antioxidant capacity through increased levels of IgM, IgG, IL-10, GSH-Px, SOD, CAT, and T-AOC. The potential of *Dioscorea alata L.* anthocyanins in improving the meat quality, antioxidant capacity, and immune function of Hainan black goats signifies their promising role as feed additives in goat production systems. Further research is needed to elucidate the mechanisms of action and determine the ideal dosage and duration of supplementation with anthocyanins derived from *Dioscorea alata L.*

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 the Ethics Committee of Hainan University (Haikou, China). The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

HF: Conceptualization, Methodology, Writing – original draft. HS: Conceptualization, Funding acquisition, Methodology, Visualization, Writing – review & editing. FY: Formal analysis, Investigation, Validation, Writing – review & editing. YY: Data curation, Project administration, Software, Writing – review & editing. XW: Conceptualization, Methodology, Resources, Supervision, Writing – review & editing.

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Conflict of interest

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

Publisher's note

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Intramuscular vitamin A injection in newborn lambs enhances antioxidant capacity and improves meat quality

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Introduction: Vitamin A (VA) and its metabolite, retinoic acid (RA) possess several biological functions. This report investigated whether neonatal intramuscular VA injection affected antioxidative activity and meat quality in longissimus dorsi (LD) muscle of lambs.

Methods: Lambs were injected with 0 (control) or 7,500 IU VA palmitate into the biceps femoris muscle on day 2 after birth. At 3, 12, and 32 weeks of age, blood samples were collected in the jugular vein for serum levels of RA and muscle samples were collected in the biceps femoris for analysis of relative mRNA expression of enzyme contributors to retinoid metabolism. All animals were harvested at 32 weeks of age and muscle samples were collected to explore the role of VA on the meat quality and antioxidant capacity of lambs.

Results and discussion: Our results indicated that VA increased the redness, crude protein, and crude fat ($p < 0.05$), without affecting moisture, ash, and amino acid composition in LD muscle ($p > 0.05$). In addition, VA increased catalase (CAT) activity and decreased malondialdehyde (MDA) levels in LD muscle ($p < 0.05$). Meanwhile, greater levels of CAT and NRF2 mRNA and protein contents with VA treatment were observed in LD muscle ($p < 0.05$), partly explained by the increased level of RA ($p < 0.05$). Collectively, our findings indicated that VA injection at birth could improve lamb meat quality by elevating the redness, crude protein, crude fat, and antioxidative capacity in LD muscle of lambs.

KEYWORDS

vitamin A, retinoic acid, antioxidant activity, meat quality, longissimus dorsi, lamb

Introduction

Meat and its related products are important sources of nutrition for human beings. Meat quality is important to ensure consumer satisfaction, which is affected by genetic factors, feeding methods, and the nutritional status of the animal (1). Traditionally, human sensory perception is used as a factor in evaluating meat quality, including appearance, color, flavor, texture (especially tenderness), juiciness/holding power, and odor. Moreover, freshness or health is also an important quality indicator that is related to the safety of meat for consumption (2). In recent years, demand for lamb meat has consistently increased in several regions of the world (3). Given the higher contents of in iron and zinc (3, 4), spoilage and color change are more easily to be happened in lamb meat (5). The oxidation of lipids and proteins is the main reason for the decrease in nutritional value and the deterioration of sensory and physicochemical properties (such as color, flavor, or tenderness) of meat (6, 7). Particularly, lambs kept in captivity for long

periods of time and fed a single variety of feed further exacerbates oxidative stress in the animals and ultimately leads to poor lamb meat quality (8). Therefore, improving the antioxidant capacity of lambs and meat quality has become a priority in meat sheep farming.

Numerous studies have investigated the biological functions of VA as a natural nutritional supplement, where antioxidant capacity (9), adipogenesis (10), myogenesis (11), mitochondrial function (12), and the regulation of muscle fiber type conversion (13) may be associated with improved meat quality. When VA enters the cell, it produces RA under the action of dehydrogenase. In the nucleus, RA binds to the heterodimer of retinoic acid receptor (RAR) and retinoid X receptor (RXR) to perform its biological function (14).

Indeed, VA as a natural antioxidant was reported as early as 1932, and their report indicated the antioxidant potential of VA and carotenoids, both of which protect lipids from rancidity (9). Retinol is a potent peroxy radical scavenger by inhibiting peroxidation in a homogeneous solution of methyl linoleate and in model phosphatidylcholine liposomes (15). Moreover, in previous study, we have shown that neonatal intramuscular VA injection effectively promoted the muscle growth of lambs (16). However, there are no relevant studies to clarify whether there is a potential relationship between VA and lamb meat quality. Traditionally, VA has been added to animal diets as a nutritional supplement (17). However, VA is not completely absorbed by livestock and does not perform its biological functions with this approach. In this experiment, we injected VA into the muscle to ensure that the muscle receives sufficient VA, and the effect is more direct. Thus, the objective of this research was to explore the effects of neonatal VA injection on lamb meat quality and antioxidant activity.

Materials and methods

Animal management

All experimental procedures were approved by the Institutional Animal Care and Use Committee of Shanxi Agricultural University (sxnd202028). Randomly selected 80 purebred Hu sheep with similar body states, all of them were on their third pregnancy. In order to avoid the influence of the sire, all ewes were artificially inseminated with semen from one Dorper ram after simultaneous estrus. Attention should be paid to operation and hygiene during insemination. After ewe pregnancy, every 3 pregnant ewes were put into one eweshed. Ewes were fed in the stalls where they were given clean water to drink, and were free to move around after feeding. The diet was formulated to consistent with National Research Council (18) requirements for the nutrition of ewes. During the experimental period, the feeding management and environment of ewes in each group were maintained at the same level and sterilized regularly to keep the eweshed dry and hygienic. At 35 d of gestation, fetal number was determined using an ultrasound monitor. For the follow-up study, only ewes carrying 2 fetuses were used. After birth, we selected twin lambs, both male, weighing 3.5 ± 0.5 kg, and distributed them to control and VA groups to ensure the same body condition of lambs ($n = 8$ in each group).

Based on previous studies (13, 19), we determined to inject 7,500 IU VA palmitate (product no. PHR1235, Sigma, Milwaukee, US) or an equivalent volume of corn oil (product no. c8267, Sigma, Milwaukee, US) into the biceps femoris muscle on day 2 after birth. The lambs were given weekly injections at a fixed point in time for 3 weeks, and paired with ewes for management. Lambs weaned at 12 weeks of age and fed a backgrounding diet for 55 days, followed by a finishing diet with free access to clean water and salt blocks. Moreover, grass hay (peanut seedlings) was added to promote lamb growth during the finishing period. The nutrient composition of concentrate feed and grass hay has been reported in another manuscript (16).

For peanut seedlings, the AOAC method (20) was used to determine DM content, and the Van Soest method was used to determine NDF and ADF contents (21). The Kjeldahl and Soxhlet extraction methods were used to measure the content of crude protein and crude fat, respectively (22). The content of total ash in samples was measured after 40 min of carbonization in a constant temperature crucible at 600°C. All animals were harvested at 32 weeks of age.

Serum and muscle biopsy collection and treatment

Blood samples of lambs were collected from the jugular vein in the 3rd, 12th, and 32nd week. The serum was separated and stored at −80°C to prevent the degradation of the components in the serum. After determining the detection method, RA in serum was detected by high performance liquid chromatography (HPLC) with a reversed-phase column. (Atlantis® dC18, 5 μm, 100 A, United States), and its content was determined from the standard curve of the standard sample (#B21287, Shanghai, China). The method was as follows: methanol was used as mobile phase with a flow rate of 1.0 mL/min, detection wavelength 325 nm, column temperature 35°C, injection 20 μL (23). Furthermore, the content of Hexokinase (HK) and Lactate dehydrogenase (LDH) in serum and the activities of total antioxidant capacity (T-AOC), total superoxide dismutase (T-SOD), catalase (CAT), glutathione peroxidase 4 (GPx4) and the content of malondialdehyde (MDA) in LD muscle were detected by kits. Information on the kits was given below: HK (#A077-3-1), LDH (#A020-2-2), T-AOC (#A015-2-1), catalase (#A007-1-1), Gpx4 (#H545-1-1), SOD (#A001-3-2), and MDA (#A003-1-2) were acquired from Nanjing Jiancheng Bioengineering Institute (Nanjing, Jiangsu, China).

Skeletal muscle samples were taken from lambs at 3 and 12 weeks of age using a biopsy needle. A small area of wool was removed from the biceps femoris muscle of the lamb's right hind leg with a shaver and wiped with iodophor. The lambs were fixed and the muscle was removed by inserting a biopsy needle into the biceps femoris and placed in liquid nitrogen for RNA extraction (q-RT-PCR).

Meat quality analysis

After harvest, the LD muscle of lambs was removed and analyzed for pH, meat color, conductivity, shear force, cooking loss, and drip loss. The pH was measured 45 min after the lamb stopped breathing (pH 45 min) and again 24 h after stopping breathing (pH 24 h) using a pH-STAT meter (SFK-Technology, Denmark). Flesh color (L*, a*, and b*) and conductivity were measured simultaneously with pH, using a carcass conductivity meter and a LAB flesh color meter (Matthäus, Klausa, Germany). For drip loss analysis, the muscle with

the fascia removed was trimmed into strips, weighed, and hung on hooks. The strips were placed in a 50 mL centrifuge tube, avoiding contact between the samples and the wall of the tube, and stored at 4°C for 48 h; the strips were removed, and the water on the surface of the strip was absorbed with filter paper and each strip was weighed. For the determination of shear force, the muscle sample was aged at 4°C for 72 h, then exposed to a thermostatic water bath at 80°C and heated until the central temperature of the muscle reaches 70°C. Muscle samples of 3 cm in length and 1 cm^2 in cross-section were cut from samples cooled to room temperature in the direction of the muscle fibers and shear force, expressed in "N," was measured using a shear gauge (Mecmesin, West Sussex, United Kingdom). Approximately 100 g of muscle was weighed for measurement of cook loss. The method was as follows: the sample was stripped of its outer membrane and attached fat, weighed and steamed in boiling water for 30 min. The cooked muscle samples were hung in a cool place for 30 min and weighed.

Meat nutrient analysis

Recognized AOAC methods (24) were used to analyze the moisture, crude protein, ether extract and total ash contents of LD muscle. Specifically, moisture content was calculated by baking constant weight samples at 105°C. The Kjeldahl and Soxhlet extraction methods were used to measure the contents of crude protein and ether extract, respectively, (22). The content of total ash in samples was measured after 40 min of carbonization in a constant temperature crucible at 600°C.

Muscle periodic acid–Schiff staining and glycogen analysis

The muscle tissue samples were fixed in 4% PFA for 2 days, dehydrated serially by gradient ethanol and xylene, then immersed in paraffin for 9 h, and embedded in paraffin. Then, a microtome (Leica, Germany) was used to slice the samples to a thickness of 5 μm and sections were sequentially dewaxed, rehydrated and stained in periodic acid–Schiff (PAS) staining solution (cat no. G1008, Servicebio, Wuhan, China), then rinsed in running water, dehydrated, transparent, sealed, and finally observed with a microscope (DMi8 microscope, Leica, Germany) for imaging acquisition and analysis (All muscle tissue samples were cut at intervals of 50 μm, with at least 3 replicates in each sample). To measure glycogen content, LD muscle samples were grinded and homogenized in 0.9% saline by a homogenizer, centrifuged (2,500 × g, 10 min, 4°C) and the supernatant was extracted to measure the glycogen concentration with a kit (#A043-1-1, Nanjing Jiancheng Bioengineering Institute, Jiangsu, China).

Muscle Masson staining and collagen analysis

Muscle samples were embedded in optimal cutting temperature (OCT) compound and sliced into 10 μm thickness sections with cryostat microtome (Leica, Germany). Frozen sections were sequentially dewaxed, rehydrated and stained in Masson staining solution (cat no. G1006, Servicebio, Wuhan, China), then rinsed, dehydrated, sealed, finally observed with a microscope (DMi8 microscope, Leica, Germany) for imaging acquisition and analysis (All muscle tissue samples were cut at intervals of 50 μm, with at least 3 replicates in each sample). Moreover, we analyzed the relative expression of type I, type III collagen fibrils and fibronectin.

Amino acid composition

Determination of amino acids contents in LD muscle of lambs using high performance liquid chromatography (HPLC). The method was as follows: mobile phase (A: CH_3COONa; B: CH_3OH, H_2O), flow rate 1.0 mL/min, detection wavelength 360 nm, column temperature 40°C, injection 20 μL. The amino acid content in the LD was determined from the standard curve based on the standard sample (AAS18-10ML, Sigma, Milwaukee, United States).

Quantitative real-time PCR

Total RNA was extracted from muscle tissue using Trizol reagent (Sigma, Saint Louis, MO) and synthesized into cDNA using a reverse transcription kit (TAKARA Co, Ltd., Dalian, China). The CFX RT-PCR detection system (Bio-Rad, Hercules, CA) and SYBR Green RT-PCR kit (TAKARA Co, Ltd.) were used for q-RT-PCR. The procedure was as follows: 95°C, 10 min; 45 2-step cycles of 95°C, 15 s, 60°C, 30 s, with at least 3 replicates per set. Primer sequences are shown in Table 1. The relative changes of gene expression were calculated by $2^{-\Delta\Delta Ct}$ method (8), and β-actin was used as housekeeping gene. Gene expression in the VA group showed a fold change compared to the control group.

Western blotting

The muscle samples were ground in liquid nitrogen and lysed with RIPA lysate (1% NaF, 1% Na_3VO_4, 1% PMSF, 2% β-mercaptoethanol, 0.1% protease inhibitor, 1 × loading buffer constant volume to 10 mL) for 30 min, then boiled at 100°C for 10 min, centrifuged at 12,000 × g for 8 min at 4°C and the supernatant removed as the isolated protein. Extracted proteins were separated by SDS-PAGE (room temperature, 80 V for 0.5 h, 120 V for 1.5 h) and then transferred to nitrocellulose membranes (4°C, 100 V for 2 h), blocked with 5% skimmed milk powder (Shanghai Sanger Biotechnology Co., Ltd., Shanghai, China) for 1 h. Finally, nitrocellulose membranes were incubated with the primary antibody (4°C, overnight) and the corresponding secondary antibody (room temperature, 1 h). The Odyssey infrared imaging system was used to visualize protein bands, and the band density was standardized to β-tubulin content.

Antibodies against SOD (no. sc-8637), GPx4 (no. sc-50497), and catalase (no. sc-34281) were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, United States). NRF2 (no. bs-1074R) and β-tubulin (bsm-33034 M) were from Biosynthesis Biotechnology Co., Ltd. (Beijing, China). A goat anti-rabbit secondary antibody (926–32,211) was from LI-COR Biosciences (Lincoln, NE)

TABLE 1 Primer sequences for real-time PCR.

Gene name	Sequence (5′ → 3′)	Product size, bp
ADH4	AGAAAATGGGCACCAAGGGA	80
	ATTCATTGCTGAGGGGCTTGT	
ALDH1A1	CGCAACCGAGGAGAAACTCT	200
	TCATAGCCTCCATTGTCGCC	
ALDH1A2	AGCTCTGTGCTGTGGCAATA	101
	GTGGAAAGCCAGCCTCCTTG	
ADH1C	TCGCTCTGGAAAGAGTGTCC	167
	TGGAAAGCTCCCATGTGCAA	
SOD	GGAGACCTGGGCAATGTGAA	182
	CCTCCAGCGTTTCCAGTCTT	
CAT	GAGCCCACCTGCAAAGTTCT	148
	CTCCTACTGGATTACCGGCG	
GPx4	TCGCTGCTGGCTATAACGTC	189
	GACCATACCGCTTCACCACA	
NRF2	TGTGGAGGAGTTCAACGAGC CGCCGCCATCTTGTTCTTG	88
Collagen I	GACATCCCACCAGTCACCTG	161
	GGGACTTTGGCGTTAGGACA	
Collagen III	AGGGCAGGGAACAACTGATG	145
	ACAGTGGGATGAAGCAGAGC	
Fibronectin	ACCCTGGGTATGACACTGGA	165
	CATTCGGCGGATACGGTCTT	
β-actin	CGGCTTTCGGTTGAGCTGAC	159
	GCCGTACCCACCAGAGTGAA	

and a donkey anti-goat secondary antibody (no. D110120) was purchased from Sangon Biotech Co., Ltd. (Shanghai, China).

Statistical analysis

Graphpad Prism 9 software (Monrovia, CA, United States) was used for statistical analysis. The lambs used in the experiment were not completely randomly assigned, so we used paired *t*-test to analyze the data. Results were shown as the Mean ± SEM. $p < 0.05$ was considered significant between control and VA groups.

Results

Retinoic acid content in serum and retinol metabolizing enzymes mRNA expression in longissimus dorsi muscle

As shown in Figure 1A, VA injection significantly elevated RA content in serum of lambs in the 3rd (Figure 1A, $p < 0.01$) and 12th (Figure 1A, $p < 0.05$) weeks. Consistently, the mRNA level of retinol metabolizing enzymes in VA group showed the same trend in LD muscle of lambs. Specifically, there was no difference in *ADH1C* between VA and control groups (Figure 1B, $p > 0.05$), while *ADH4* expression in VA group was higher than that of control group at the age of 3 weeks (Figure 1C, $p < 0.05$). Moreover, VA increased the level of *ALTH1A1* at the age of 3 (Figure 1D, $p < 0.01$) and 12 weeks (Figure 1D, $p < 0.05$), and VA increased the level of *ALTH1A2* at the age of 3, 12 weeks and harvest (Figure 1E, $p < 0.05$).

Meat quality and nutritional composition in longissimus dorsi muscle

There were no alterations in pH, meat color (L* and b* values), conductivity, shear force, cook loss, and drip loss in LD muscle between VA and control group of lambs (Table 2, $p > 0.05$). However, lambs in VA group exhibited a higher a^*_{24h} value than that in the control group (Table 2, $p < 0.05$). Moreover, VA injection had no effects on moisture and ash content of LD muscle (Table 3, $p > 0.05$), but raised the content of crude protein and crude fat (Table 3, $p < 0.05$).

Glycogen and collagen abundance in longissimus dorsi muscle and hexokinase, lactate dehydrogenase content in serum

As shown in Figure 2 and Table 4, VA did not affect the content of glycogen in LD muscle (Figure 2 and Table 4, $p > 0.05$) and the content of HK and LDH in serum (Table 4, $p > 0.05$) of lambs. For collagen content in LD muscle of lambs, there was also no difference between control and VA groups (Figures 3A,B, $p > 0.05$).

Amino acid composition in longissimus dorsi muscle

Compared with control group, VA injection had no effects on amino acid composition and total amino acid in LD muscle of lambs (Table 5, $p > 0.05$).

Antioxidant enzyme activities and malondialdehyde content

Compared with control group, VA increased the CAT activity and reduced the MDA content in LD muscle of lambs (Table 6, $p < 0.05$). Moreover, the data revealed that *CAT* and *NRF2* mRNA expression in LD muscle were higher in VA group, and protein abundance were also significantly elevated (Figures 4A–E, $p < 0.05$).

Discussion

VA has been widely used as a nutritional supplement in the animal husbandry. In the current study, we found that intramuscular injection of VA increased the content of RA in serum of lambs at 3 weeks of age and weaning. RA is known to be the most active metabolite of VA, which mediates the metabolic functions of VA except vision. To explain this phenomenon, we examined the mRNA expression of enzymes that catalyze the synthesis of RA, because RA synthesis is

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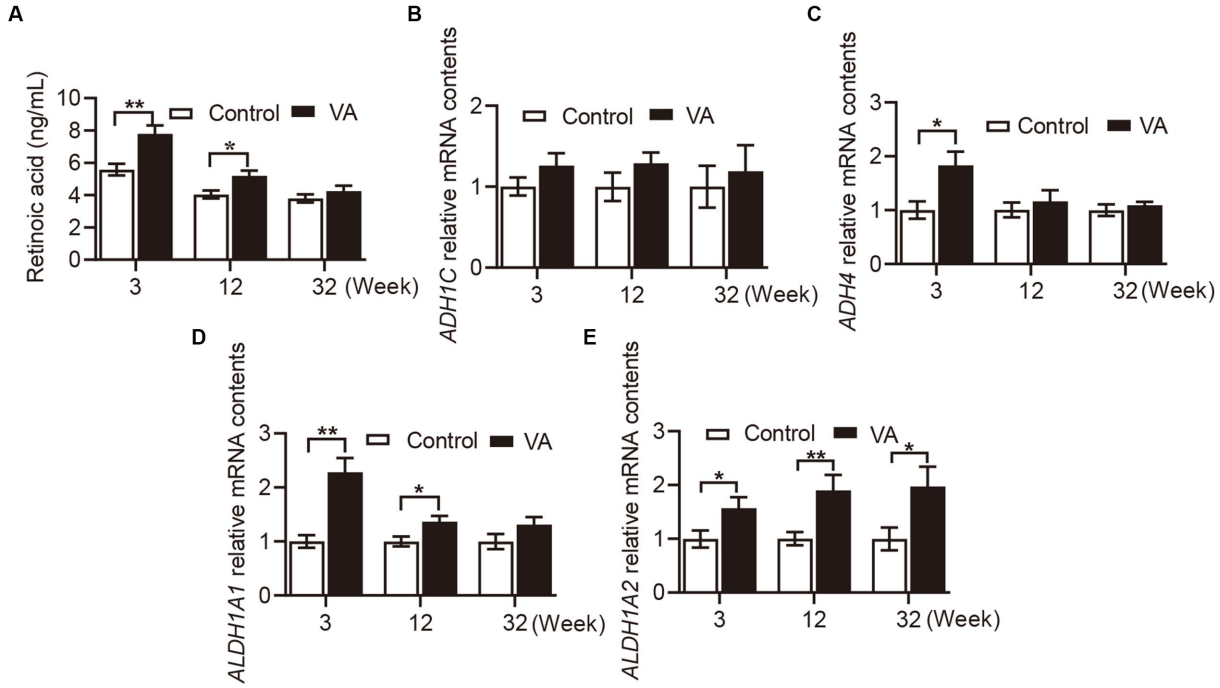

FIGURE 1
Effects of vitamin A injection on RA content in serum and retinol metabolizing enzymes mRNA expression in LD muscle of lambs. **(A)** RA content in serum. **(B)** Relative mRNA of *ADH1C*. **(C)** Relative mRNA of *ADH4*. **(D)** Relative mRNA of *ALDH1A1*. **(E)** Relative mRNA of *ALDH1A2*. (Mean ± SEM; $n = 8$ in each group, $^{*}p < 0.05$ and $^{**}p < 0.01$).

TABLE 2 **Effects of vitamin A injection on meat quality of lambs.**

Meat quality		Groups		SEM	*p* value
		Control	VA		
pH	45 min	6.55	6.64	0.14	0.55
	24 h	5.55	5.57	0.05	0.76
L*	45 min	27.10	25.82	0.96	0.21
	24 h	33.18	32.46	0.89	0.44
a*	45 min	9.31	10.24	0.74	0.23
	24 h	9.58	10.90	0.54	0.03
b*	45 min	8.34	8.20	0.32	0.66
	24 h	9.54	9.45	0.29	0.77
Conductivity	45 min	1.86	1.91	0.12	0.69
	24 h	2.53	2.31	0.42	0.61
Shear Force (N)		25.18	23.95	3.07	0.70
Cook Loss (%)		36.57	36.99	0.76	0.58
Drip Loss (%)		18.26	17.51	0.02	0.73

VA, Vitamin A.

mainly dependent on the catalytic action of alcohol dehydrogenase (ADHS) and aldehyde dehydrogenase (ALDH). However, the data showed that the expression of the enzymes did not seem to be clearly related to RA content, as they exhibited different levels at different stages between control and VA groups, which were consistent with the findings of Harris et al. (19). Indeed, RA has been shown to decrease the sensitivity of chicken embryonic neurons (25, 26), PC12 (27) cells and mesangial cells (28) to oxidative stress. Hence, these results suggested that part of the effect of VA on meat quality might be attributed to the synthesis of high levels of RA.

The pH of the carcass after harvest is dependent on glycogen metabolism in skeletal muscle, and the content of glycogen is positively correlated with the pH value of meat (29). In addition, LDH is a key oxidoreductase in the glycolytic pathway of organisms that catalyzes the formation of lactate acid, and its activity is negatively correlated with pH. In the present study, VA injection did not alter either glycogen in LD muscle or HK and LDH in serum in this trial, indicating that VA did not affect the ultimate pH of lamb meat. Considering that ultimate pH is an important factor affecting meat quality, which is strongly associated with water holding capacity and tenderness (30–32), these data might explain why intramuscular VA injection did not affect holding capacity and tenderness. Indeed, dietary supplementation of natural antioxidants in animals does not affect pH of meat. For example, addition of grape seed extract to the diet had no effect on the final pH of swine muscle (33). In lambs, dietary addition of buckwheat extract also did not affect the pH of the Longissimus thoracis et lumborum muscle (8). Furthermore, intramuscular VA injection did not affect the conductivity, cook loss, and drip loss of LD muscle, which may be attributed to the constant pH value.

Meat color is considered as one of the indicators to judge the health of fresh meat, and flesh redness plays a key role in determining consumers' decisions (34). In the current study, VA injection significantly increased the LD muscle redness of lambs, as evidenced by an increase in a* value. Similarly, previous studies have shown that

TABLE 3 Nutritional composition in longissimus dorsi muscle of lambs.

Items	Groups		SEM	*p* value
	Control	VA		
Moisture, %	72.17	72.20	0.01	0.96
CP, %	20.19	21.62	0.66	0.04
EE, %	2.84	3.56	0.22	0.01
Ash, %	4.65	4.62	0.12	0.83

VA, Vitamin A; CP, crude protein; EE, ether extract.

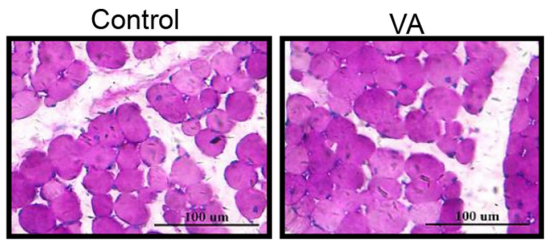

FIGURE 2
Effects of vitamin A injection on glycogen content in LD muscle of lambs. Glycogen PAS staining of LD muscle. (Mean ± SEM; $n = 8$ in each group).

TABLE 4 The glycogen content in longissimus dorsi muscle, hexokinase, and lactate dehydrogenase contents in serum of lambs.

Item	Groups		SEM	*p* value
	Control	VA		
Glycogen (mg/g)	4.30	4.65	0.60	0.57
HK (U/L)	38.68	40.76	3.29	0.54
LDH (U/L)	658.25	644.25	19.51	0.49

VA, Vitamin A; HK, hexokinase; LDH, lactate dehydrogenase.

supplementation with natural antioxidants increases the redness of meat. For example, feeding pasture abundant in natural antioxidants improved the meat redness (35). Supplementation of finishing pigs with resveratrol or grape seed proanthocyanidin extract improved the pork redness (36, 37). Indeed, meat color is closely related to the level of myoglobin. Kim et al. reported that the increased redness in pork was caused by the high concentration of myoglobin in the muscle (38). When the fresh meat was exposed to air, myoglobin in LD muscle was oxidized to the oxymyoglobin, giving the meat a bright cherry red color, which is what consumers perceive as the color of freshness (39). In addition, meat color is highly correlated with muscle fiber type composition (37). Muscle fibers in adult mammals are divided into four main types: MyHC I, MyHC IIa, MyHC IIb, and MyHC IIx (40, 41). It has been suggested that the higher a* value was always attributed to a high proportion of type I myofiber (38). Consistently, type I myofiber is oxidized muscle fiber with redder flesh color and tend to have higher myoglobin concentrations. However, whether VA injection affected the muscle fiber type composition needs to be further explored.

In beef cattle, intramuscular fat content greatly affected the tenderness and flavor of beef (42, 43), while intramuscular VA

TABLE 5 Amino acid composition in longissimus dorsi muscle of lambs (mg/g).

Amino Acid	Groups		SEM	*p* value
	Control	VA		
Asp	11.02	12.47	1.44	0.34
Glu	20.29	20.74	2.43	0.34
Ser	6.88	7.34	0.65	0.5
Arg	10.64	11.18	0.93	0.58
Gly	6.48	6.73	0.56	0.67
Pro	7.57	7.64	0.51	0.9
Ala	9.97	10.48	0.98	0.62
Thr	5.87	6.26	0.59	0.53
Val	2.57	2.7	0.3	0.67
Met	4.51	4.47	0.38	0.94
Ile	9.67	9.41	0.5	0.61
Leu	11.81	12.46	1.21	0.6
Phe	7.92	8.12	0.62	0.75
His	4.36	4.26	0.33	0.76
Lys	8.97	9.49	1.26	0.69
Tyr	4.67	4.68	0.46	0.99
TAA	133.19	140.43	12.63	0.58

VA, Vitamin A; TAA, total amino acid.

TABLE 6 Antioxidant enzymes activities and malondialdehyde content in longissimus dorsi muscle of lambs.

Items	Groups		SEM	*p* value
	Control	VA		
T-AOC, U/mg protein	0.19	0.21	0.44	0.65
CAT, U/mg protein	5.93	8.87	1.3	0.04
T-SOD, U/mg protein	37.66	38.04	5.45	0.94
GPx4, U/mg Protein	249.52	295.72	42.66	0.3
MDA, nmol/mg protein	0.5	0.34	0.07	0.04

VA, Vitamin A; T-AOC, total antioxidant capacity; CAT, catalase; T-SOD, total superoxide dismutase; GPx4, glutathione peroxidase 4; MDA, malondialdehyde.

injection increased intramuscular fat content through up-regulation of *Zfp423* expression, a key transcription factor regulating adipogenesis (19). Consistently, a higher EE was observed in LD muscle of VA-injected lambs. However, we did not observe the alteration in muscle shear force, implying that VA did not affect lamb meat tenderness. Indeed, meat tenderness is determined by numerous factors, including animal's breed and age (44), intramuscular collagen content and the length of the sarcomere (45), intramuscular fat content and ultimate pH (46). Interestingly, we observed that VA increased the content of crude protein in LD muscle, in line with a

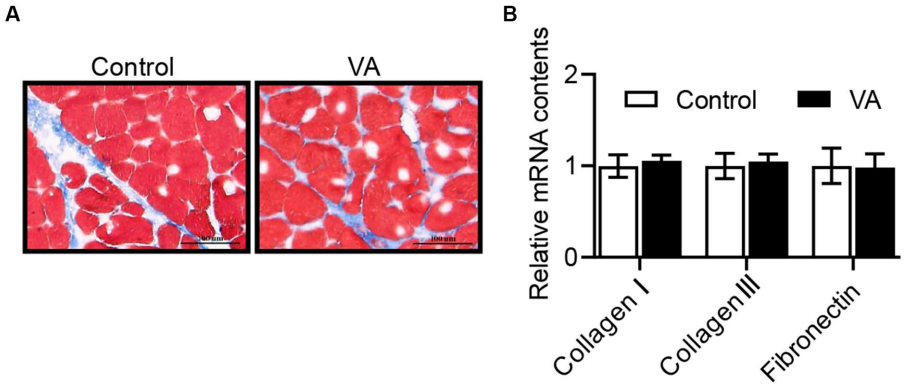

FIGURE 3
Effects of vitamin A injection on collagen content in LD muscle of lambs. **(A)** Masson staining of LD muscle. **(B)** Relative mRNAs of *collagen I*, *collagen III*, and *fibronectin*. (Mean ± SEM; $n = 8$ in each group).

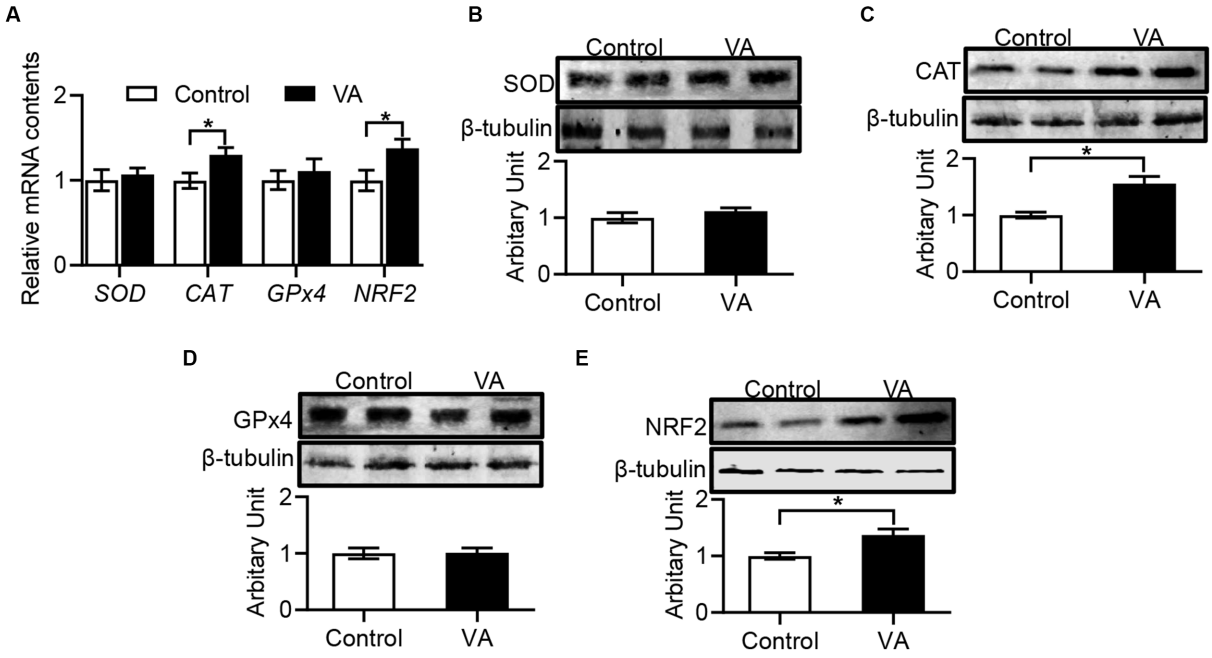

FIGURE 4
Effects of vitamin A injection on antioxidative activity in LD muscle of lambs. **(A)** Relative mRNAs of *SOD*, *CAT*, *GPx4*, and *NRF2*. **(B)** SOD protein abundance. **(C)** CAT protein abundance. **(D)** GPx4 protein abundance. **(E)** NRF2 protein abundance. (Mean ± SEM; $n = 8$ in each group, $^*p < 0.05$).

previous study, showing that VA promoted skeletal muscle growth (16).

Lipid oxidation is one of the main causes of meat and meat products spoilage and deterioration (47). The antioxidant system is essential to provide protection against oxidative damage and can be activated by a variety of biologically active substances and antioxidant-related genes (48). Several studies have shown that natural antioxidants can improve meat quality by reducing lipid peroxidation and improving the antioxidant status (49, 50). For example, dietary addition of soy isoflavones improves meat quality by increasing antioxidant capacity in male broilers (51). In pigs, dietary resveratrol supplementation also improves pork quality by increasing antioxidant capacity (37). In this trial, VA treatment significantly increased CAT activity and decreased MDA content in the LD muscle of lambs. Catalase exists in almost all oxygen-exposed organisms (e.g., bacteria, plants and animals), and catalyze the breakdown of hydrogen peroxide into water and oxygen (52). Indeed, catalase have been used to make food wrapping paper to prevent food oxidation in the food industry (53). A previous research has shown that the overexpression of catalase reduced oxidative stress in aging mice (54). The production of MDA has also been used as a bio-marker to assess organismal oxidative stress (55). In addition, Keap1-NRF2 signaling is also important for regulating the antioxidant system, and Keap1 plays a negative regulatory role

on activating the antioxidant genes expression such as *CAT*, *SOD1*, and *NRF2* (48, 56). Zhao et al., showed that dietary supplementation of tartary buckwheat extract significantly increased the protein abundance of Gpx4 and Nrf2 and decreased the malondialdehyde content in skeletal muscle, thus improving the antioxidant capacity of lamb muscle (8). Xu et al., found that inclusion of grape seed proanthocyanidin extract in feed of finishing pigs affected mRNA expression of antioxidant related genes, including *SOD1*, *CAT*, *GPX1*, *GST*, *Keap1*, and *NRF2* in LD muscle (36). As expected, our data shown that VA injection enhanced the abundances of NRF2 and CAT, suggesting that VA activating the antioxidant system and protecting the body from oxidative damage. Considering that the shelf life is a major economic constraint for the lamb meat industry (57), the improvement of antioxidant potential in VA-injected lambs may extend the lamb meat's shelf life.

Conclusion

In summary, intramuscular VA injection in neonatal stage improved the meat quality of lambs, including postmortem a* value, crude protein and crude fat content, and enhanced the antioxidant capacity in LD muscle.

Data availability statement

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

Ethics statement

The animal studies were approved by Institutional Animal Care and Use Committee of Shanxi Agricultural University (sxnd202028). 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

PS: Conceptualization, Software, Validation, Visualization, Writing – original draft. GH: Data curation, Formal analysis, Writing – original draft. JF: Investigation, Writing – original draft. WZ: Methodology, Writing – original draft. XL: Methodology, Writing – original draft. JZ: Funding acquisition, Project administration, Resources, Supervision, Writing – review & editing.

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Conflict of interest

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

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Effects of alkaline mineral complex supplementation on production performance, serum variables, and liver transcriptome in calves

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Calf diarrhea causes huge economic losses to livestock due to its high incidence and mortality rates. Alkaline mineral complex water is an alkaline solution containing silicon, sodium, potassium, zinc, and germanium, and has biological benefits and therapeutic effects. This study aimed to evaluate the impact of alkaline mineral complex water supplementation on the health of calves and to investigate the effect of Alkaline mineral complex water supplementation on neonatal calf serum variables and the liver transcriptome. Sixty Holstein calves (age 1.88 ± 0.85 days, weight 36.63 ± 3.34 kg) were selected and randomly divided into two groups: the T group (treatment group with alkaline mineral complex water supplemented during the experiment) and C group (control group without alkaline mineral complex water supplementation). Alkaline mineral complex water supplementation significantly increased the body weight for calves aged 60 d and average daily gain during the experimental period (1–60 d). In addition, Alkaline mineral complex water supplementation could significantly decrease the diarrhea rate for calves aged 16–30 d, enhance the T-AOC, IgG, IGF-1, and IGFBP-2 in concentrations. The results of KEGG enrichment analysis in transcriptomics indicate that Alkaline mineral complex water supplementation inhibited the target IL-1B gene of the NF-kappa B signaling pathway of liver. Alkaline mineral complex water supplementation decreased calf diarrhea and improved partial immune function, anti-inflammatory activity, antioxidant capacity, and health of calves. Alkaline mineral complex is a candidate to replace medicated feed additives. Alkaline mineral complex waterAlkaline mineral complex waterAlkaline mineral complex waterAlkaline mineral complex waterAlkaline mineral complex waterAlkaline mineral complex water.

KEYWORDS

calf, stress, anti-inflammatory activity, antioxidant capacity, liver transcriptome

Introduction

In contemporary farm production, it is well-established that farm animals undergo a series of stressors post-birth, significantly impacting their overall welfare. These stressors encompass a range of factors, including separation from maternal animals (1), the stress of transportation (2), challenges posed by the farm environment (3), the stress of weaning (4), antibiotic usage (5), heat stress (6) and adjustments in their dietary regimen (6). Newborn animals, in particular, exhibit heightened vulnerability to these stressors and require a higher level of care and attention to ensure their well-being (7, 8). Adequate nutrition of calves is a fundamental requirement for efficient production in later life. Suboptimal nutrition before weaning could have detrimental long-term effects on metabolic health and could thereby decrease production efficiency (7).

Water is an essential component of the diet and plays an important role in animal nutrition (9). Natural alkaline mineral water usually contains sodium, potassium, zinc, metasilicic acid, and other rare minerals (10). These alkaline minerals are rich in inorganic components in biota and play key roles in physiological metabolism and catalysis (11). Alkaline mineral water or mineral supplementation can improve the quality of life of patients with diarrhea-predominant irritable bowel syndrome and improve the gut health of young animals (12, 13). Calf diarrhea (CD) is a common stress disease associated with livestock production (14). Calf diarrhea causes huge economic losses to livestock due to its high incidence and mortality rates (15, 16). Antibiotics are routinely used to treat CD (17); however, excessive use of antibiotics can lead to various side effects, the most significant of which is infection by multidrug-resistant bacteria, which is increasing world-wide (18). Therefore, it is critical to find a low-cost, convenient and without side effect method to improve CD and calf immunity.

Alkaline mineral complex (AMC) water has biological benefits and therapeutic effects, including improving the quality of life of patients with cancer, conferring anti-oxidative effects, promoting intestinal health, and treating intestinal inflammatory diseases and diarrhea (12, 19, 20). The Alkaline mineral complex water used in this study was an alkaline solution (pH 9.1) containing silicon, sodium, potassium, zinc, and germanium, and its functions were based on its mineral composition (21). These minerals are essential for various physiological functions [such as digestion and immune biosynthesis in animals (22, 23)]. The liver is a key metabolic and central immune organ (24). Research has shown that the liver responds to these minerals, affecting their immune function (25). Recent research has shown that Alkaline mineral complex water can improve piglet diarrhea. Study of Chen et al. (26) showed that Alkaline mineral complex water (as used in this study) can improve intestinal morphology and inflammatory reactions, promote intestinal health, and accelerate the growth performance of weaned piglets. In addition, Alkaline mineral complex waterAlkaline mineral complex water can improve the structure and function of intestinal microflora, maintain intestinal epithelial regeneration, and improve the ability of piglets to recover from diarrhea (21, 27). In this experiment, we expected Alkaline mineral complex water supplementation to improve CD, enhance partial immune function, and promote their growth, development, and health. As calves are born with an esophageal sulcus reflex, their digestive mode is similar to that of non-ruminants (28). At present, it is known that Alkaline mineral complex water could improve diarrhea and intestinal immune function. However, as the largest immune organ in the liver, it is unclear how the expression of liver function genes and participation in immunity can be enhanced by Alkaline mineral complex water supplementation. In addition, the value of AMC in ruminants has not yet been explored. There has been no research on AMC in ruminants, and the effect of Alkaline mineral complex water on the liver transcriptome has not yet been studied. Hence, our study aimed to evaluate the impact of Alkaline mineral complex water supplementation on the health of calves and to investigate the effect of Alkaline mineral complex water supplementation on calf serum variables and the liver transcriptome.

Materials and methods

All experimental methods and humane endpoints for decreasing pain in animals were performed after approval by the Experimental Animal Welfare and Animal Experiment Ethics Review Committee of China Agricultural University (Approval No. AW01103202-1-32). Animal research was conducted in accordance with State Council Order No. 676 of the People's Republic of China (Regulations on the Administration of Experimental Animals).

Experimental animals and treatment headings

The experiment was conducted on a commercial dairy farm in Yinchuan, Ningxia, China. According to the principle of similar body weight (BW), and age (1–3 d), 60 neonatal Holstein calves aged 1.88 ± 0.85 d (mean ± SD) and weighing 36.63 ± 3.34 kg BW were selected. Briefly, according to a completely randomized design, 60 neonatal calves were randomly divided into two groups in the experimental cattle farm: the T group (treatment group with alkaline mineral complex water supplemented during the experiment) and C group (control group without alkaline mineral complex water supplementation), with 30 calves (including 10 male calves and 20 female calves) in each group. All experimental calves were housed in individual hutches (each hutch has an area of at least 2.5 × 1.6 m). Calves could have direct visual and tactile contact, and have hay bedding. Calves were fed at 06:00 and 16:00 daily on the farm, and the daily liquid feed rate during the experimental period was 4 L/calf at 1–7 d of age, 6 L/calf at 8–14 d, 8 L/calf at 15–25 d, and 10 L/calf at 26–50 d; this was reduced by 2 L/calf every 2 d between 51 and 60 days of age until weaning. Pelleted feed was added to calves at 31 days of age. The water and pellets were changed daily during feeding to ensure the pellets were clean and fresh. To ensure that there was no interference from other mineral elements in this experiment, pure drinking water was used. The experiment included 7 d of adaptation, followed by 53 d (until weaning) of formal experimentation. During the 16–30 d experimental period, two calves in the C group died because they were unable to recover from diarrheal symptoms.

Experimental feed

Experimental diet according to Nutrient Requirements of Dairy Cattle (NRC) (29). The ingredients and nutrient compositions of the experimental diets and liquid feed are listed in Supplementary Tables S1–S3. AMC is a liquid colloidal substance that is soluble in water, and it contains Na_2SiO_3, ZnO, Ge-132. Alkaline

mineral complex water was supplemented using a continuous syringe during feeding in the afternoon during the experimental period (Supplementary Figure S1). The calves were fed twice at 06:00 and 16:00 and had free access to water. The liquid feed for 1–7 d of age of calves was fed in bottles with rubber nipples, and the calves were guided to use buckets for liquid feed. Calves after 8 days of age consume liquid feed in buckets. According to Chen et al. (26), based on the nutritional needs of calves, Alkaline mineral complex water supplementation should be 5 mL per cow per day. All the raw materials for AMC were provided by Nail Biotechnology Co., Ltd. (Beijing, China). The compositions of the AMC are listed in Table 1. Both pellet feed (Borui Technology Co., Ltd., Changchun, China) and feeding milk (Nutrifeed Co., Ltd., Veghel, Netherlands) were commercial feed. The mixing ratio of feeding milk was: (milk replacer 1: water 7): normal milk =1:1. All feed implemented Current National Standards and Industry Standards for Feed Industry of China. The feed provided in this study was carefully monitored to ensure that aflatoxin levels were well below the established safety limits for animal feed.

Growth performance measurements and diarrhea rate statistics

BW was measured at birth and every month thereafter. Body weight was measured by weighing the calves 2 h before the morning feeding (when the calves' stomachs were empty). The weight of the pellet feed was measured before feeding (06:00) every day, and the pellet feed intake of the previous day for calves was calculated. The severity of diarrhea in the experimental calves was graded according to the consistency of feces. The fecal scores were as follows:

1 = normal (retains shape), 2 = soft (flows across a surface), 3 = muddy (liquid), and 4 = severe diarrhea (very watery) (14).

Diarrhea rate (%) = (total number of calves with diarrhea × days of diarrhea)/(total number of experimental calves × experimental period days) × 100 (30).

One independently trained observer collected the fecal score data. Calves were defined as having diarrhea with scores of 3 or 4.

Blood samples collection and analysis

Ten calves from each experimental group were randomly selected for blood analysis. Blood samples were collected as described by Liu et al. (30). On days 1, 15, 30, 45, and 60 of the experimental period, blood was collected through jugular puncture using a vacuum blood collection vessel without an anticoagulant 2 h after morning feeding. Blood samples were centrifuged at 3,000 × g for 15 min at 4°C to collect serum and stored in 1.5 mL microcentrifuge tubes at −80°C for later analysis.

TABLE 1 The composition of alkaline mineral complex (AMC) concentrate.

Ingredient	Content	Chemical formula
Sodium metasilicate pentahydrate	200 g/L	$5H_2O{\cdot}Na_2SiO_3$
Potassium bicarbonate	100 g/L	$KHCO_3$
Zinc oxide	10 mg/L	ZnO
Bis-(carboxyethylgermanium) sesquioxide	1 mg/L	Ge-132

According to the radioimmunoassay method, insulin (INS) and growth hormone (GH) were analyzed with a measuring instrument (BFM-96, Zhongcheng Electromechanical Technology Development Co., Ltd., Hefei, China). Total cholesterol (TC), blood urea nitrogen (BUN), alkaline phosphatase (ALP), total protein (TP), albumin (ALB), globulin (GLB), malondialdehyde (MDA), glutathione peroxidase (GSH-PX), superoxide dismutase (SOD) and total antioxidant capacity (T-AOC) were determined according to the manufacturer's instructions, using commercial reagent kits (Nanjing Jiancheng Co., Ltd., Nanjing, China) and an automatic biochemical analyzer (CLS880, Zecen Biotechnology Co., Ltd., Jiangsu, China). The β-hydroxybutyric acid (BHBA), nonestesterified fatty acid (NEFA), immunoglobulin G (IgG), insulin-like growth factor-1 (IGF-1), insulin-like growth factor binding protein-2 (IGFBP-2), zonula occludens-1 (ZO-1), endothelin-1 (ET-1), diamine oxidase (DAO) and D-lactic acid (D-LA) were analyzed using ELISA kits (Abcam, Cambridge, U K) with a microplate reader (Thermo Multiskan Ascent, Thermo Fisher Scientific, Shanghai, China).

Collection, RNA extraction, sequencing and differential expression gene and function analysis of liver samples

Ten calves were randomly selected for humane slaughter at 60 d of the experimental period (five calves in Group C and five calves in Group T). After slaughtering, liver samples were collected from the same areas of calves in the C and T groups. Liver sample collection was done according to the method of Zhao et al. (31). Briefly, liver samples were quickly sectioned, washed with precooled PBS (4°C), placed in 2 mL sterile cryopreservation tubes, quickly frozen in liquid nitrogen, and then stored at −80°C for further analysis.

According to the manufacturer's instructions (Invitrogen, Carlsbad, CA, United States), total RNA was extracted from liver tissue using TRIzol® Reagent and genomic DNA was removed using DNase I (TaKara, Kusatsu, Japan). RNA degradation and contamination were monitored by 1% agarose gel electrophoresis. RNA quality was determined using a 2,100 Biological Analyzer (Agilent Technologies, Santa Clara, CA, United States) and quantified using an ND-2000 (NanoDrop Technologies, Wilmington, DE, United States). Only high-quality RNA samples (OD260/280 = 1.8–2.2, OD260/230 ≥ 2.0, RIN ≥ 8.0, 28S:18S ≥ 1.0 > 1 μg) were used to construct the sequencing library.

RNA purification, reverse transcription, library construction, and sequencing were performed at Shanghai Majorbio Bio-pharm Biotechnology Co., Ltd. (Shanghai, China), according to the manufacturer's instructions (Illumina, San Diego, CA, United States). The transcriptome library was prepared using the TruSeqTM RNA sample preparation Kit from Illumina (San Diego, CA, USA) using 1 μg of total RNA. Briefly, mRNA was isolated according to the poly(A) selection method using oligo(dT) beads and then fragmented using fragmentation buffer. Next, double-stranded cDNA was synthesized using a SuperScript double-stranded cDNA synthesis kit (Invitrogen) with random hexamer primers (Illumina). Then the

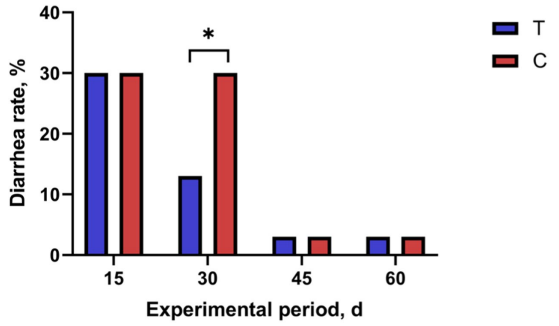

FIGURE 1
Effects of alkaline mineral complex water supplementation on diarrhea rate of calves. C is the control group; T is the treatment group. *$p < 0.05$. $n = 30$ per experimental group.

TABLE 2 Effects of alkaline mineral complex water supplementation on pellets feed intake in calves.

Items	Experimental period	C[a]	T[b]	SEM	*p*-value
Pellets feed intake, g	30–40 d	190.23	203.23	29.96	0.66
	40–50 d	493.60	498.60	28.42	0.86
	50–60 d	963.00	964.80	20.09	0.95
Total pellets feed intake, g	30–60 d	548.94	555.55	16.71	0.69

[a]C, Control group.
[b]T, Treatment group. Data are presented as the means ± SEM ($n = 30$ per experimental group).

synthesized cDNA was subjected to end-repair, phosphorylation and 'A' base addition, according to Illumina's library construction protocol. Libraries were selected for cDNA target fragments of 300 bp on 2% low-range ultra-agarose, followed by PCR amplification using Phusion DNA polymerase (NEB) for 15 PCR cycles. After quantification using TBS380, the paired-end RNA-seq library was sequenced using an Illumina NovaSeq 6000 sequencer (2 × 150 bp read length).

Data analysis

The data displayed in the tables and figures represent the mean ± standard error of the mean (SEM). Before conducting statistical analysis, the normality and homogeneity of data of average daily gain, feed intake and serum variables were tested based on the description of Marta et al. (32). The effects of Alkaline mineral complex water supplementation on serum variables in neonatal calves were assessed using SAS software (version 9.4; SAS Institute Inc., Cary, NC, United States). According to the following model, a block experimental design was used for the time, group, and interaction effects between treatment and group:

$$Y = \mu + T_i + G_j + \mathrm{TG_{ij}} + \mathrm{E_{ijl}}$$

Y is the dependent variable, μ is the overall mean, T_i is the time effect, G_j is the treatment effect, $\mathrm{TG_{ij}}$ is the interaction effect between T and G, E_{ijl} is the random residual error. Statistical significance was set at $p < 0.05$. different. One-way analysis of variance using SAS 9.4, the statistical significance of the effect of Alkaline mineral complex water supplementation on se-rum variables. Differences were considered statistically significant at $p < 0.05$, and trends were declared at $0.05 \leq p \leq 0.10$.

The source of the reference gene was *Bos taurus*, the version of the reference genome was ARS-UCD1.2, and the source of the reference genome was http://asia.ensembl.org/Bo. ctaurus/Info/Index. The Clean Reads of each sample were sequenced with the specified reference genome. Transcriptome analysis of liver samples was performed using the Majorbio Cloud Platform (www.majorbio.com) (33). Differential expression analysis was performed using DESeq2, and DEGs with log2(fold change) ≥ 1 and P-adjusted ≤0.05 (DESeq2) were considered to be significant DEGs. Kyoto En-cyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis was per-formed using R software (version 3.3.1). The KEGG pathway function was considered significantly enriched when the *p*-value was <0.05. All other statistical analyzes were performed using GraphPad Prism version 9 (GraphPad Software).

Results

Diarrhea rate of calves

The effect of alkaline mineral complex water supplementation on the diarrhea rate is shown in Figure 1. Alkaline mineral complex water supplementation significantly decreasedthe diarrhea rate for calves aged 16–30 d ($p < 0.05$).

Average daily gain and feed intake of calves

Alkaline mineral complex water supplementation for pellet feed intake is presented in Table 2 and Figure 2. Alkaline mineral complex water supplementation for BW and average daily gain (ADG) are presented in Table 3. AMC supplementation significantly increased the BW for calves aged 60 d ($p < 0.05$) and ADG during the experimental period (1–60 d) ($p < 0.05$). In addition, due to the natural characteristics of calves, they prefer milk more. All liquid feed calves fed daily can be consumed.

Serum variables of calves

The effects of alkaline mineral complex water supplementation on serum variables are presented in Table 4. Compared to calves in the T and C groups, alkaline mineral complex water supplementation significantly reduced BUN levels during the experimental period ($p < 0.05$) and significantly increased T-AOC and IgG levels ($p < 0.05$). A significant interaction was observed between the experimental groups and AMC supplementation time for GLB, IGF-1, IGFBP-2, and ET-1 ($p < 0.05$). Further analysis of serum variable data revealed that alkaline mineral complex water supplementation significantly increased IGF-1 at 30 and 45 days of the experimental period and IGFBP-2 at 45 d of experimental period ($p < 0.05$) (Figure 3), while significantly decreasing GLB and IGFBP-2 at 15 d and ET-1 at 30 d of experimental period ($p < 0.05$) (Figure 3).

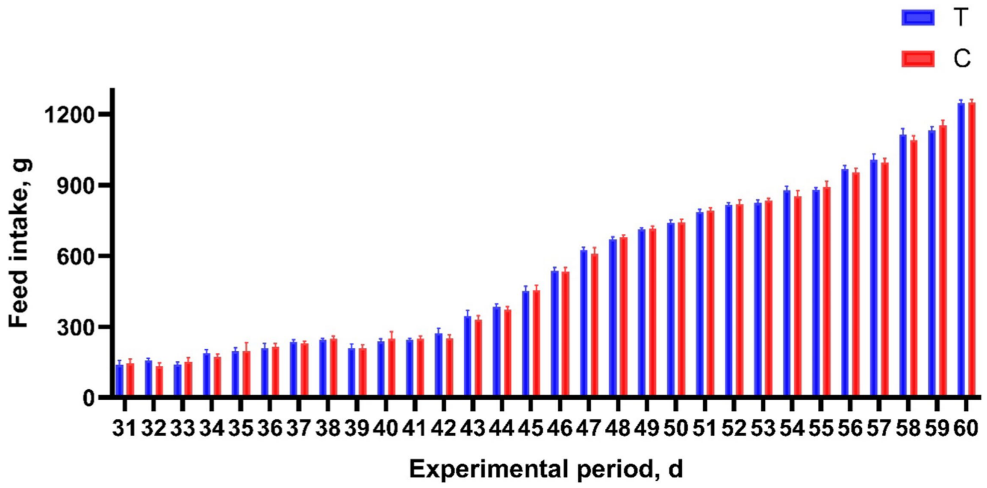

FIGURE 2
Effects of alkaline mineral complex water supplementation on pellets feed intake of calves. C is the control group; T is the treatment group. $n = 30$ per experimental group.

TABLE 3 Effects of alkaline mineral complex water supplementation on body weight and average daily gain in calves.

Items	Experimental period	C[a]	T[b]	SEM	*p*-value
Body weight, kg	1 d	36.00	36.65	2.00	0.75
	30 d	65.13	67.38	1.49	0.40
	60 d	103.40	107.50	1.92	0.03
Average daily gain, kg/d	1–30 d	0.81	1.00	0.11	0.08
	30–60 d	1.22	1.23	0.05	0.95
	1–60 d	1.11	1.22	0.04	0.01

[a]C, Control group.
[b]T, Treatment group. Data are presented as the means ± SEM ($n = 30$ per experimental group).

Differentially expressed genes in the liver of C and T groups

A total of 19,019 genes were detected and tested for differential expression in the liver samples, and 30 were differentially expressed between the T and C groups ($p < 0.05$) (Figure 4). Table 3 lists the significant DEGs in the liver samples of the calves; some unnamed genes are not listed. Respectively, DDIT4, TAT, NOCT, FOSB, SPRY3, LU-RAP1L, ARHGEF26, DUSP1, FHIT, et al. and GSTT4, IL1B, DUSP6, MID1IP1, OSGIN1, COBL, TFAP4, et al. transcripts were up and downregulated in the T group compared to the C group (Table 5).

KEGG enrichment analysis

The KEGG enrichment analysis results for Alkaline mineral complex water supplementation are presented in Figure 5. The significant enrichment pathway was the NF-kappa B signaling pathway (P-adjusted <0.05). The MAPK signaling pathway and transcriptional mis-regulation in cancer tended to be significantly enriched, although these changes were not statistically significant ($0.05 \leq p \leq 0.10$).

Discussion

Alkaline mineral complex water is rich in multiple trace elements. Although the role of AMC in non-ruminant is generally considered positive, its effectiveness in cattle farming has not yet been confirmed. In this study, we evaluated the impact of alkaline mineral complex water supplementation on the health of calves and investigated the effect of alkaline mineral complex water supplementation on production performance, serum variables, and the liver transcriptome. Our results indicated that alkaline mineral complex water supplementation could improve the health and promote the growth of calves.

The CD rate is related to age; calves are at a high risk for CD and death in early life, and CD in the first 3 weeks of life is a major cause of death (34, 35). With the growth and development of calves, their immunity improves and their CD rate decreases (36, 37). Alkaline mineral complex water contains silicates, which have anti-inflammatory properties and can effectively treat diarrhea (38). In this study, alkaline mineral complex water supplementation decreased the CD rate at 16–30 d of the experimental period. Alkaline mineral complex water supplementation had no significant effect in other experimental periods, possibly because Alkaline mineral complex water the immunity of calves improved with their growth and development after 30 d.

Feeding and management of calves at a young age are crucial for the growth, health, and future production performance of dairy cows. The BW of calves reflects their growth and development and is related to the incidence of diseases, reproductive performance, and milk yield (39). CD could have an impact on the BW of calves, which is negatively correlated with BW even after recovery from CD, and the ADG of calves could decrease (40). In this study, Alkaline mineral complex water supplementation increased BW and ADG. Feed palatability is a critical

TABLE 4 Effects of alkaline mineral complex water supplementation on serum variables in calves.

Item	Group		SEM	*p*-value	
	T[a]	C[b]		Group	Group*Time
INS, mIU/mL	28.09	27.79	1.34	0.82	0.37
GH, ng/mL	1.34	1.24	0.07	0.16	0.29
GLU, mmol/L	7.46	7.43	0.15	0.84	0.07
TC, mmol/L	2.87	3.05	0.12	0.13	0.86
BUN, mmol/L	2.94	3.30	0.14	0.01	0.35
TP, g/L	55.20	55.39	0.90	0.84	0.09
ALB, g/L	27.69	27.89	0.27	0.46	0.63
GLB, g/L	26.36	27.75	0.86	0.11	0.02
MDA, nmol/mL	1.52	1.50	0.03	0.54	0.83
GSH-PX, umol/L	7.06	6.87	0.28	0.49	0.48
SOD, U/mL	46.48	47.33	0.90	0.35	0.64
T-AOC, U/mL	8.30	7.87	0.18	0.02	0.15
NEFA, umol/L	44.92	44.71	0.76	0.77	0.19
BHBA, mmol/L	0.36	0.35	0.01	0.63	0.07
IgG, mg/mL	21.09	20.09	0.31	<0.01	0.13
IGF-1, ng/mL	132.16	123.81	4.56	0.07	0.02
IGFBP-2, ng/mL	156.45	151.87	3.14	0.15	0.01
ZO-1, ng/mL	27.79	27.80	0.01	0.99	0.67
ET-1, pg./mL	19.60	20.11	0.14	0.27	0.03
DAO, U/mL	3.87	3.95	0.08	0.67	0.17
D-LA, umol/L	6.31	6.35	0.05	0.69	0.05

[a]C, Control group.
[b]T, Treatment group. Data are presented as the means ± SEM ($n = 10$ per experimental group).

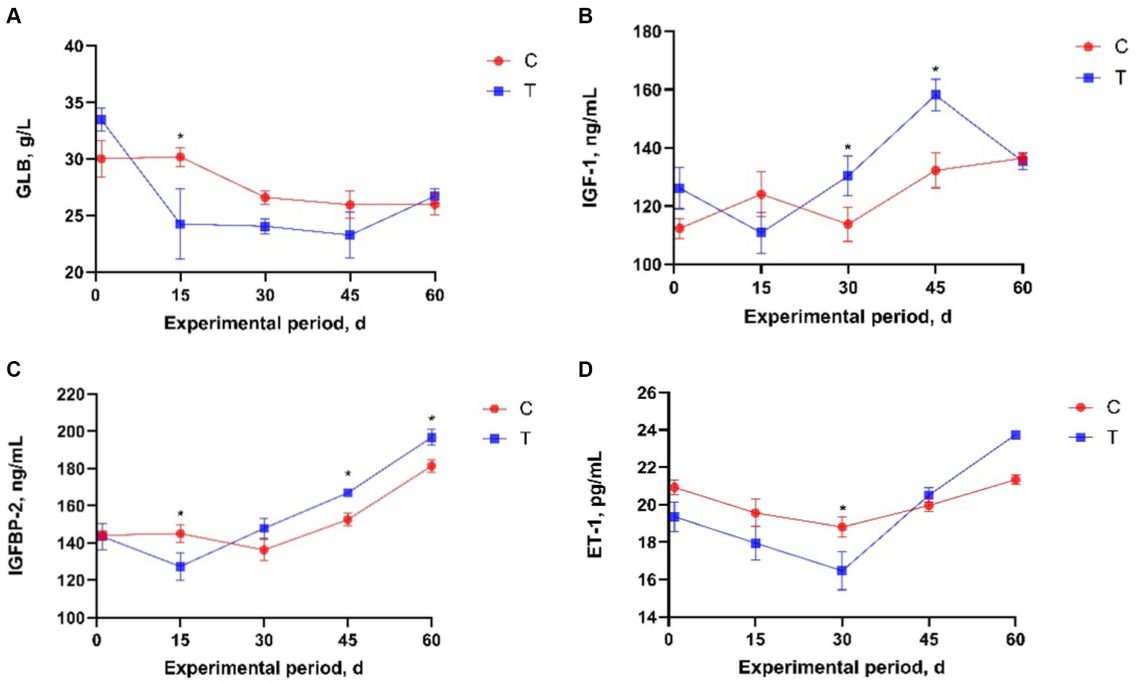

FIGURE 3
Effects of alkaline mineral complex water supplementation on serum variables in calves. **(A)** GLB, **(B)** IGF-1, **(C)** IGFBP-2, **(D)** ET-1. C is the control group; T is the treatment group. Data are presented as the means ± SEM ($n = 10$ per experimental group). *$p < 0.05$.

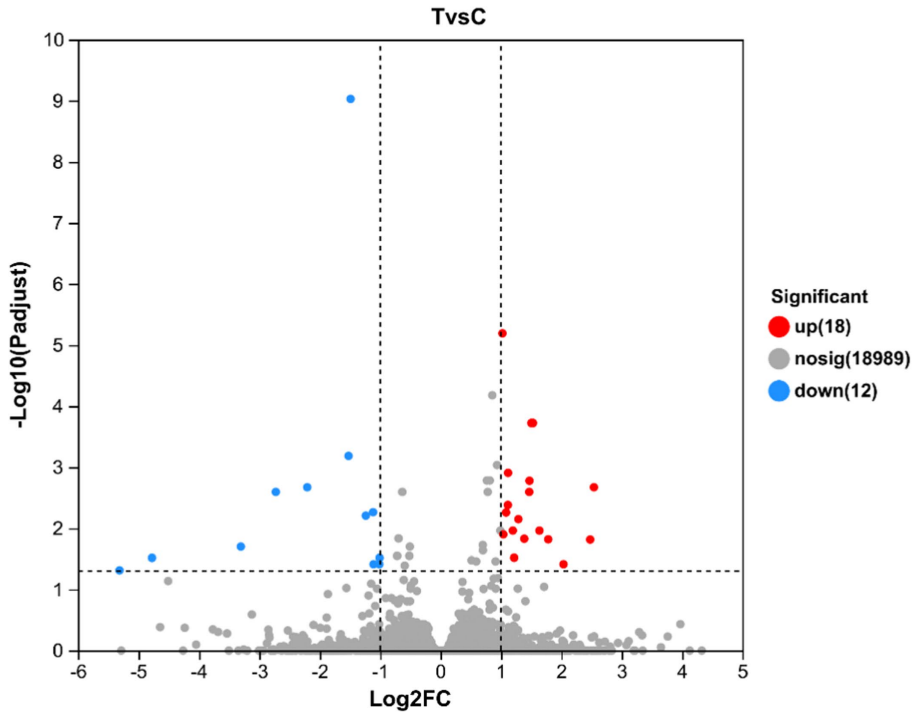

FIGURE 4
Volcano map of differentially expressed genes in the liver of calves at 60 d of the experimental period. The horizontal coordinate is the multiple of gene/transcript expression difference between the two samples, namely the value obtained by dividing the amount expressed by treated samples by the expression amount of control samples; the vertical coordinate is the statistical test value of gene expression difference, namely the *p* value. The larger the −log10(*p*-value) is, the more significant the difference in ex-pression is. Both the horizontal and vertical values are logized. Each dot in the figure represents a specific gene, with red, blue, and gray dots representing significantly up-regulated, significantly downregulated, and non-significant genes, respectively. The points on the left and right are genes with downregulated and upregulated expression, respectively. The closer to the two sides and the point on the top, the more significant the expression difference.

factor influencing feed intake and, subsequently, animal performance (41). In this experiment, calves could quickly consume all liquid feed every day due to their preference for milk. And there was no difference in the intake of pellet feed for calves. Therefore, AMC supplementation did not interfere with the normal feeding of calves in this experiment. In particular, Alkaline mineral complex water supplementation significantly increased the BW of calves aged 60 d and significantly increased the ADG during the experimental period (1–60 d). In this experiment, the decrease in CD and the increase in feed intake may be the reasons for the increase in calf BW and ADG. Increases in BW and ADG are of great significance for future calf production.

BUN is an important indicator of the balance between amino acid and protein metabolism in animals. Coma et al. (42) showed that animals with lower BUN concentrations had normal amino acid metabolism and higher protein synthesis rates. In this study, AMC supplementation significantly reduced BUN levels during the experimental period, which may indicate that AMC supplementation can improve the utilization rate of dietary protein in calves. This may be one of the reasons for the increase in BW and ADG during the experimental period.

Alkaline mineral complex water is an excellent source of silicon and zinc; these elements are related to the immune system and have anti-inflammatory and antioxidant effects (23, 43–45). Excessive serum globulin levels are associated with inflammation (46). Endothelial damage may increase ET-1 production (47). Alkaline mineral complex water supplementation significantly reduced GLB and ET-1, especially on days 15 and 30 of the experiment. Calves were most vulnerable and had the highest rates of CD in the period within 30 d after their birth. IgG can indicate the strength and weakness of the immune system, and the survival and well-being of calves are strongly dependent on IgG (48). Total antioxidant capacity represents the total antioxidant levels of various antioxidant substances and enzymes (49). In this study, AMC supplementation significantly increased T-AOC and IgG levels. Changes in these serum variables could imply that Alkaline mineral complex water has immunomodulator, anti-inflammatory, and antioxidant effects on calves and that alkaline mineral complex water supplementation improved their health. In addition, AMC supplementation promoted calf growth. AMC supplementation significantly increased IGF-1 and IGFBP-2 levels, particularly in the later period of the experiment. IGF-1 and IGFBP-2 are growth-promoting factors that costimulate osteoblast differentiation (50, 51); increases in BW and ADG confirmed this result. IGF signal transduction is regulated by conserved members of the IGFBP family. IGFBP-5 is a multifunctional protein that conditionally regulates IGF signaling as a molecular switch (52). In the present study, the increase in IGF-1 and IGFBP-2 levels in the treatment group may have been due to the significant upregulation of IGFBP-5. However, the specific regulatory mechanism of IGFBP-5 gene is still unclear and warrant further investigation.

The liver is the largest organ in the body and plays an important role in the immune system (53). NF-kappa B signaling pathway is a highly conserved evolutionary pathway that plays important functions in regulating immune and inflammatory responses (54). In

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TABLE 5 Significantly differentially expressed genes in the liver samples of calves at 60 d of the experimental period.

Gene id	Gene name	Log2FC (T/C)	*p* value	P-adjust	Regulate
ENSBTAG00000000163	DDIT4	1.50	6.00E-08	1.89E-04	Up
ENSBTAG00000000170	GSTT4	−3.31	5.59E-05	0.02	Down
ENSBTAG00000001321	IL1B	−1.12	9.87E-06	0.01	Down
ENSBTAG00000002214	TAT	1.02	8.03E-10	6.42E-06	Up
ENSBTAG00000004587	DUSP6	−1.49	5.87E-14	9.38E-10	Down
ENSBTAG00000005998	NOCT	2.04	1.53E-04	0.04	Up
ENSBTAG00000008182	FOSB	2.47	3.92E-05	0.02	Up
ENSBTAG00000010245	SPRY3	1.20	2.24E-05	0.01	Up
ENSBTAG00000010431	LURAP1L	1.47	1.67E-06	1.66E-03	Up
ENSBTAG00000011463	MID1IP1	−1.52	2.85E-07	0.65E-03	Down
ENSBTAG00000012508	OSGIN1	−1.01	1.02E-04	0.03	Down
ENSBTAG00000013439	ARHGEF26	1.04	2.90E-05	0.01	Up
ENSBTAG00000013863	DUSP1	1.53	7.09E-08	1.89E-04	Up
ENSBTAG00000014418	FHIT	2.54	2.65E-06	2.12E-03	Up
ENSBTAG00000017162	STK39	1.64	2.38E-05	0.01	Up
ENSBTAG00000021672	RGS1	1.47	3.70E-06	2.53E-03	Up
ENSBTAG00000023806	COBL	−1.24	1.20E-05	6.20E-03	Down
ENSBTAG00000033174	TFAP4	−1.01	1.54E-04	0.04	Down
ENSBTAG00000039819	RPH3AL	1.39	3.61E-05	0.01	Up
ENSBTAG00000047103	IDNK	1.29	1.41E-05	0.01	Up
ENSBTAG00000052499	RNF39	−2.21	2.64E-06	2.12E-03	Down
ENSBTAG00000054218	IGFBP5	1.22	9.81E-05	0.03	Up

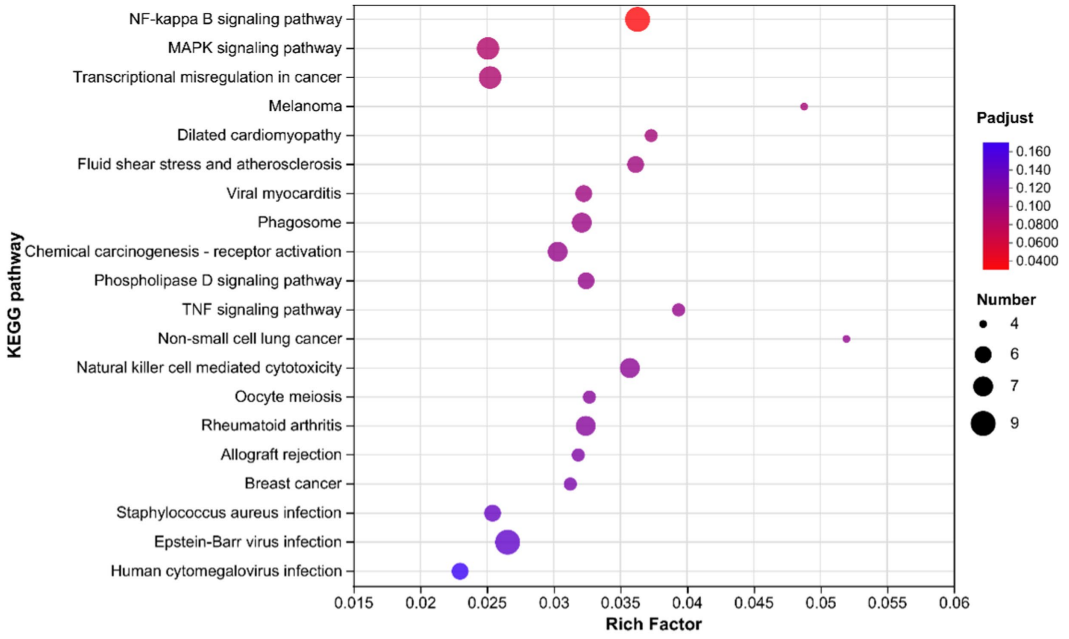

FIGURE 5
Kyoto Encyclopedia of Genes and Genomes enrichment analysis in the liver samples of 60 days of the experimental period of calves. The vertical axis represents the pathway names, and the horizontal axis represents the ratio of the enrichment factor (sample number of genes/transcripts enriched in this pathway to the background number of annotated genes/transcripts). The larger the enrichment factor, the greater the degree of enrichment. The size of dots indicates the number of genes in this pathway, and the colors of the dots correspond to different P-adjusted ranges. The enrichment results of top 20 were displayed when P-adjusted <0.05.

the liver, NF-kappa B signaling pathway is an important transcriptional regulator of inflammatory response and plays an essential role in regulating liver inflammatory signaling pathways (55). In this study, alkaline mineral complex water supplementation significantly regulated the NF-kappa B signaling pathway in the liver. Chen et al. found that Alkaline mineral complex water could target the inhibition of NF-kappa B signaling pathway through microbial-intestinal interaction (27). IL-1B, one of the main cytokines involved in the pathogenesis of many inflammation-related diseases, and it can regulate the NF-kappa B signaling pathway (56, 57). In this study, alkaline mineral complex water supplementation significantly downregulated the IL-1B gene Research showed that trace elements that are abundant in alkaline mineral complex water could inhibit the IL-1B gene (58). This study showed that alkaline mineral complex water supplementation could inhibit the target genes of the NF-kappa B signaling pathway, affected the immune function of the liver, and improved the health of calves.

Calves, especially female calves, play a crucial role in the future of any herd, making them an important component of dairy farms. Raising calves requires a significant investment of resources and time to achieve profitability (59). Therefore, raising calves requires consideration of cost. Some limitations need to be acknowledged. Although alkaline mineral complex can be proven beneficial for calves, it is necessary to continuously supplement alkaline mineral complex before weaning. If large-scale fed can reduce the number of days for alkaline mineral complex supplementation, it can save costs. The cost of commercial alkaline mineral complex supplementation in calves does not exceed 0.04 dollars per calf per d, indicating that alkaline mineral complex is more economical compared to regular antibiotics. In the future, emphasis should be placed on determining the number of days calves are supplemented with alkaline mineral complex to save more costs.

Conclusion

Under the experimental conditions applied, Alkaline mineral complex water supplementation promoted the growth and health of the calves, possibly by enhancing T-AOC, IgG, IGF-1, and IGFBP-2, thereby improving growth-promoting factors, antioxidant status, and partial immune function in calves. These effects decrease CD and promote the growth of calves. It is worth noting that Alkaline mineral complex water supplementation could inhibit the target IL-1B gene of the NF-kappa B signaling pathway, affect the immune and anti-inflammatory functions of the liver, and improve the health of calves. Overall, alkaline mineral complex water supplementation decreased calf diarrhea and improved, partial immune function, anti-inflammatory activity, antioxidant capacity, and health of calves; thus, it is a candidate to replace medicated feed additives.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found at: https://www.ncbi.nlm.nih.gov/bioproject; PRJNA1008432.

Ethics statement

The animal study was approved by Experimental Animal Welfare and Animal Experiment Ethics Committee of China Agricultural University. The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

CG: Conceptualization, Data curation, Investigation, Methodology, Writing – original draft. XW: Investigation, Methodology, Validation, Writing – review & editing. DD: Investigation, Resources, Writing – review & editing. FK: Writing – review & editing. SW: Writing – review & editing. XS: Data curation, Software, Writing – review & editing. SL: Funding acquisition, Project administration, Supervision, Writing – review & editing. XX: Methodology, Project administration, Resources, Writing – review & editing. LZ: Methodology, Resources, Writing – review & editing.

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Conflict of interest

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

Publisher's note

Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2023.1282055/full#supplementary-material

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Evaluation of a novel dipotassium phosphate bolus for treatment of metabolic disorders in dairy cattle

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A dipotassium phosphate bolus (K Phos-Boost) has been developed to treat both hypophosphatemia and hypokalemia, as the clinical signs of both conditions are similar and occur in the early post-partum period. The objective of this research was to evaluate the efficacy and application of the bolus for prevention and treatment of metabolic diseases that are common in dairy production systems. *Study 1 (Pharmacokinetic study)*: Healthy post-partum cows were either untreated or received two K Phos-Boost boluses at times 0, 24, and 48 h. Blood was taken at $t = 0$, 2-, 4-, 6-, 8-, 10-, 24-, and 52-h post-treatment for analysis of total serum minerals. There was an increase in serum phosphorous to normal levels within 2 h of treatment with the bolus, but control cows remained hypophosphatemic. Serum potassium was significantly elevated 2 h after bolus administration relative to control, while calcium, magnesium, sodium, and chloride levels were not affected by the K Phos-Boost bolus. *Study 2 (Downer Cow Treatment)*: K Phos-Boost boluses were provided to cows that were unresponsive to intravenous calcium therapy and had been unable to stand for over 24 h ("downer cows"). Most cows (16 of 19) treated with two boluses were standing without assistance between 1 and 24 h after treatment and the serum phosphorous was increased to normal levels in five of five tested animals. *Study 3 (Ketosis Treatment)*: cows with clinical ketosis were provided with propylene glycol and K Phos-Boost boluses ($n = 29$) or only propylene glycol ($n = 23$). Cows treated with the K Phos-Boost bolus showed a more rapid recovery by increased milk production (3.9 kg/day) and rumination rate (97 min/day). *Study 4 (Health Promotion)*: cows in herds with >40% post-partum hypophosphatemia received K Phos-Boost boluses ($n = 130$) or no treatment ($n = 146$) following calving. There was a trend for treated 2nd-lactation animals to have higher milk production after 30 DIM (49.1 vs. 46.2 kg/day; $P = 0.09$). There were no significant differences between control and bolus treated animals in the incidence of subclinical ketosis, post-calving total health events, or culling rates. The K Phos-Boost bolus is a novel product and has the potential to treat and prevent several important metabolic diseases in dairy cattle. The studies described in this paper are early investigations and further research should be conducted to demonstrate the applications of a dipotassium phosphate bolus in dairy cows.

KEYWORDS

dipotassium phosphate, K Phos-Boost, downer cows, hypophosphatemia, hypokalemia, ketosis, periparturient

1 Introduction

It is recognized that inadequate blood phosphorus and potassium is associated with downer cows as well as reduced feed intake, reduced rumen and intestinal motility, and an increased susceptibility to other metabolic and infectious diseases (1). Early treatment of such metabolic disorders is beneficial for both positive clinical outcomes and economic returns (2, 3). The incidence of periparturient hypophosphatemia in dairy cattle has been reported to be 50% on the day of parturition and 10%−15% during the first 2 weeks post-partum (4). It has been shown that periparturient supplementation of phosphate in the ration does not prevent post-parturient hypophosphatemia (5), so phosphate deficiencies must be addressed at the time of calving. Subnormal serum or plasma phosphorous levels have been associated with downer cow syndrome and alert downer cows following treatment for hypocalcaemia (milk fever). Hypophosphatemia is also associated with hemoglobinuria, incoordination, reduced milk production, reduced feed intake, and increased risk of post-partum disease (mastitis, ketosis, abomasum displacement, and abomasal volvulus) (1, 4, 5). Intravenous administration of phosphate salts has been shown to provide only transient correction of hypophosphatemia (2–4 h) and is therefore an unsuitable treatment for hypophosphatemia, which can persist for several days during the post-partum period (4). Oral phosphate drenches with phosphate salts, sodium phosphate (NaH_2PO_4) or disodium phosphate (Na_2HPO_4) equivalent to 60 g of phosphorus have been used to correct hypophosphatemia for at least 24 h (6–8). Longer term correction for cows may require daily drenching with a gastric tube, as a diet adjustment for the entire group to which the cow belongs is not practical. Repeated drenching is stressful to the cow and is labor intensive. A novel bolus containing phosphate salts may be an appropriate alternative.

Cows receive a potassium-rich diet and readily excrete excessive potassium through the kidney (9). The normal physiological blood plasma potassium levels of a cow ranges between 3.9 and 5.8 mmol/L, however the ratio of the intracellular to the extra cellular potassium contents are more relevant (9–12). A sudden decrease in food intake can result in hypokalemia, as the kidneys may not be able to respond to the decrease in dietary potassium (10–12). Hypokalemia is also associated with retained placenta, clinical mastitis and abomasal displacement (10–12). The clinical signs of hypokalemia are muscle weakness and recumbency (1, 10–12). Hypokalemia has been treated with oral drenching of potassium chloride (KCl) since intravenous therapy carries a risk of cardiac arrest (11). The optimum amount to be delivered per day has been reported to be 60–250 g/100 kg body weight, although most practitioners recommend 250 g per cow per day for 3–5 days (11).

There has been limited research into prevention and treatment of post-parturient hypophosphatemia and hypokalemia. One of the reasons is that a readily available and easy to deliver therapeutic product has not been available. A dipotassium phosphate (K_2PO_4) bolus ("K Phos-Boost") has been developed by Solvet Animal Health (Calgary, Alberta, Canada) to treat both hypophosphatemia and hypokalemia, as the clinical signs of both conditions are similar and occur in the early post-partum period. Each K Phos-Boost Bolus (Solvet Animal Health, Calgary, Alberta, Canada) weighs 230 g and consists of dipotassium phosphate (K_2HPO_4), providing 100 g of phosphate and 83 g of potassium per bolus. Each bolus is individually vacuum sealed, and the bolus retains its shape under storage temperatures below 40°C (unpublished results). The bolus completely dissolves in the rumen within 30 min (based on unpublished *in vivo* studies using fistulated cattle). The recommended published dose for phosphorous and potassium deficient dairy cows is 198 g of phosphate and 131 g of potassium per day for 1–5 days (4, 11). When two dipotassium phosphate boluses are provided, cows receive 200 g of phosphate and 166 g of potassium per day. This closely matches the published recommended daily dosages. The suggested dose is therefore two boluses, once per day, for up to 3 days.

The rationale for providing a bolus that supplements both potassium and phosphorous is that, although hypophosphatemia is easy to identify in post-partum dairy cattle using blood levels, hypokalemia is not (5). A serum potassium concentration of <2.5 mEq/L indicates severe hypokalemia which occurs after intracellular potassium is depleted. Early lactation cows are reported to be in a negative potassium balance as they excrete a significant amount of potassium in the milk (13). If the cow is not deficient in intracellular potassium, it is able to readily eliminate the excess potassium in the urine (9, 12).

This paper provides early studies investigating the biochemical, pharmacological, and clinical effects of a dipotassium phosphate (K Phos-Boost) bolus in post-partum dairy cows where hypophosphatemia and hypokalemia are most common.

2 Materials and methods

2.1 Pharmacokinetics in post-partum dairy cows

A 600-cow Holstein dairy herd in the province of Alberta, Canada was selected for the pharmacokinetic study where cows were milked in a rotary milking system three times daily. The post-partum ration consisted of 7.85 kg of grass silage, 8 kg of corn silage, 3 kg of dry hay, and 4 kg of a supplement (calcium 0.56% D.M., phosphorus 0.31% D.M., magnesium 0.50% D.M., and potassium 1.41% D.M.). Only clinically healthy cows in their second and third lactations were enrolled in this study and were allocated using a randomization chart to one of two treatment groups: A = Two K Phos-Boost boluses at times 0, 24, and 48 h (i.e., treated group, $n = 4$); B = Untreated (i.e., control group, $n = 5$). Blood was taken from the coccygeal vein pre-treatment at time 0 (time of first treatment) and at 2, 4, 6, 8, 10, 24 (time of second treatment), 30, 48 (time of third treatment), and 52 h. A *post-hoc* power calculation at the 2-h time point indicated that, at $\alpha = 0.05$, power = 92.9% and 99.3% for P and K, respectively. The first ($t = 0$) bolus administration was within 12 h of calving. The blood was immediately centrifuged on the farm (3,000 rpm, 10 min) and the serum transferred into micro-centrifuge tubes for storage at −18°C and transport to the laboratory, where total serum phosphorous, potassium, calcium, magnesium, sodium, and chloride were analyzed on an Element DC5x Veterinary Chemistry Analyzer (HESKA, Barrie, Ontario, Canada).

2.2 Treatment of hypophosphatemic downer cows

The hypothesis was that cows that have been unable to stand for over 24 h, after going down with hypophosphatemia, or hypophosphatemia and hypocalcemia, will respond to treatment by increasing serum phosphorous and intracellular potassium. Veterinary practitioners in the provinces of Ontario and Quebec were provided K Phos-Boost boluses and enrolled cases of post-partum cows that met the criteria of a downer for over 24 h that were refractory to intravenous calcium treatments and in sternal recumbency. Seventeen cows that were downers for ∼24 h and two cows that were down for ∼48 h were enrolled into the study. Blood was collected from the coccygeal vein and serum calcium and phosphorous was measured before treatment with the K Phos-Boost Bolus. Cows received two K Phos-Boost boluses daily until they were standing (maximum 3 days of treatment). Hypocalcemic cows also received intravenous calcium borogluconate. Serum calcium and phosphorous were measured ∼24 h following K Phos-Boost Bolus treatment in only 5 of the 19 cases. Response to treatment was considered a recovery when the cow stood and did not revert to the downer state. There were no untreated controls in this study as it was considered unethical not to treat downer cows. It is well-known that post-partum cows that have been recumbent for over 24 h have very poor outcomes (14).

2.3 Treatment of clinical ketosis

The study hypothesis was that phosphorous and potassium supplementation using dipotassium phosphate bolus will stimulate appetite and improve recovery from the ketotic state in dairy cows. The study was conducted with cows from nine commercial dairy facilities in Quebec, Canada. Cows that exhibited decreased milk production, reduced feed intake, and depression were subjected to a blood ketone test (Precision Xtra™ meter using the Precision Xtra™ Blood Ketone test strips, Abbott Laboratories, Abbott Park, IL). Cows with a ketone level ≥1.4 mM BHB (beta hydroxybutyrate) were enrolled in the study and randomly allocated to one of two treatment groups: A = two dipotassium phosphate boluses immediately following the diagnosis of ketosis and for two more consecutive days (treatment group; $n = 29$); B = no bolus (control group; $n = 23$). All cows received the treatment for clinical ketosis according to the site protocol which included intravenous dextrose and oral propylene glycol. Daily rumination and milk production data were collected from 2 days prior to the ketosis diagnosis through to 5 days post-diagnosis. Ketone levels were checked again at Day 5.

2.4 Treatment of hypophosphatemia within 12 h after calving

The hypothesis of the study was that post-partum phosphorous supplementation in herds with hypophosphatemia will stimulate appetite and recovery from calving in the transition period. This will increase milk production and decrease post-partum health events. As hypocalcemia is common in post-partum cows, supplementing cows with calcium after calving is often a routine practice. A total of 276 Holstein dairy cows, in their second lactation and greater, from four dairy herds were enrolled. Enrolled herds were located in the provinces of Alberta (two herds), Ontario (one herd), and Québec (one herd). In these herds, 50% of cows were below the 1.3 mmol/L study selection threshold, with phosphorous levels varying from 0.66 to 2.16 mmol/L. The Phosphorous was analyzed on an Element DC5x Veterinary Chemistry Analyzer (HESKA, Barrie, Ontario, Canada). All cows that calved in both the treated and control groups received 2 calcium boluses (Cal-Boost, Solvet, Calgary, AB) within 12 h of calving. Even numbered cows received an additional 2 boluses of K Phos-Boost (Treatment) while odd numbered cows did not receive the K Phos-Boost boluses (Control). All cows were tested for subclinical ketosis between 7 and 14 days in milk using blood taken from the coccygeal vein for evaluation of ketone levels (Freestyle Precision Xtra ketone strip, Abbott Labs). Animals with a blood ketone result of 1.2 mmol/L or greater were considered to have subclinical ketosis. Health events (milk fever, retained placenta, clinical ketosis, displaced abomasum, mastitis, respiratory disease) were recorded for the first 60 days in milk. Milk production on days 30 and 60 as well as peak milk was obtained from the dairy herd management software.

2.5 Statistical analyses

Statistical analyses were performed essentially as described elsewhere (15, 16). The experimental unit was defined as each individual animal. Health events for mildly hypophosphatemic cows were analyzed by Fisher's Exact Test. BHB data for ketotic cows was analyzed using a two-tailed, unpaired T-test; normality was confirmed with a Shapiro-Wilk test. All other data were analyzed using a mixed-effects model, wherein time, treatment, and the time-by-treatment interaction effects were considered fixed, while animal and residual effects were considered random. In all cases, the significance level was $P < 0.05$, while a trend was defined as a P-value between 0.05 and 1.0. Statistical analyses were conducted in Prism v 9.5.1 (GraphPad Software, San Diego, CA, USA).

2.6 Ethics approval and consent

The present field-based study was conducted in compliance with the best practice of veterinary care in accordance with the research guidelines set forth by the Canadian Council on Animal Care and each study was reviewed by an animal ethics committee. The owners of the cattle provided informed consent for their animals to be used in the present study.

3 Results

3.1 Study 1: pharmacokinetics in post-partum dairy cows

The serum phosphorus, potassium, calcium, magnesium, sodium, and chloride levels in treated cows (two K Phos-Boost

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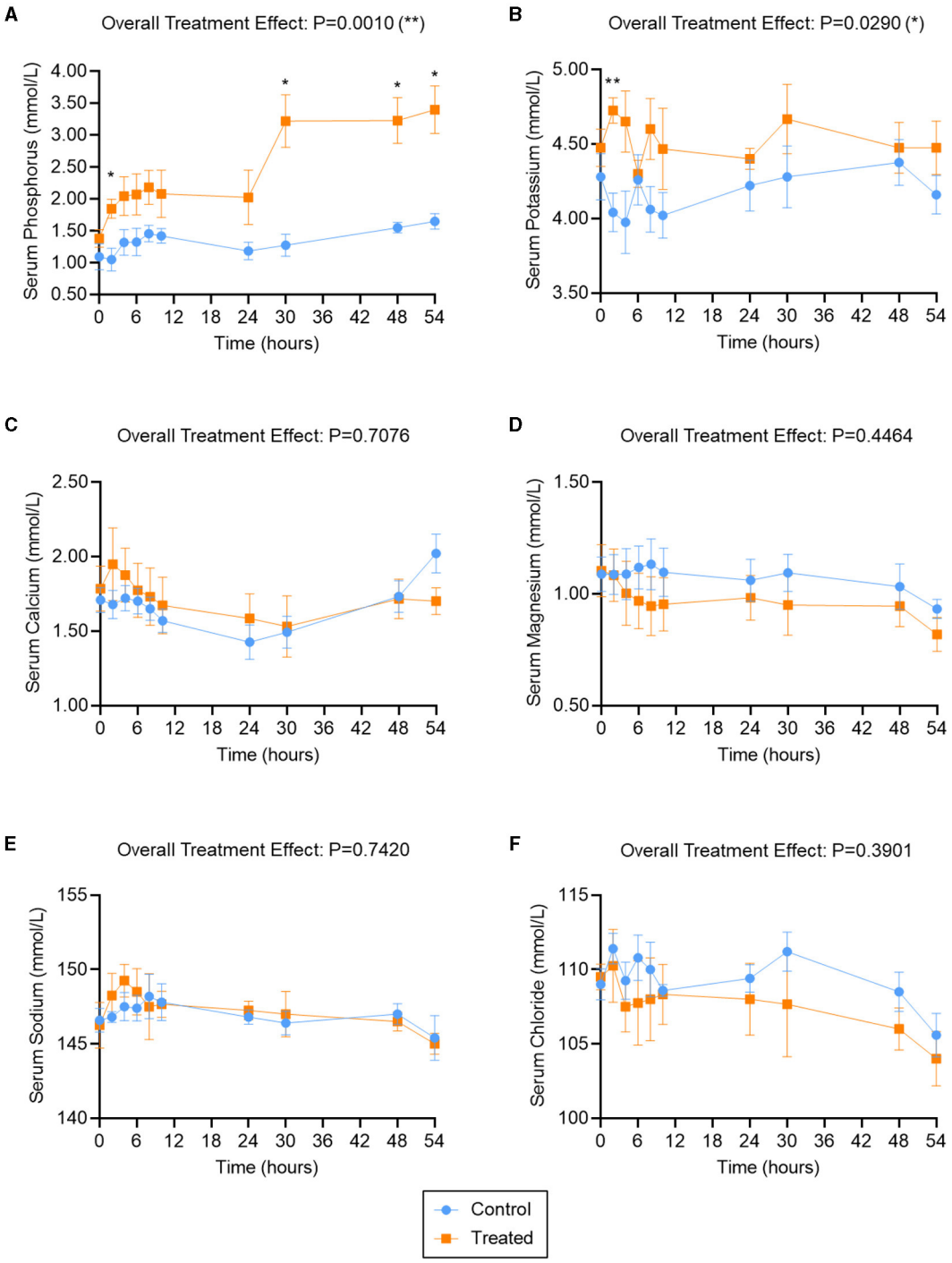

FIGURE 1
Serum ion levels in second- and third-lactation cows treated with K Phos-Boost boluses (Treated) vs. untreated (Control). Serum Phosphorous **(A)**, Potassium **(B)**, Calcium **(C)**, Magnesium **(D)**, Sodium **(E)**, or Chloride **(F)** were measured at the indicated time points; two boluses were administered to the Treated group at times 0, 24, and 48 h. Bars represent the mean ± SEM (Standard Error of the Mean) for 5 (Control) or 4 (Treated) animals. Data were analyzed for statistical significance using a mixed model with repeated measures. *$P < 0.05$; **$P < 0.01$.

boluses at times 0, 24, and 48 h) and control (untreated control cows) are provided in Figure 1. Overall, a significant ($P = 0.001$) increase in serum phosphorous was observed in bolus-treated cows, relative to untreated cows, over the 54-h time course (Figure 1A). The increase in serum phosphorous was significant as early as 2 h after administration of the first boluses and increased sharply after administration of the second set of boluses (at $t = 24$ h), and was significantly elevated, relative to untreated animals, at $t = 30$, 48, and 54 h (Figure 1A). Serum potassium levels were also significantly elevated overall ($P = 0.029$) in bolus-treated vs.

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TABLE 1 Effects of K Phos-Boost bolus treatment on downer cows.

Case	P (mmol/L) ($T = 0$ h)	P (mmol/L) ($T = 24$ h)	Ca (mmol/L) ($T = 0$ h)	Ca (mmol/L) ($T = 24$ h)	Outcome	P and Ca status
1	1.25	N/A	2.40	N/A	Recovered	HypoP
2	1.00	N/A	1.26	N/A	Recovered	HypoP + HyoCa
3	0.68	N/A	2.61	N/A	Recovered	HypoP
4	1.14	N/A	1.53	N/A	Recovered	HypoP + HyoCa
5	0.85	N/A	0.78	N/A	Recovered	HypoP + HyoCa
6	0.95	N/A	2.47	N/A	Recovered	HypoP
7	0.72	3.29	1.00	1.53	Recovered	HypoP + HyoCa
8	1.06	N/A	1.29	N/A	Recovered	HypoP + HyoCa
9	0.88	N/A	1.86	N/A	Recovered	HypoP + HyoCa
10	0.61	N/A	0.77	N/A	Recovered	HypoP + HyoCa
11	0.43	N/A	1.10	N/A	Recovered	HypoP + HyoCa
12	0.70	N/A	2.95	N/A	Recovered	HypoP
13	1.20	N/A	2.30	N/A	Deceased	HypoP
14	0.79	1.09	2.81	2.18	Deceased	HypoP
15	0.63	N/A	2.11	N/A	Deceased	HypoP + HyoCa
16	0.54	3.02	1.22	2.58	Recovered	HypoP + HyoCa
17	1.23	2.02	2.17	2.30	Recovered	HypoP
18	0.20	2.61	0.83	2.51	Recovered	HypoP + HyoCa
19	0.79	N/A	1.32	N/A	Recovered	HypoP + HyoCa

untreated cows, with a sharp and significant increase at $t = 2$ h after administration of the first boluses (Figure 1B). Potassium levels did not remain elevated (compare Figures 1A, B) and returned to normal levels within 12 h. Subsequent treatments with the K Phos-Boost bolus did not yield a significant increase in potassium levels 6 h after receiving the bolus. None of the other ions tested (calcium, magnesium, sodium, or chloride) were significantly different in bolus-treated vs. untreated cows over the 54-h time course (Figures 1C–F).

3.2 Study 2: treatment of hypophosphatemic downer cows

The study yielded data for 19 Holstein cows from 17 different herds (Table 1). Blood samples collected immediately before K Phos-Boost bolus treatment indicated that 7 of the 19 cows were hypophosphatemic (serum phosphorus <1.30 mmol/L) with normal serum calcium (calcium >2.12 mmol/L), and 12 of the cows were both hypophosphatemic (phosphorus <1.30 mmol/L) and hypocalcemic (calcium <2.12 mmol/L). Sixteen cows fully recovered and were standing between 1 and 24 h following treatment with two K Phos-Boost boluses. The serum phosphorous and calcium were collected 24 h after treatment with K Phos-Boost boluses in five cows and demonstrated that serum phosphorus was normal (4 out of 5 cows) or returning to normal (one out of five cows). Three cows (13, 14, and 15) remained as downer cows and were euthanized. Two of the cows that died (13 and 14) had been downers for over 2 days despite having normal serum calcium levels but were hypophosphatemic. Cow 15 was both hypophosphatemic and hypocalcemic but did not respond to calcium and phosphorous supplementation.

3.3 Study 3: effects of the K Phos-Boost bolus on rumination, milk production, and ketosis in cows with clinical ketosis

No statistically significant differences in rumination rate, milk production, and BHB were observed between treatment and control groups at day 0 (Supplemental Figure S1). The change in rumination rate, milk production, and BHB, relative to their respective day 0 levels, are reported for each treatment group in Figure 2.

The rumination rate increased for both treatment groups over the 5-day observation period (Figure 2A). Overall, mixed effects analysis indicated that rumination tended to be improved in dipotassium phosphate bolus-treated vs. untreated animals over the observation period (treatment effect: $P = 0.09$). Rumination rate was significantly different between treatments and controls on day 4 (91 min/day; $P = 0.04$) and day 5 (97 min/day; $P = 0.02$; Figure 2A).

Milk production also increased for both treatment groups over the 5-day observation period (Figure 2B). Overall, milk production was significantly improved in bolus-treated vs. untreated animals

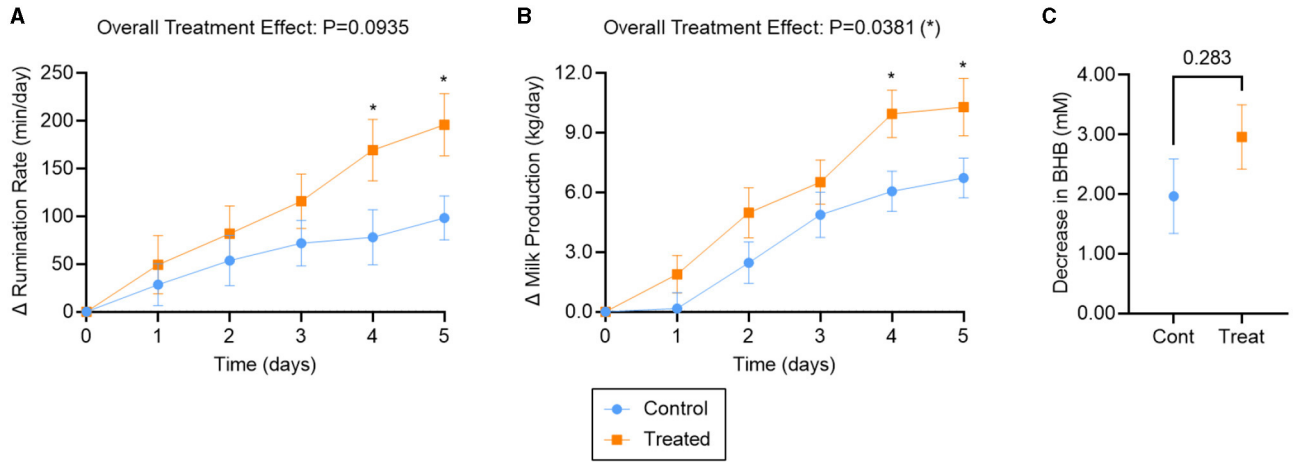

FIGURE 2
Outcomes for ketotic cows treated with K Phos-Boost boluses (Treated) vs. untreated (Control). **(A)** Increase in Rumination Rate (min/day) relative to Day 0, **(B)** increase in Milk Production (kg/day) relative to Day 0, **(C)** decrease (by Day 5) in serum Beta-Hydroxybutyrate (mM) relative to Day 0. Two boluses were administered to the Treated group at times 0, 24, and 48 h. Bars represent the mean ± SEM (Standard Error of the Mean). For **(A)**, $n = 22$ (Control) or 27 (Treated); for **(B)**, $n = 23$ (Control) or 29 (Treated) animals. A subset of those animals was examined for BHB **(C)**, such that $n = 9$ (Control) or 20 (Treated). Data in **(A)** and **(B)** were analyzed for statistical significance using a mixed model with repeated measures; data in **(C)** were analyzed with an unpaired, two-tailed *T*-test. *$P < 0.05$.

TABLE 2 **Health events for mildly hypophosphatemic cows treated with K Phos-Boost at calving vs. untreated controls.**

Event	Control	K Phos-Boost	*P*-value[a]
SCK	21/146 (14.4%)	12/126 (9.5%)	0.27
Health Event	34/146 (23.3%)	24/130 (18.5%)	0.38
Culled/Died	3/146 (2.1%)	6/130 (4.6%)	0.31

[a] *P*-values were determined using Fisher's Exact Test.

(treatment effect: $P = 0.04$), with significantly higher milk production by days 4 (3.9 kg/day; $P = 0.02$) and 5 (3.6 kg/day; $P = 0.04$; Figure 2B).

BHB levels decreased back to normal levels by Day 5 for both treatment groups (Supplemental Figure S1C). No statistically significant difference in BHB was observed between bolus-treated and untreated animals over the 5-day observation period ($P = 0.28$; Figure 2C).

3.4 Study 4: effects of the bolus on subclinical ketosis, milk production, and post-parturient health events when administered to hypophosphatemic herds within 12 h after calving

Relative to the control group, dipotassium phosphate supplemented cows had a lower incidence of subclinical ketosis (9.5% vs. 14.4%) and post-calving total health events (18.5% vs. 23.3%), although these did not reach statistical significance ($P = 0.27$ and 0.38, respectively). Control and treated animals also had a statistically similar culling rate (4.6% vs. 2.1%, $P = 0.31$; Table 2).

Overall, there was no statistically significant difference in milk production between the two groups (Figure 3), although 2nd-lactation animals tended to have higher production after 30 days in milk (DIM) for the treated vs. control group (mean: 49.1 vs. 46.2 kg/day; SEM: 0.9 vs. 1.4 kg/day; $P = 0.09$; Figure 3A).

4 Discussion

Metabolic disorders are associated with disturbances of one or more blood metabolites of cattle which result in a group of diseases that most commonly affect dairy cows during the first month after parturition (2, 3, 17). These diseases include milk fever, downer cows, retained placenta, ketosis, left displaced abomasum, rumen acidosis, laminitis, liver abscesses, and bloat. There is a strong association of these diseases with each other, although the mechanisms are not well understood. Approximately 75% of the metabolic diseases occur within the periparturient period (3 weeks before calving and 3 weeks after calving). This period is associated with decrease serum concentrations of macrominerals and glucose (1, 18).

It has been suggested that approaches to metabolic diseases need to be updated because of new findings about these disorders (2). The studies presented in this paper demonstrate the need for further research in post-parturient phosphorus and potassium supplementation to address metabolic diseases in dairy cattle. The dipotassium phosphate bolus provides a convenient tool to further investigate the benefits of oral supplementation of potassium and phosphorus.

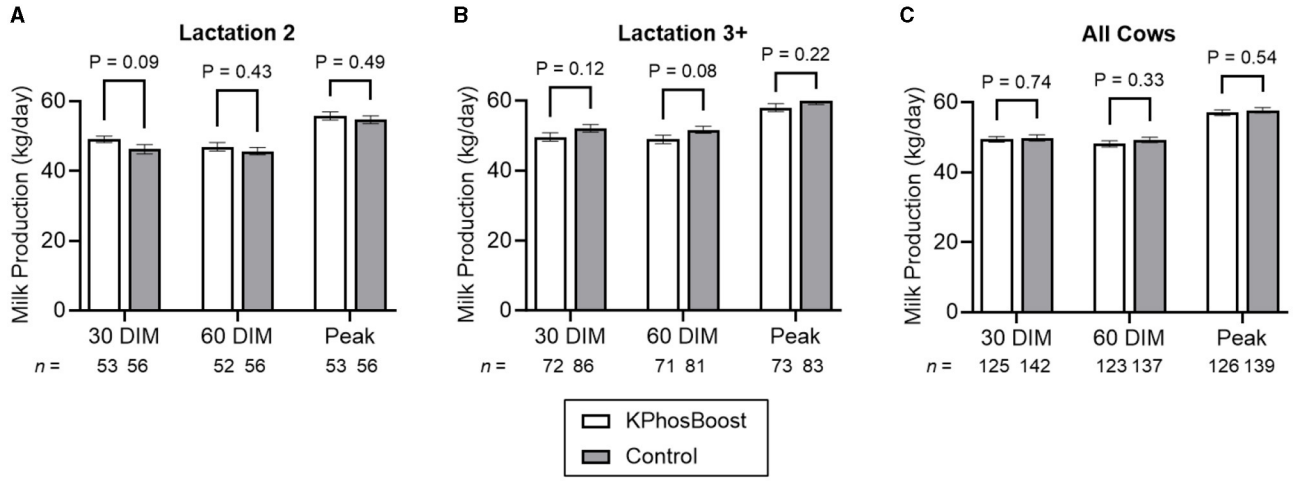

FIGURE 3
Milk production in hypophosphatemic cows treated with K Phos-Boost, vs. untreated Controls, within 12 h of calving. **(A)** Second lactation animals only. **(B)** Animals in their third or higher lactation. **(C)** All animals. Bars represent the mean ± SEM (Standard Error of the Mean) for the indicated number of animals. Data were analyzed for statistical significance using an unpaired, two-tailed *T*-test.

4.1 Pharmacokinetics

Within 2 h of treatment with the potassium phosphate bolus (62 g of phosphorus), serum phosphorus was significantly increased compared to controls (Figure 1A) and within the normal range (>1.3 mmol/L). This rapid response is similar to that observed when cattle were drenched with 60 g of phosphorous as sodium phosphate (NaH_2PO_4), disodium phosphate (Na_2HPO_4), or mono potassium phosphate (KH_2PO_4) (6–8). Previous studies have shown that calcium phosphate ($CaHPO_4$) or magnesium phosphate ($MgHPO_4$) had a delayed and less pronounced change in serum phosphorus compared to corresponding sodium or potassium salts (6–8). The dipotassium phosphate boluses maintained an elevated serum phosphorous for over 24 h and a second treatment with 2 boluses increased these levels from 2.0 mmol/L to 3.0 mmol/L. The third treatment at 48 h did not elicit a significant increase in serum phosphorous. This study provides evidence that the dipotassium phosphate bolus provides the necessary phosphorous supplementation to address the hypophosphatemic state in dairy cows.

Treatment with two dipotassium phosphate boluses (166 g of potassium) rapidly increased serum potassium within 2 h of administration. This increase was transient, and the serum potassium was at pre-treatment levels within 6 h of administration. A similar response was elicited following the 24- and 48-h treatments. The reduction in serum potassium is most likely associated with either movement of potassium into the intracellular compartment, or it being eliminated in the urine or in the milk. Oral administration of potassium chloride or potassium propionate has shown a similar rapid increase in serum potassium with peak levels at ∼6 h (10, 12). It was shown that there was no difference in serum response when administration of potassium chloride at 0.5 g/kg BW was delivered as multiple small doses or two individual doses (10). This suggests that a single daily treatment with the dipotassium phosphate bolus meets the daily potassium requirements.

There is a concern that oral phosphate supplementation may cause transient hypocalcemia and hypomagnesemia due to interference of rumen absorption of minerals (2, 5, 10, 19, 20). In this study, there was no significant change in serum calcium or magnesium up to 5 days following treatment with dipotassium phosphate bolus. There was probably insufficient phosphate in the bolus or the duration of treatment was too short to cause hypocalcaemia or hypomagnesemia.

4.2 Treatment of downer cows

Both veterinarians and producers are frustrated that, despite treatment, downer cows are often still unable to get up. Parturient paresis is common in high-producing dairy cows. Affected cows are frequently hypocalcemic and hypophosphatemic. Most cattle respond clinically by standing following intravenous and/or subcutaneously administered calcium borogluconate. However, some cows do not respond and are defined as downer cows. Cows that are unable to stand with normal serum calcium (>2.12 mmol/L) but low serum phosphorus (<1.3 mmol/L) are referred to as alert downer cows. In this study, we defined downer cow syndrome as "a cow that is in sternal recumbency for more than 24 h that is bright and alert." Downer cow syndrome has been poorly understood and may be associated with hypophosphatemia and hypokalemia that results in muscle weakness, muscle necrosis and myoglobinuria (2, 14, 21). Phosphorous is necessary for the synthesis of adenosine triphosphate for membrane integrity and a source of energy (1, 2, 4). Potassium is a principal ion involved in maintenance of muscle function and nerve impulse transmission which makes supplementation of downer cows with potassium a rational therapeutic measure (2, 11, 22).

Survival percentage of downer cows is generally poor and has been reported to be between 16 to 50% (14, 21). Any intervention that improves outcomes would be welcomed. In this study, 16 of the 19 downer cows (84%) survived following treatment with the dipotassium phosphate boluses.

It is believed that both persistent hypophosphatemia and hypokalemia contribute to prolonged recovery (2, 22, 23) and the dipotassium phosphate bolus addresses these deficiencies. In this study, it was shown that the bolus increased serum phosphorous in all five treated animals that were analyzed. The literature supports that oral administration of phosphate and potassium is the method of choice for treatment of clinical hypophosphatemia and hypokalemia (2, 4, 11). As noted previously, this field study had inherent limitations by using historical response rates to downer cows, but the high survival rate in animals treated with dipotassium phosphate boluses was a remarkable finding and warrants additional research.

4.3 Treatment of ketosis

Ketosis occurs in early lactation when energy demands exceed dietary energy intake with the mobilization of adipose tissue (2). Early treatment is beneficial for positive clinical outcomes and economic returns (2, 3). The most common clinical sign of hypophosphatemia is reduced feed intake (4, 5) which may initiate or exacerbate the clinical ketosis. Oral phosphate is an appetite stimulant (5, 8) and therefore has the potential to improve energy intake and may therefore be valuable in the treatment of ketosis. This study suggests that oral supplementation with dipotassium phosphate boluses results in an improved recovery from clinical ketosis as indicated by increasing rumination rate and milk production. The suggested mechanism of action was increased feed intake due to appetite stimulation by phosphate supplementation, thereby correcting the energy imbalance. It has been shown that the most common clinical sign of hypophosphatemia is depressed feed intake (24).

Decrease in feed intake that occurs in clinical ketosis can also result in hypokalemia (10–12, 25). The provision of supplemental potassium may also have acted to stimulate gastrointestinal motility and appetite stimulation, thereby improving the recovery rate from clinical ketosis.

4.4 Post-partum treatment with K Phos-Boost boluses

Dairy cattle are extremely vulnerable to the development of metabolic disorders in the post-partum period where levels of macro minerals and glucose are rapidly changing. Over 50% of dairy cattle are deficient in phosphorous during the early post-partum period (1, 4, 5) when metabolic disease such as hypocalcemia and ketosis are common (3). Metabolic health is also important for decreasing the risks of infectious diseases such as mastitis and metritis (3). This study attempted to demonstrate a beneficial effect of post-partum treatment with dipotassium phosphate boluses on metabolic health and disease prevention. Although we were unable to demonstrate a statistical reduction of subclinical ketosis and health events, there was a tendency toward a beneficial effect in milk production in second-lactation cows in the first 30 days in milk ($P = 0.09$) in dipotassium phosphate treated cows which may indicate an improvement in metabolic health in this group of cattle. Further research with larger sampling sizes is recommended. This study provides the variances that are necessary to perform power calculations required for future study design.

5 Conclusion

The dipotassium phosphate bolus is a novel, easy to administer product and has the potential to treat and prevent several important metabolic diseases in dairy cattle. The studies described in this paper are early investigations and further research will be required to further demonstrate the applications of a dipotassium bolus in dairy cows.

Data availability statement

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

Ethics statement

The animal studies were approved by Alberta Veterinary Laboratories' Animal Ethics Committee. The studies were also reviewed and approved by Alberta Veterinary Laboratories' field veterinarian in consultation with the practicing veterinarian associated with the study site. 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

WV: Conceptualization, Formal analysis, Investigation, Methodology, Project administration, Writing—original draft, Writing—review & editing. SZ: Conceptualization, Formal analysis, Investigation, Methodology, Project administration, Writing—original draft, Writing—review & editing. JR: Data curation, Formal analysis, Methodology, Visualization, Writing—original draft, Writing—review & editing. KB: Data curation, Formal analysis, Investigation, Methodology, Visualization, Writing—review & editing. MO: Conceptualization, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Supervision, Writing—original draft, Writing—review & editing.

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Conflict of interest

JR and KB are employed by Chinook Contract Research Inc. WV and SZ were employed by Alberta Veterinary Laboratories, Ltd./Solvet Animal Health (AVL/Solvet, Calgary, Alberta, Canada). MO is employed by AVL/Solvet. The authors declare that this study received funding from AVL/Solvet. The funder had the following involvement in the study: manufacturing of the dipotassium phosphate boluses used in this study.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2023.1274183/full#supplementary-material

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Enhancing rumen microbial diversity and its impact on energy and protein metabolism in forage-fed goats

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Introduction: This study explores if promoting a complex rumen microbiota represents an advantage or a handicap in the current dairy production systems in which ruminants are artificially reared in absence of contact with adult animals and fed preserved monophyte forage.

Methods: In order to promote a different rumen microbial diversity, a total of 36 newborn goat kids were artificially reared, divided in 4 groups and daily inoculated during 10 weeks with autoclaved rumen fluid (AUT), fresh rumen fluid from adult goats adapted to forage (RFF) or concentrate (RFC) diets, or absence of inoculation (CTL). At 6 months of age all animals were shifted to an oats hay diet to determine their ability to digest a low quality forage.

Results and discussion: Early life inoculation with fresh rumen fluid promoted an increase in the rumen overall microbial diversity which was detected later in life. As a result, at 6 months of age RFF and RFC animals had higher bacterial (+50 OTUs) and methanogens diversity (+4 OTUs) and the presence of a complex rumen protozoal community (+32 OTUs), whereas CTL animals remained protozoa-free. This superior rumen diversity and presence of rumen protozoa had beneficial effects on the energy metabolism allowing a faster adaptation to the forage diet, a higher forage digestion (+21% NDF digestibility) and an energetically favourable shift of the rumen fermentation pattern from acetate to butyrate (+92%) and propionate (+19%) production. These effects were associated with the presence of certain rumen bacterial taxa and a diverse protozoal community. On the contrary, the presence of rumen protozoa (mostly *Entodinium*) had a negative impact on the N metabolism leading to a higher bacterial protein breakdown in the rumen and lower microbial protein flow to the host based on purine derivatives urinary excretion (-17% to -54%). The inoculation with autoclaved rumen fluid, as source of fermentation products but not viable microbes, had smaller effects than using fresh inoculum. These findings suggest that enhancing rumen microbial diversity represents a desirable attribute when ruminants are fed forages in which the N supply does not represent a limiting factor for the rumen microbiota.

KEYWORDS

energy metabolism, forage digestion, multi-kingdom, protein metabolism, protozoa, rumen microbiota, ruminants

Introduction

Evolution has allowed ruminants to develop a complex, multi-chambered forestomach and a system of regurgitation and rumination in order to establish an enhanced rumen microbial fermentation. This anaerobic fermentation is conducted by rumen bacteria, methanogenic archaea, anaerobic fungi, protozoa and phages and provides several competitive advantages but also some drawbacks. Increased rumen microbial protein synthesis and fiber digestion are thought to represent important overall evolutionary advantages for wild ruminants grazing (or browsing) highly diverse forages and bushes (1). However, in modern ruminant production systems ruminants are often fed elevated proportions of highly fermentable feeds in which the concentrate can represents up to 55, 85, and 92% of the total diet for dairy cows, dairy goats and feedlot systems, respectively (2). This type of diets often leads to digestive disorders such as rumen acidosis and diarrhea which often require the use of feed additives to maintain productivity and health (3). On the contrary, in meat-orientated systems or low nutrient requiring situations, ruminants are often fed low quality diets with a high proportion of forage (up to 100%). Additionally, there is an increasing trend to feed ruminants with preserved forages (i.e., hay and silage) which are often made with a single botanical species, aspect that represents an over-simplification of the diets which ruminants were originally developed for.

In relation to the rearing system, it has been demonstrated that a progressive rumen microbial colonization and functional development occur when young ruminants are reared with the dam or adult companions, allowing a natural rumen microbial transfer to the offspring (4) and feeding behavior learned from the adults (5) resulting on a superior forage digestion and animal growth than artificially reared ruminants (6). On the contrary, in modern intensive dairy systems, the newborns are usually separated from their dams after birth and fed milk replacer or whole milk. This absence of contact with adult animals often leads to a delay in the rumen microbial and physiological development that can persist as long as the animals are not in contact with adult ruminants (7, 8). In the last decades, important efforts have been made to optimize the artificial rearing of young ruminants such as improvements of the colostrum feeding, the development of high-quality liquid feed, texturized starter feeds, feed additives with biologically active substances or the implementation of high quality forages (9). This has allowed to optimize the anatomical and physiological development of the rumen mucosa and associated papillae which is key for a successful post-weaning process (10). Moreover, several authors have evaluated the effects of inoculating young ruminants with fresh (11–13) or lyophilized rumen fluid from adult ruminants (14, 15). Most of these studies have reported an acceleration of the rumen microbial and functional development. In a previous publication related to the present experiment, it was noted that the inoculation with fresh rumen fluid had positive effects during the weaning process (16), however, the persistency of these effects later in life and the potential effects on the productive performances remain unknown.

The objectives of this study were to investigate the long-term effects of enhancing the rumen microbial diversity in forage-fed goats by early-life inoculation with rumen fluid from adult ruminants. A multi-kingdom meta-taxonomic community analysis including bacteria, methanogens, protozoa and anaerobic fungi was performed to have a detailed description for the rumen microbiome, and to identify the key microbes associated with changes in the rumen fermentation, feed utilization and productive performance.

Materials and methods

Inocula preparation

This study involved the inoculation of young goat kids with various types of rumen inocula obtained from adult goats to enhance rumen microbial diversity. A comprehensive description of the inocula preparation process has been published in previous studies (13, 16). Briefly, four adult goats, each equipped with a permanent rumen fistula, were fed two different diets. Four of them were fed a forage diet consisting of oats hay, while the remaining four received a high-concentrate diet (75% concentrate feed and 25% oats hay) to generate distinct rumen microbial inocula. After 2 weeks of adaptation to the diet, rumen fluid from the donor goats fed forage (RFF) or concentrate diet (RFC) were collected daily 2 h after the morning feeding, pooled by diet, strained through a cheesecloth, maintained in anaerobic conditions in a thermal flask and orally inoculated as fresh inoculum to young goat kids. Additionally, autoclaved inoculum (AUT) was generated weekly by combining equal volumes of RFF and RFC inocula. This mixture was autoclaved at 115°C for 30 min to lyse all microbes while preserving the rumen fermentation products. Samples from each type of inocula were collected for inocula characterization.

Inoculation

A total of 36 newborn Murciano-Granadina goat kids were nourished with natural colostrum as previously described (16). These kids were randomly allocated into 4 experimental treatments ($n = 9$) which were kept physically separated during the entire experiment. The experiment consisted in three periods (Figure 1): (1) the inoculation period (from birth to week 10 of age) to promote a different degree of rumen microbial development, (2) the fattening or wash out period (from week 10 to 25 of age), and (3) the sampling period after the animals were shift to a full forage diet (from week 26–28 of age).

Experimental treatments involved oral and daily inoculation (2.5 mL/animal during week 1 and 5 mL/animal thereafter) with AUT, RFF, RFC or absence of inoculation (CTL). All goat kids were fed a commercial milk replacer (Univet Spray, Cargill, Barcelona, Spain, declared composition in DM: 92.8% OM, 24% CP and 22% EE) which was freshly prepared by mixing with warm water (170 g/L) twice per day (at 09:00 and 17:00 h) and offered *ad libitum*. From 2 weeks of age, all animals had *ad libitum* access to a pelleted concentrate (0–14 Rumiantes Transición, Macob, Granada, Spain, chemical composition in g/kg DM, OM 938, CP 199, NDF 323, ADF 132, ADL 15) and oats hay (OM 930, CP 79, NDF 634, ADF 280, ADL 55). At 7 weeks of age, all animals were weaned by gradually decreasing the solids concentration of milk replacer during 4 consecutive days (−20, −40%, −60 and −80%, respectively). During the fattening or wash out period the animals remained physically separated in four groups and were fed the same concentrate feed and oats hay as described before (both *ad libitum*), but they did not

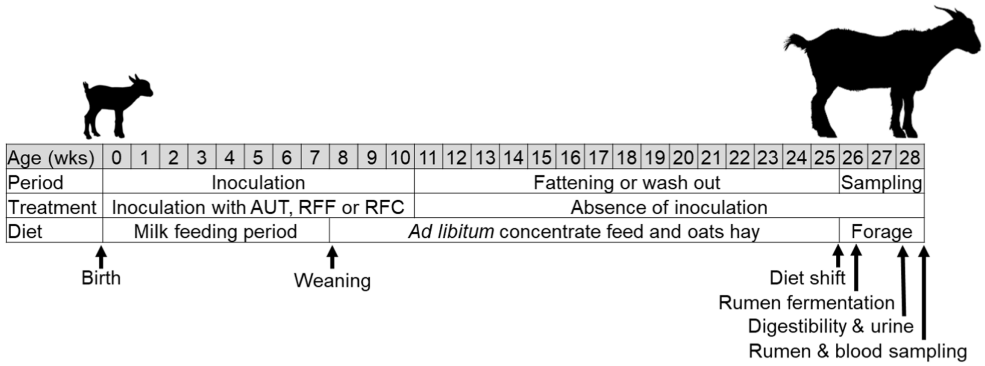

FIGURE 1
Ilustration of the diferent stages of the experimental set up. Animals received an daily and oral inoculation with autoclaved rumen fluid (AUT), rumen fluids from adult animals fed forage (RFF), or fed concentrate diets (RFC) or without inoculation (CTL).

receive any further inoculation. This allowed to assess the persistency of the treatment effects later in life. Finally, at 26 weeks of age, an abrupt dietary shift was implemented, involving the removal of access to concentrate feed while providing access to forage (oats hay) as the sole dietary ingredient. This abrupt change in diet served as a means to assess the animals' ability to adapt to a forage diet.

Rumen and blood sampling

Animals were housed in individual pens (2 × 2 m) and daily feed intake and BW change was monitored during 21 days after the dietary change. Rumen fermentation was evaluated at 4 and 21 d after this dietary shift. Rumen content (approximately 50 mL) was withdrawn for each animal by oro-gastric intubation at 0900 h as previously described (17). Rumen samples were filtered through a cheesecloth and solids discarded given their small and variable proportion in the samples. Then, pH was immediately measured and 3 subsamples were taken for volatile fatty acids (VFA), ammonia and lactate determinations, as previously described (18). Rumen VFA concentrations were determined by a GC system coupled with a Flame Ionization Detector (Auto-System, Perkin Elmer, Waltham, MA) whereas ammonia (19) and lactate concentrations (20) were measured using a colorimetric methods. Moreover, two additional sub-samples were collected on day 21, one was snap frozen in liquid N to describe the rumen microbiota, while the other sample (12.5 mL) was mixed with 37.5 mL of anaerobic buffer and incubated at 39°C for 24 h in 120-ml Wheaton bottles to determine gas and CH_4 production *in vitro*. Blood samples (4 mL) were collected at 0900 h from the jugular vein (on day 21), placed in tubes without anticoagulant, centrifuged at 2,000 x *g* for 15 min. Serum samples were frozen and sent to the "Laboratorio de Técnicas Instrumentales" from University of Leon (Spain) to determine concentrations of glucose, β-hydroxybutyrate, blood pH, pCO_2, tCO_2, Na^+, K^+, $HCO3^-$, Cl^- and anion gap using an auto-analyzer (BA400, Bio-Systems, Barcelona, Spain). To assess the potential effects of the experimental treatments on the stress levels induced by the dietary shift, cortisol concentration in hair was measured as previously reported (16). Briefly, a surface of 25 cm^2 in the dorsal neck was shaved before (day 0) and after 21d the dietary shift and cortisol concentration was measured using a commercial kit (Cortisol ELISA Saliva, ALPCO, Salem, NH).

Digestibility and microbial protein synthesis

After 17d of adaptation to the diet, goats were placed in metabolic crates to determine feed digestibility during 4 consecutive days. Due to the limited number of metabolic crates, goats were divided into two periods with equal number of goats per treatment within each period. Feed intake and fecal excretion were daily monitored and feed refusals were sampled, pooled per animal and analyzed to calculate the feed nutrients intake and digestibility. Water consumption was recorded and urine excretion was collected in buckets containing 50 mL of H_2SO_4 (10% vol/vol). Aliquots representing 10% of the daily fecal and urinary production were pooled and stored at −20°C for further analyses.

Chemical composition

The starter concentrate used in this study was freshly made in a commercial mill (Cereales Macob, Granada, Spain) and consumed before the expiration date to prevent the potential presence of mycotoxins and its negative effects on productivity and health (21). Dry matter (DM) and organic matter (OM) concentrations were determined using the method 934.01, 942.05, respectively (22). Nitrogen (N) content was also measured in solid (method 990.03) and urine samples (method 993.13). Neutral detergent fiber (NDF) and acid detergent fiber (ADF) were measured (23) using an Ankom 220 fiber analyzer unit (Ankom Technology Crop., Macedon, NY) with α-amylase and expressed without residual ash. The concentration of purine derivatives (PD) and creatinine in urine samples were determined using a HPLC system (24) using allopurinol as an internal standard.

Rumen microbiota

For rumen microbial characterization frozen samples were freeze-dried, physically disrupted by bead-beating for 1 min and DNA was extracted using a commercial kit (QIAamp DNA Stool Mini Kit, Qiagen LTd., Barcelona, Spain). Rumen concentration of the different microbial groups were determined by qPCR using serial dilutions of microbial standards (6) and specific primers for the 16S rRNA gene

for bacteria, the mcrA gene for methanogens and 18S rRNA genes for protozoal and anaerobic fungi (Supplementary Table S1).

For rumen meta-taxonomic analyses, DNA samples were sent to University of Illinois Biotechnology Center (Urbana, IL, USA) for amplicon sequencing using Miseq V3 (Ilumina Inc., San Diego, CA, USA) as previously described (13). Briefly, specific primers sets were used to amplify bacterial 16S (V3-V5 region), methanogens 16S (V3-V4 region), protozoal 18S (V4-V6 region) and anaerobic fungi ITS3-ITS4 regions (Supplementary Table S1). For each of the 4 major microbial groups, samples were primer-sorted, demultiplexed and paired-end reads were merged into one file. Downstream analysis was performed using QIIME2 (Version 2021.4) for bacteria and methanogens (25), PIPITS for fungi (26) and IM-Tornado for protozoa (27). Low-quality reads (<Q25) were trimmed and chimeras were removed using chimera.vsearch (28). All sequences were grouped into operational taxonomic units (OTUs) with a similarity cut-off of 97%. The resulting OTUs were taxonomically classified using the Silva_138 database (29) for bacteria and protozoa, RIM-DB for methanogens (30) and UNITE for fungi (31). After taxonomical classification, data from each of the 4 major microbial groups was processed separately. The number of sequences per sample was normalized for each microbial group and singletons were removed. The relative abundance of each OTU was determined along with the Good's coverage, and alpha diversity.

Calculations and statistical analyses

Statistical calculations were performed using SPSS software (IBM Corp., Version 21.0, NY, USA). For rumen fermentation data were analyses based on a repeated measures mixed-effects (residual maximum likelihood) as follows:

$$Y_{ijkl} = \mu + I_i + T_j + \left(I \times T\right)_{ij} + G_k + A\left(G\right)_l + e_{ijkl}$$

Where Y_{ijkl} is the dependent, continuous variable, μ is the overall population mean, I_i is the fixed effect fo the inoculation (I = CTL vs. AUT vs. RFF vs. RFC), T_j is the fixed effect of the sampling time (j = 4d vs. 21d), $(I \times T)_{ij}$ is the interaction term, G_k is the random effect of the period (k = 1 vs. 2), $A(G)_l$ is the random effect of the animal nested to the period (l = 1–36), and e_{ijkl} is the residual error. For blood metabolites, digestibility, urinary PD excretion and qPCR only one sample time was considered (21d) and data were analyzed by ANOVA excluding the time as a factor. Fermentable organic matter (FOM) was calculated according to (32). Taxa abundances (in %) were tested for normality using the Shapiro–Wilk test and data were analyzed using the Kruskal-Wallis non-parametric test. False discovery rate was minimized by using the Bonferroni *post hoc* test. Significant effects were declared at $p < 0.05$ and tendency to difference at $p < 0.1$.

Treatment effects on the bacterial, methanogens, protozoal and anaerobic fungi communities were assessed based on the Bray-Curtis distance metrics using the UPGMA function (PRIMER-6 software, PRIMER-E Ltd., Plymouth, UK). Abundances of each OTU were log10-transformed and data were analyzed by PERMANOVA after 999 random permutations of residuals under the reduced model using the Montecarlo test. When significances were detected, pair-wise comparisons were performed across treatments. For each microbial group, a principal Coordinate analysis (PCoA) was conducted to illustrate the impact of the treatments on the overall community structure, and tripod vectors were included to indicate the relationships between the community structure and the metadata consisting in 31 variables including rumen fermentation, digestibility, purine derivatives, blood metabolites and microbial diversity. A multi-kingdom analysis was conducted including rumen abundances from all microbial groups in order to assess the treatments effects on the overall rumen microbiome. Spearman correlations were calculated to identify relationships between the microbial taxa abundances and the metadata but only strong correlations were considered ($\rho \geq 0.4$ or ≤ -0.4 and $p < 0.001$).

Results

Inoculum and rumen fermentation

Fermentative and microbiological differences across rumen fluids used as inoculum has been previously described [Supplementary Table S2; (13)]. All animals required an adaptation process after the abrupt dietary shift from a high-concentrate to a full-forage diet as noted in the rumen fermentation data from 4 and 21 days after the dietary shift (Table 1). This adaptation included an increase in the DMI (p = 0.098), total VFA (p < 0.001), FOM (p < 0.001), butyrate (p = 0.042) and iso-acids molar proportions (i.e., iso-butyrate and iso-valerate) and lower rumen ammonia (p = 0.042). However, RFF and RFC tended to have a higher DMI (p = 0.094) at day 4 after the dietary shift. This inoculation with RFF or RFC inocula also promoted higher rumen ammonia concentration (p = 0.003) and proportions of butyrate (p < 0.001) and propionate (p = 0.023) in detriment to acetate (p < 0.001) in comparison to the CTL treatment across both sampling times. Animals inoculated with autoclaved rumen fluid showed intermediate rumen fermentation values between the CTL and the RFF or RFC animals.

Blood metabolites, digestibility and microbial protein synthesis

All animals remained in good health and no differences were noted neither on the hair cortisol levels in hair nor in the serum metabolite concentrations (Table 2). No differences between AUT, RFF and RFC were noted in terms of apparent feed digestibility, but these three treatments had substantially higher digestibility values for DM (p = 0.014), OM (p = 0.007), N (p = 0.092), NDF (p < 0.001) and ADF (p < 0.001) than CTL animals. *In vitro* gas production and CH_4 production were higher for AUT, RFF and RFC than for the CTL treatment, whereas no differences were noted when CH_4 emission was normalized for FOM.

Similar urinary and fecal N excretions were observed across treatments (Table 2), whereas CTL animals had higher creatinine excretion than RFF and RFC animals (p = 0.034). The inoculation with fresh rumen fluid promoted a negative effect on the microbial protein synthesis as noted by the lower PD excretion (p < 0.001) and PD / creatinine ratio (p = 0.014) than observed for CTL animals. The efficiency of microbial protein synthesis (EMPS), measured as PD excretion divided by the digestible OM intake, was also lower for inoculated than for the CTL animals (p < 0.001).

TABLE 1 Feed intake and rumen fermentation in 26-week-old goats measured at 4 and 21 days after a shift from a high-concentrate to a full forage diet (oats hay).

	Day	Treatments[1]					*p*-values		
		CTL	AUT	RFF	RFC	SED	Ino.	Time	IxT
DMI, g/d	4d	432^{b}	500^{ab}	502^{a}	516^{a}	39.37	0.642	0.098	0.094
	21d	517	535	493	500				
Rumen pH	4d	6.83	6.88	6.68	6.83	0.118	0.164	0.339	0.879
	21d	6.95	6.91	6.76	6.83				
N-NH_3, mg/dL	4d	1.56^{b}	2.61^{ab}	4.60^{a}	3.16^{a}	0.960	0.003	0.042	0.720
	21d	0.42^{b}	1.82^{a}	2.77^{a}	2.75^{a}				
Lactate, ng/L	4d	189	180	172	197	18.20	0.188	<0.001	0.198
	21d	66.0	110	93.6	116				
Total VFA, mmol/L	4d	44.7	43.9	54.0	49.9	10.36	0.485	<0.001	0.927
	21d	62.8	71.4	75.1	75.0				
Proportion, %									
Acetate	4d	75.3^{a}	70.3^{b}	67.5^{b}	68.5^{b}	1.373	<0.001	0.083	0.279
	21d	74.6^{a}	70.3^{b}	69.4^{b}	70.7^{b}				
Propionate	4d	16.2^{b}	19.2^{b}	20.2^{a}	18.4^{ab}	1.347	0.023	0.293	0.861
	21d	16.5	18.9	19.2	17.3				
Butyrate	4d	4.97^{c}	7.30^{b}	9.27^{a}	9.87^{a}	0.833	<0.001	0.042	0.175
	21d	7.00^{c}	8.34^{b}	9.07	9.91^{ab}				
Isobutyrate	4d	0.84	0.97	1.08	1.07	0.119	0.173	<0.001	0.321
	21d	0.67^{b}	0.92^{a}	0.79^{ab}	0.78^{ab}				
Valerate	4d	1.67^{a}	1.23^{b}	0.99^{b}	1.22^{b}	0.157	0.009	<0.001	0.190
	21d	0.93	0.79	0.72	0.78				
Isovalerate	4d	1.01	0.99	0.99	0.98	0.194	0.352	0.001	0.291
	21d	0.30^{b}	0.74^{a}	0.82^{a}	0.56^{ab}				
FOM, mmol/L	4d	23.9	23.9	29.7	28.0	5.782	0.398	<0.001	0.928
	21d	33.8	38.9	41.0	41.6				

[1]During the first 10 weeks of age goats ($N = 36$) received a daily and oral inoculation with autoclaved rumen fluid (AUT), rumen fluid from adult animals fed forage (RFF), or fed concentrate diets (RFC) or without inoculation (CTL). Means within a raw with different superscript differ ($p < 0.05$).

Rumen multi-kingdom microbiota

The multi-kingdom microbiota included all microbial OTUs from bacteria (87.4%), methanogens (2.7%), protozoa (2.1%) and anaerobic fungi (7.8%). Inoculation with fresh rumen fluid led to a substantial increase in the rumen microbial diversity (Figures 2A,B) in terms of richness ($p < 0.001$) and Shannon's index ($p < 0.001$) than CTL and AUT animals. Principal coordinate analysis showed clear differences in the structure of the rumen microbiota according to the treatments (Figure 2C, $p < 0.001$). Pair-wise comparisons identified significant differences across all treatments except for RFF and RFC which showed a similar multi-kingdom microbial community structure. Tripod vectors showed that the structure of the rumen microbiota in RFF and RFC animals positively correlated with higher diversity values (for bacteria, methanogens, protozoa, anaerobic fungi and multi-kingdom) and negatively with PD to creatinine ratio. On the contrary, the multi-kingdom community structure in CTL animals positively correlated with PD excretion, EMPS and acetate molar proportion and negatively with feed digestibility and rumen protozoa, fungi, ammonia, butyrate and lactate concentrations. For a more comprehensive description of the rumen microbiota, the main microbial groups were studied separately.

Rumen bacteria

Quantitative PCR showed that the bacterial community represented the most abundant microbial group in the rumen and its concentration was similar across treatments (Table 3). Bacterial sequencing yielded 9,758 ± 2,657 high-quality sequences per sample and diversity analysis showed that treatments RFF and RFC had the highest bacterial richness ($p < 0.001$), Shannon's ($p < 0.001$) and Simpson's indexes ($p = 0.061$). The AUT animals had the lowest bacterial richness across treatments but similar Shannon and Simpson index than CTL animals. The bacterial community structure (Figure 3A) showed a clear separation between inoculated (RFF, RFC and AUT, left) and non-inoculated animals (right) according to the PCO1. PERMANOVA analysis showed that the structure of the rumen bacterial community was significantly affected by the treatments ($p < 0.001$) and pair-wise comparisons

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TABLE 2 Blood parameters, feed digestibility and microbial protein synthesis in 26-week-old goats measured at 21 days after a shift from a high-concentrate to a full forage diet (oats hay).

Treatments[1]	CTL	AUT	RFF	RFC	SED	*p*-value
BW, kg	24.3	25.1	24.2	23.6	1.383	0.757
Cortisol in hair, ng/mg	1.50	1.53	1.49	1.54	0.144	0.982
Blood metabolites, mmol/L						
Glucose	2.86	3.20	2.99	2.89	0.240	0.482
β-hydroxybutyrate	2.63	2.50	2.84	2.17	0.381	0.327
Blood pH	7.30	7.33	7.35	7.33	0.028	0.222
pCO_2, mm of Hg	56.0	48.4	46.6	46.8	3.960	0.088
tCO_2, mm of Hg	27.0	24.8	25.2	24.3	1.299	0.410
Na^+	145	146	146	144	0.817	0.121
K^+	4.75	5.03	4.91	4.84	0.174	0.417
HCO_3^-	25.3	23.4	23.8	22.8	1.217	0.513
Cl^-	110	112	111	111	0.758	0.212
Anion Gap[1]	14.6	16.2	15.2	15.1	0.814	0.450
Apparent digestibility, %						
DM	58.0^b	63.3^a	62.4^a	64.1^a	1.856	0.014
OM	59.0^b	64.7^a	64.1^a	65.8^a	1.883	0.007
N	45.4	53.1	53.9	52.0	3.790	0.092
NDF	50.6^b	59.6^a	60.2^a	62.7^a	2.470	<0.001
ADF	37.3^b	48.7^a	49.6^a	52.8^a	3.190	<0.001
Fecal N excretion, g/d	3.33	3.18	2.81	3.04	0.418	0.579
Urinary excretion						
Total volume, L/d	2.05	2.49	1.62	1.79	0.271	0.133
N, g/d	9.15	8.65	9.92	8.13	3.840	0.605
Creatinine, μmol/kg $BW^{0.75}$	195^a	133^{ab}	102^b	107^b	23.70	0.034
PD, mmol/d	13.8^a	7.27^b	6.80^b	5.90^b	1.758	<0.001
PD/Creatinine ratio	0.95^a	0.94^a	0.86^{ab}	0.73^b	0.085	0.043
EMPS, mmol PD/kg DOMI	43.1^a	23.8^b	24.5^b	19.0^b	4.48	<0.001
In vitro fermentation						
Gas production, mmol/d	40.3^b	52.6^a	51.6^a	50.3^a	4.02	0.002
CH_4, mmol/d	0.22^b	0.31^a	0.27^a	0.28^a	0.03	0.006
CH_4, mol/mol FOM	6.69	9.17	6.71	7.31	1.257	0.106

BW, body weight; PD, purine derivatives; EMPS, efficiency of microbial protein synthesis; DOMI, digestible OM intake.
[1]During the first 10 weeks of age, goats ($N = 36$) received a daily and oral inoculation with autoclaved rumen fluid (AUT), rumen fluid from adult animals fed forage (RFF), or fed concentrate diets (RFC) or without inoculation (CTL). Means within a raw with different superscript differ ($p < 0.05$).

detected significant differences across all treatments except for RFF and RFC animals. Moreover, tripod vectors showed that the bacterial community structure was intimately correlated with rumen fermentation and productive variables. In particular, the bacterial community in RFF and RFC animals positively correlated with higher feed digestibility (DM, OM, NDF and ADF), rumen ammonia, lactate, butyrate, methanogens, protozoa and anaerobic fungal levels as well as protozoa and methanogens diversities, whereas in CTL animals it was positively correlated with higher acetate molar proportion and microbial protein synthesis (in terms of PD, PD to creatinine ratio and EMPS). Bacterial community structure in AUT animals was negatively correlated with the bacterial and fungal diversities.

The analysis of the relative abundances of the most predominant bacterial taxa showed a strong effect of the treatments (Table 3). Control animals showed the highest abundances for the phyla Actinobacteriota, Desulfobacterota and Firmicutes (including the families *Christensenellaceae, Clostridia_UCG-014* and *Oscillospiraceae*), whereas AUT animals had the highest abundances for the phyla Bacteroidota (including the family Prevotellaceae) and Synergistota and the family Selenomonadaceae, resulting on the lowest Firmicutes / Bacteroidota ratio across treatments. Inoculation with RFF in early life led to the highest abundances for Fibrobacterota, Acholeplasmataceae, Izemoplasmatales and UCG-010, whereas inoculation with RFC did it for Actinobacteriota, p-251-o5, Rikenellaceae, Cyanobacteria and Elusimicrobiota. Moreover, all animals inoculated with fresh rumen

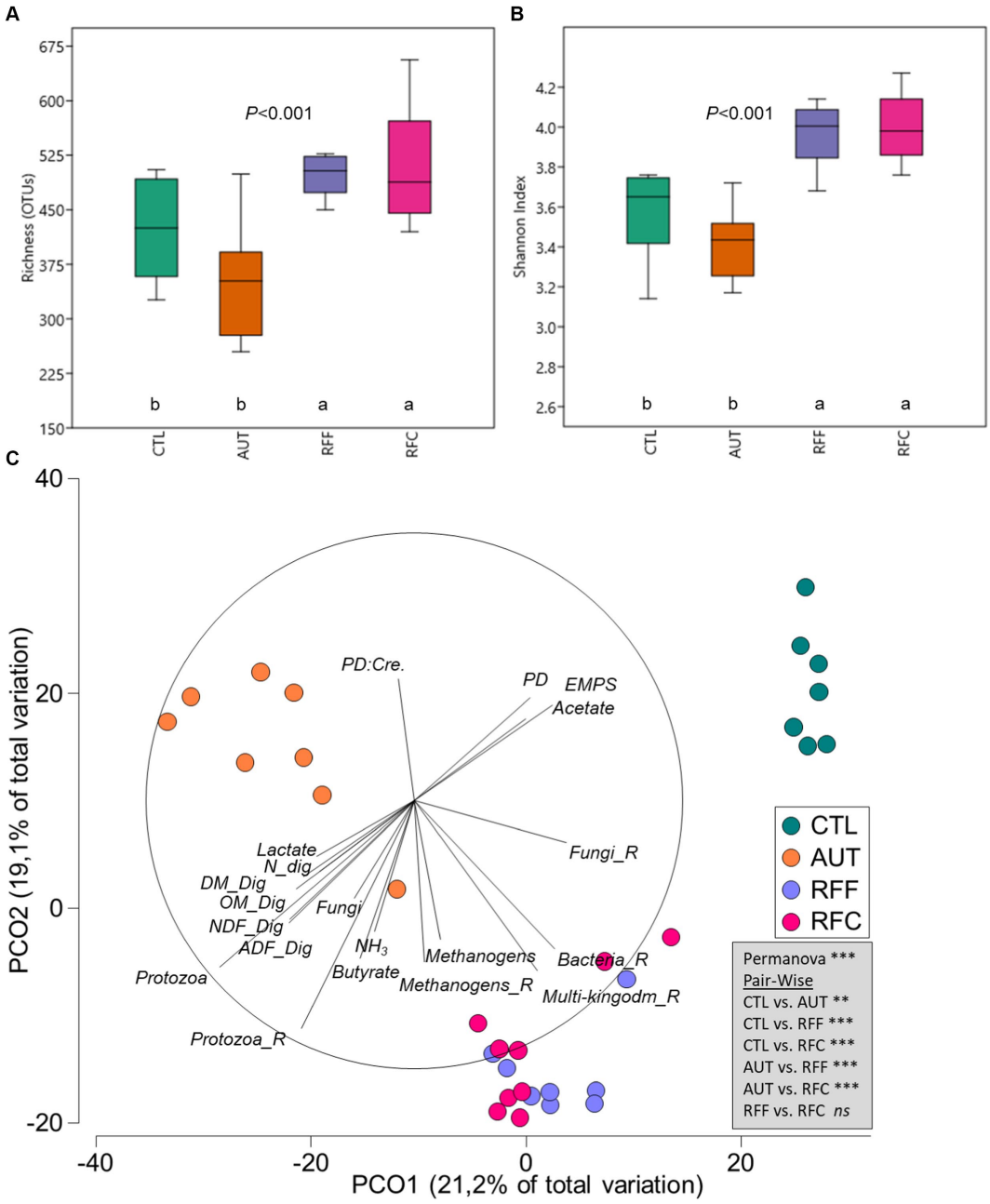

FIGURE 2
Box plot illustrating the rumen multi-kingdom diversity in terms of richness **(A)** and Shannon's index **(B)** in 26-week-old goats measured at 21 days after a shift from a high-concentrate to a full forage diet (oats hay). During the first 10 weeks of age, goats ($N = 36$) received a daily and oral inoculation with autoclaved rumen fluid (AUT), rumen fluid from adult animals fed forage (RFF), or fed concentrate diets (RFC) or without inoculation (CTL). Principal coordinates analysis **(C)** illustrating relationships ($\rho > 0.4$) between the structure of the rumen microbiota and productive data. PERMANOVA values are provided based on the Bray–Curtis dissimilarity.

fluid (RFF or RFC) had the highest abundances for Bacteroidetes_RF16_ group, Erysipelatoclostridiaceae and Paracaedibacteraceae.

Rumen methanogens

No differences were noted in the rumen methanogens concentrations across treatments (Table 4). Methanogens sequencing yielded 7,147 ± 702 high quality sequences per sample and treatment AUT led to the lowest methanogens richness ($p = 0.008$), Shannon's ($p = 0.005$) and Simpson's indexes ($p = 0.009$) across treatments. Control animals also had lower methanogens richness than that observed for RFF and RFC animals, but similar for Shannon's and Simpson's indexes, suggesting the presence of fewer species but with a higher homogeneity in their abundance. The methanogens community structure was affected by the treatments (Figure 3B, $p < 0.001$). Pair-wise comparisons showed that AUT animals had a different methanogens community than the rest of the animals ($p < 0.05$) whereas RFF and RFC tended to differ to the CTL animals ($p < 0.1$). A similar methanogens community structure was observed for RFF

TABLE 3 Rumen bacterial diversity and taxa abundances in 26-week-old goats measured at 21 days after a shift from a high-concentrate to a full forage diet (oats hay).

Treatments[1]	CTL	AUT	RFF	RFC	SED	*p*-value
Concentration, log10 copy/mg DM	10.5	10.3	10.0	10.4	0.263	0.367
Diversity						
Richness, OTUs	362^{b}	283^{c}	409^{a}	415^{a}	29.80	<0.001
Shannon	3.63^{b}	3.49^{b}	4.22^{a}	4.26^{a}	0.199	<0.001
Simpson	0.91^{b}	0.91^{b}	0.95^{a}	0.96^{a}	0.023	0.061
Abundance, %						
Ratio Firmicutes/Bacteroidota	0.67^{a}	0.28^{b}	0.70^{a}	0.53^{a}	0.105	0.003
p_Actinobacteriota	0.01^{b}	0.00^{b}	0.00^{b}	0.05^{a}	0.019	0.003
p_Bacteroidota	57.8^{b}	72.2^{a}	50.7^{b}	57.2^{b}	4.226	<0.001
f_Bacteroidales_RF16_group	3.56^{bc}	10.2^{a}	1.81^{c}	5.35^{b}	1.682	<0.001
f_Bacteroidetes_BD2-2	0.12^{a}	0.01^{b}	0.18^{a}	0.13^{a}	0.047	0.021
f_p-251-o5	0.00^{b}	0.01^{b}	0.30^{ab}	0.68^{a}	0.255	0.001
f_p-2534-18B5_gut_group	4.38^{a}	0.01^{b}	4.27^{a}	0.64^{ab}	1.709	0.008
f_Prevotellaceae	34.1^{b}	50.0^{a}	31.3^{b}	35.4^{b}	4.495	0.006
f_Rikenellaceae	5.87^{ab}	2.95^{b}	6.05^{ab}	8.96^{a}	1.915	0.031
p_Chloroflexi	0.00^{b}	0.41^{a}	0.02^{b}	0.01^{b}	0.140	<0.001
p_Cyanobacteria	1.79^{b}	0.93^{b}	2.85^{ab}	5.20^{a}	1.371	0.035
p_Desulfobacterota	0.04^{a}	0.00^{b}	0.00^{b}	0.00^{b}	0.008	<0.001
p_Elusimicrobiota	0.09^{ab}	0.02^{b}	0.37^{ab}	0.39^{a}	0.148	0.010
p_Fibrobacterota	1.38^{b}	0.48^{b}	6.03^{a}	2.40^{b}	1.249	<0.001
p_Firmicutes	36.2^{a}	19.8^{b}	33.2^{b}	29.4^{b}	3.653	0.005
f_Acholeplasmataceae	0.00^{b}	0.01^{b}	0.30^{a}	0.08^{ab}	0.094	0.005
f_Christensenellaceae	5.52^{a}	1.45^{b}	0.77^{b}	0.83^{b}	1.004	0.002
f_Clostridia_UCG-014	1.62^{a}	0.24^{b}	0.74^{b}	0.92^{ab}	0.327	0.006
f_Clostridia_vadinBB60_group	0.27^{bc}	0.16^{c}	0.62^{a}	0.54^{ab}	0.134	0.031
f_Erysipelatoclostridiaceae	0.17^{b}	0.19^{b}	12.1^{a}	8.90^{a}	3.353	<0.001
f_Izemoplasmatales	0.02^{ab}	0.00^{b}	0.03^{a}	0.00^{b}	0.012	0.033
f_Oscillospiraceae	16.7^{a}	0.50^{b}	0.99^{b}	3.61^{b}	3.626	0.001
f_Selenomonadaceae	1.16^{b}	10.2^{a}	5.13^{b}	4.40^{b}	1.846	<0.001
f_UCG-010	0.04^{b}	0.06^{b}	0.33^{a}	0.16^{b}	0.061	0.002
p_Proteobacteria	1.50	1.71	2.52	1.87	0.853	0.194
f_Paracaedibacteraceae	0.00^{b}	0.00^{b}	0.08^{a}	0.08^{a}	0.042	0.011
p_Spirochaetota	0.91	0.45	1.65	1.12	0.383	0.066
p_Synergistota	0.14^{b}	3.65^{a}	2.35^{ab}	2.06^{ab}	0.977	0.023
p_Verrucomicrobiota	0.01^{b}	0.28^{a}	0.27^{a}	0.22^{a}	0.059	0.002

[1]During the first 10 weeks of age, goats ($N = 36$) received a daily and oral inoculation with autoclaved rumen fluid (AUT), rumen fluid from adult animals fed forage (RFF), or fed concentrate diets (RFC) or without inoculation (CTL). Means within a raw with different superscript differ ($p < 0.05$). Only families (f) with an average abundance higher than 0.01% and *p*-values <0.1 are shown.

and RFC animals and it was positively correlated with the bacteria, methanogens and anaerobic fungal richness. Interestingly, the structure of the methanogens community did not correlate with the metadata considered in this study.

The abundances of the main methanogens families were unaffected by the treatments (Table 4), but moderate differences were noted within the Methanomassiliicoccaceae family. The methanogens species *Group10_sp* and *Group9_sp ISO4-G1* showed the highest abundances in the CTL and AUT animals, respectively. Inoculation with RFF and with RFC increased the abundance of *Group8_sp WGK1* and *Group9_sp CH1210*, respectively, whereas this later group was undetected in the rest of the treatments.

Rumen protozoa

Control animals remained protozoa-free during the entire duration of this experiment, while no differences in the rumen

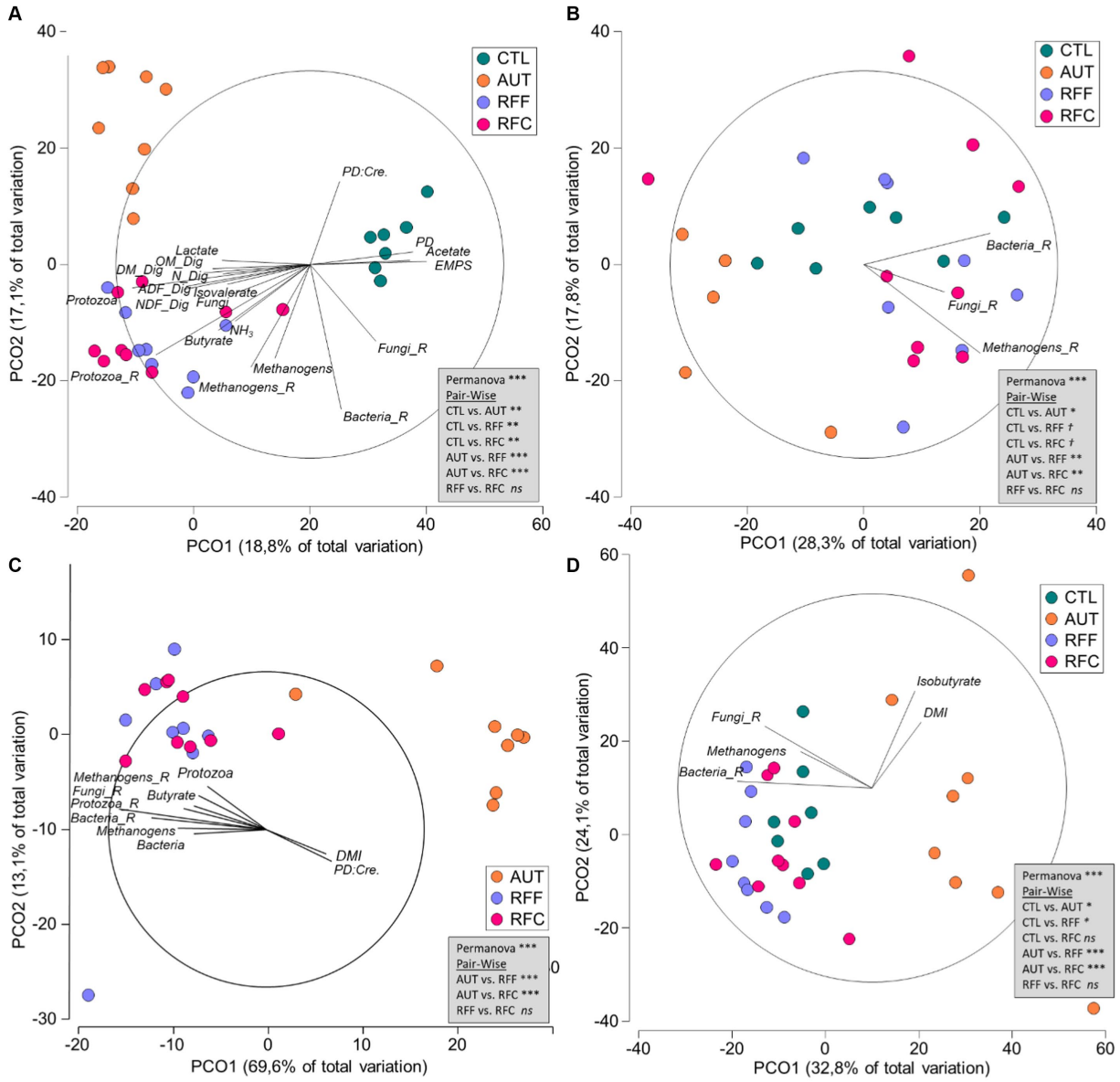

FIGURE 3
Principal Coordinate Analysis illustrating the structure of the rumen bacteria **(A)**, methanogens **(B)**, protozoa **(C)** and anaerobic fungi community **(D)** in 26-week-old goats measured at 21 days after a shift from a high-concentrate to a full forage diet (oats hay). During the first 10 weeks of age, goats ($N = 36$) received a daily and oral inoculation with autoclaved rumen fluid (AUT), rumen fluid from adult animals fed forage (RFF), or fed concentrate diets (RFC) or without inoculation (CTL). Relationships ($\rho > 0.4$) between the structure of the rumen microbiota and productive data are shown along with PERMANOVA values based on the Bray–Curtis dissimilarity.

protozoal concentration were detected across the other three treatments (Table 5). Protozoal sequencing yielded 20,121 ± 4,504 high quality sequences per sample and a substantially lower protozoal diversity indexes were noted for AUT than for RFF or RFC animals ($p < 0.001$). Similarly, PCoA and PERMANOVA analyses also showed a different protozoal community structure for AUT than for RFF or RFC animals ($p < 0.001$, Figure 3C). The protozoal community structure in RFF and RFC was similar ($p > 0.1$) and positively correlated with the rumen concentration of bacteria, methanogens and protozoa, the butyrate molar proportion and the bacteria, methanogens, protozoa and anaerobic fungal diversity. On the contrary, the protozoal structure in AUT animals positively correlated with DMI and PD to creatinine ratio. The protozoal community in the AUT animals was more abundant in the subfamily Entodiniinae ($p < 0.001$, average 96.2%) whereas animals inoculated with fresh rumen fluid had higher abundances of *Enoploplastron_triloricatum*, *Ophryoscolex_sp_LDK-2011*, and the family Isotrichidae, including *Dasytricha*, *Isotricha prostoma* and *Isotricha intestinalis*.

Anaerobic fungi

Anaerobic fungal concentration in the rumen was similar across treatments (Table 6). Fungal sequencing yielded 21,298 ± 6,920 high quality sequences per sample, but only the anaerobic fungal sequences

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TABLE 4 Rumen methanogens diversity and taxa abundances in 26-week-old goats measured at 21 days after a shift from a high-concentrate to a full forage diet (oats hay).

Treatments[1]	CTL	AUT	RFF	RFC	SED	*p*-value
Concentration, log10 copy/mg DM	6.60	6.85	6.83	7.24	0.343	0.218
Diversity						
Richness, OTUs	8.67^{b}	9.00^{b}	13.5^{a}	11.0^{ab}	1.452	0.008
Shannon	1.32^{a}	0.92^{b}	1.37^{a}	1.32^{a}	0.128	0.005
Simpson	0.66^{a}	0.47^{b}	0.62^{a}	0.64^{a}	0.056	0.009
Abundance[1], %						
f_Methanobacteriaceae	17.4	22.2	26.4	23.6	3.768	0.764
f_Methanocaldococcaceae	0.00	0.00	0.15	0.28	0.091	0.187
g_Methanocaldococcus	0.00	0.00	0.00	0.28	0.077	0.058
f_Methanomicrobiaceae	3.73	0.00	0.00	3.57	1.657	0.534
f_Methanomassiliicoccaceae	78.9	77.8	73.2	72.6	3.639	0.838
s_Group8_sp WGK1	0.00	0.05	1.81	0.71	0.521	0.088
s_Group10_sp	18.5	3.99	4.63	6.34	2.446	0.053
s_Group11_sp ISO4-G11	19.1^{a}	0.00^{b}	18.8^{a}	18.5^{a}	4.697	0.018
g_Group9	34.5^{b}	71.4^{a}	43.8^{ab}	40.4^{ab}	6.052	0.041
s_Group9_sp CH1270	0.00^{b}	0.00^{b}	0.01^{b}	0.43^{a}	0.125	0.046
s_Group9_sp ISO4-G1	34.3^{b}	71.4^{a}	43.8^{ab}	40.0^{ab}	6.049	0.035

[1]During the first 10 weeks of age, goats ($N = 36$) received a daily and oral inoculation with autoclaved rumen fluid (AUT), rumen fluid from adult animals fed forage (RFF), or fed concentrate diets (RFC) or without inoculation (CTL). Means within a raw with different superscript differ ($p < 0.05$).

TABLE 5 Rumen protozoal diversity and taxa abundances in 26-week-old goats measured at 21 days after a shift from a high-concentrate to a full forage diet (oats hay).

Treatments[1]	CTL	AUT	RFF	RFC	SED	*p*-value
Concentration, log10 copy/mg DM	ND	8.79	8.38	9.02	0.430	0.348
Diversity						
Richness, OTUs	ND	20.5^{b}	31.3^{a}	32.9^{a}	1.085	<0.001
Shannon	ND	1.68^{b}	2.24^{a}	2.28^{a}	0.062	<0.001
Simpson	ND	0.76^{b}	0.86^{a}	0.86^{a}	0.014	<0.001
Abundance, %						
f_Ophyoscolecidae	ND	97.5^{a}	77.5^{b}	81.6^{b}	3.087	<0.001
sf_Entodiniinae; *g_Entodinium*	ND	96.2^{a}	74.9^{b}	79.1^{b}	3.312	<0.001
sf_Diplodiniinae	ND	1.33	2.34	2.36	0.423	0.138
g_Enoploplastron_triloricatum	ND	0.00^{b}	0.49^{a}	0.32^{a}	0.114	0.003
g_Polyplastron	ND	1.32	1.73	1.57	0.397	0.600
g_Diplodinium	ND	0.01	0.12	0.47	0.171	0.073
sf_Ophryoscolecinae	ND	0.00^{b}	0.23^{a}	0.14^{a}	0.053	0.003
s_Ophryoscolex_sp_LDK-2011	ND	0.00^{b}	0.22^{a}	0.14^{a}	0.052	0.003
f_Isotrichidae	ND	1.14^{b}	21.85^{a}	16.6^{a}	3.099	<0.001
g_Dasytricha	ND	0.92^{b}	12.15^{a}	12.8^{ab}	2.063	0.001
g_Isotricha	ND	0.23^{b}	9.70^{a}	3.84^{a}	2.372	<0.001
s_Isotricha_prostoma	ND	0.15^{b}	6.00^{a}	1.66^{a}	1.484	<0.001
s_Isotricha_intestinalis	ND	0.06^{b}	2.63^{a}	1.15^{a}	0.585	<0.001

[1]During the first 10 weeks of age, goats ($N = 36$) received a daily and oral inoculation with autoclaved rumen fluid (AUT), rumen fluid from adult animals fed forage (RFF), or fed concentrate diets (RFC) or without inoculation (CTL). Means within a raw with different superscript differ ($p < 0.05$).

TABLE 6 Rumen anaerobic fungal diversity and taxa abundances in 26-week-old goats measured at 21 days after a shift from a high-concentrate to a full forage diet (oats hay).

Treatments[1]	CTL	AUT	RFF	RFC	SED	*p*-value
Concentration, log10 copy/mg DM	6.62	7.20	7.18	7.33	0.352	0.234
Diversity						
Richness, OTUs	52.5^{a}	36.6^{c}	43.9^{bc}	49.2^{ab}	3.910	0.001
Shannon	2.47^{a}	2.02^{b}	2.48^{a}	2.55^{a}	0.143	0.002
Simpson	0.84^{a}	0.77^{b}	0.87^{a}	0.86^{a}	0.036	0.041
Abundance, %						
g_Anaeromyces	0.77	0.91	0.75	0.00	0.292	0.096
g_Caecomyces	25.3^{ab}	30.9^{a}	20.8^{ab}	6.82^{b}	4.903	0.047
g_Neocallimastix	16.6^{b}	6.23^{c}	25.2^{ab}	33.6^{a}	3.207	<0.001
Unclassified	57.4	61.9	53.3	59.6	3.763	0.820

[1]During the first 10 weeks of age, goats ($N = 36$) received a daily and oral inoculation with autoclaved rumen fluid (AUT), rumen fluid from adult animals fed forage (RFF), or fed concentrate diets (RFC) or without inoculation (CTL). Means within a raw with different superscript differ ($p < 0.05$).

(phylum Neocallimastigomycota) were further considered (19,267 ± 5,647 per sample). Animals inoculated with AUT showed the lowest anaerobic fungal diversity in terms of richness, Shannon's and Simpson's indexes (Table 6) across treatments. Treatment AUT promoted a particular rumen fungal community structure (Figure 3D) which was positively correlated with the DMI and rumen isobutyrate molar proportion. On the contrary, similar fungal community structure was observed for the treatments RFF, RFC and CTL having a positive correlation with the bacterial and fungal richness and the rumen methanogens concentration. Over half of the anaerobic fungal sequences were unclassified at the genus level. Treatment AUT tended to increase the rumen abundances of *Caecomyces* and *Anaeromyces* while led to the lowest levels of *Neocallimastix*. On the contrary, treatment RFC led to the highest *Neocallimastix* abundance, the lowest *Caecomyces* abundance and the absence of *Anaeromyces*. Moreover, CTL animals also had lower *Neocallimastix* abundance than RFC animals.

Correlation analysis

Total VFA concentration was not associated with relevant microbiological changes (Table 7), however the acetate molar proportion was positively correlated with seven (including *Coprococcus, Desulfobacteria* and *CAG-352*) and negatively correlated with six bacterial taxa (e.g., *Lachnospiraceae_ND3007_group and Prevotellaceae_UCG-001*) and two protozoal taxa (e.g., *Ophryoscolex*) as well as with the protozoal richness. Propionate molar proportion only correlated positively and negatively with two and three bacterial species, respectively. On the contrary butyrate molar proportion was highly associated with changes in the rumen microbiota and had positive correlations with several protozoal features (including protozoal concentration, richness and abundances of Isotricha, Dasytricha, Enoploplastron and Ophryoscolex), methanogens richness, multi-kingdom richness and eight bacterial taxa (including *Lachnospiraceae_ND3007_group*), whereas negative correlation were detected with seven bacterial and one fungal taxa. Although rumen ammonia concentration and butyrate molar proportion had a small correlation coefficient ($\rho = 0.32$, $p = 0.072$), 15 out of the 19 microbial variables that correlated with the ammonia were the same (and in the same sign) than those that correlated with butyrate (including 11 protozoal variables). Rumen lactate concentration was negatively correlated with the abundance of 10 bacterial taxa but positively correlated with *Dasytricha* and *Entodinium* abundances. Urinary PD excretion and the EMPS were negatively correlated with *Entodinium* and several bacterial taxa (*Oligosphaeraceae, Anaeroplasma* and *Lachnospiraceae_ND3007_group, Paracaedibacteraceae* and *Selenomonas*) but also had a positive correlation with other bacterial taxa (including, *Oscillospira*, *Prevotella ruminicola* and *Oscillospira_guilliermondii*). The N digestibility correlated with the same bacterial taxa than described for the rumen NH_3 concentration, however this was not the case for the protozoal taxa since the abundances of *Diplodinium* and *Entodinium* positively correlated to N digestibility but not with NH_3 concentration. Finally, DM digestibility and NDF digestibility mostly correlated with the same rumen microbes given the high proportion of fiber in the diet. Several bacterial (including *Prevotellaceae_UCG-003*, *Lachnospiraceae_ND3007_group*, *Quinella*, *Fretibacterium*), methanogens (*Group9_spCH1270*) and protozoal taxa (including *Diplodinium* and *Entodinium*) as well as the protozoal concentration and richness had a positive correlation with NDF digestibility, whereas up to 11 bacterial taxa showed a negative correlation, being most of them amylolytic species.

Discussion

This study demonstrated that the inoculation of young ruminants during the pre-weaning period with rumen fluid from adult ruminants has long-term effects on the rumen microbiota and certain physiological implications in terms of feed efficiency and health when animals are fed forage.

Effects of different microbial inocula

Adult animals generally show a high host specificity which makes difficult to permanently modify their rumen microbiota (33). As a result, Weimer et al. (34) demonstrated that after a near-total exchange

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TABLE 7 Spearman's correlations ($\rho > 0.4$, $p < 0.001$) between the rumen microbes and productive data.

Kingdom	Taxa	Ace.	Prop.	But.	VFA	NH_3	Lact	PD	DMd	Nd	NDFd	EMPS
Multiple	Multi-kingdom richness			0.49								
Bacteria	*p_Desulfobacterota*	0.52		−0.44		−0.40					−0.45	0.42
Bacteria	*p_Verrucomicrobiota*					0.49				0.53		
Bacteria	*f_Christensenellaceae*			−0.58		−0.48						
Bacteria	*f_Erysipelatoclostridiaceae*			0.51		0.49						
Bacteria	*f_Oligosphaeraceae*							−0.53				−0.56
Bacteria	*f_Oscillospiraceae*	0.41					−0.52				−0.40	
Bacteria	*f_p-251-o5*			0.45			0.47					
Bacteria	*f_Paracaedibacteraceae*			0.59								−0.42
Bacteria	*f_RF39*						−0.54		−0.62		−0.63	
Bacteria	*g_Acetitomaculum*		−0.42		−0.40		−0.43					
Bacteria	*g_Anaeroplasma*	−0.45		0.50	0.44			−0.40				
Bacteria	*g_CAG-352*	0.59	−0.41	−0.40			−0.58	0.43		−0.40	−0.55	0.44
Bacteria	*g_Coprococcus*	0.63	−0.5	−0.45				0.44			−0.54	0.45
Bacteria	*g_Fretibacterium*	−0.48		0.40							0.40	
Bacteria	*g_Lachnospiraceae_ND3007*	−0.53		0.63				−0.45			0.41	−0.40
Bacteria	*g_Oscillospira*	0.47		−0.35				0.55				0.56
Bacteria	*g_p-2534-18B5_gut_group*						−0.36				−0.42	
Bacteria	*g_Prevotellaceae_Ga6A1_group*						−0.48	0.42			−0.54	0.44
Bacteria	*g_Prevotellaceae_UCG-001*	−0.56	0.45									
Bacteria	*g_Prevotellaceae_UCG-003*								0.42		0.44	
Bacteria	*g_Quinella*	−0.48	0.53						0.40		0.43	
Bacteria	*g_RF39*						−0.54		−0.62		−0.63	
Bacteria	*g_Selenomonas*					0.47						−0.40
Bacteria	*g_Succinivibrionaceae_UCG-002*	0.40					−0.43					
Bacteria	*g_UCG-002*						−0.59				−0.52	0.44
Bacteria	*g_vadinBE97*					0.73						
Bacteria	*g_Veillonellaceae_UCG-001*	−0.41		0.53		0.59						
Bacteria	*s_bacterium_FB2012*						−0.44		−0.43	−0.48	−0.53	
Bacteria	*s_Fibrobacter_succinogenes*			0.59						0.43		
Bacteria	*s_Oscillospira_guilliermondii*	0.42		−0.46				0.47				0.46
Bacteria	*s_Prevotella_ruminicola*							0.53			−0.41	0.44
Methanogen	Methanogens richness			0.43						0.44		
Methanogen	*Group9_spCH1270*								0.41		0.43	
Protozoa	Protozoal concentration			0.66		0.49					0.52	
Protozoa	Protozoal richness	−0.46		0.62		0.66					0.44	
Protozoa	*g_Dasytricha*			0.67		0.49						
Protozoa	*g_Diplodinium*						0.55		0.5	0.46	0.49	
Protozoa	*g_Enoploplastron*			0.55		0.49						
Protozoa	*g_Entodinium*							−0.51	0.45	0.40	0.48	−0.54
Protozoa	*g_Isotricha*			0.61		0.58						
Protozoa	*g_Ophryoscolex*	−0.50		0.41		0.54						
Protozoa	*g_Polyplastron*	−0.41				0.51			0.45	0.48		
Protozoa	*s_Enoploplastron_triloricatum*			0.55		0.49						
Protozoa	*s_Entodinium_sp_LDK-2011*					0.53	0.44		0.47		0.46	−0.40
Protozoa	*s_Isotricha_intestinalis*	−0.45		0.66		0.55						
Protozoa	*s_Isotricha_prostoma*			0.56		0.58						
Fungi	*g_Caecomyces*			−0.50								

Ace, Acetate; Pro, Propionate; But, Butyrate; Lact, Lactate; PD, Purine derivatives; d, digestibility; EMPS, Efficiency of microbial protein synthesis. Data across all experimental treatments measured in 26-week-old ($N = 36$) measured at 21 days after a shift from a high-concentrate to a full forage diet (oats hay).

of rumen content among adult cows, they were able to re-establish their initial rumen bacterial community and fermentation pattern after 14–61 days. On the contrary, it has been suggested that nutritional interventions in early life can represent an opportunity to modulate the rumen microbial colonization having short and potentially long-term effects on the rumen microbial community structure and animal productivity (33). A companion publication of the present experiment (13) and similar studies described the positive short-term effects of inoculating fresh (11, 12) or lyophilized (14, 15) rumen fluid from adult ruminants to young ruminants. In the present study we demonstrated that these effects have a prolonged persistency since after a substantial time elapsed (28 weeks of age) the RFF and RFC animals still had a higher rumen multi-kingdom microbial community richness (503 OTUs) as a result of a higher bacterial and protozoal diversity than the CTL animals (423 OTUs) which remained protozoa-free. An incomplete rumen microbial colonization has been described in artificially-reared ruminants without physical contact with adult animals (8), and characterized by absence of rumen protozoa, as they are highly sensitive to oxygen, making it necessary to have direct contact between animals for an effective transmission (35). The AUT animals also retained low bacterial, anaerobic fungi and protozoal richness, which was dominated by a single protozoa genus (*Entodinium*, 96.2%). This observation indicated an incomplete protozoal colonization, possibly as a result of a cross-faunation that may have accidentally occurred when inoculating animals from different groups (13). However, the presence of few protozoal species in AUT animals, along with the potential positive effects derived from the inoculation with rumen metabolites such as VFA (36), micro-nutrients and microbial extracts (37) could explain the moderate positive impact on the energy metabolism (feed digestibility and gas production) along with the negative effect on the protein metabolism (lower microbial protein synthesis than CTL goats).

In relation with the type of microbial inocula, De Barbieri et al. (38) showed a different rumen bacterial colonization when young lambs were inoculated with rumen fluid from adult sheep supplemented with coconut oil or protected fat. Similarly, our companion paper showed that inoculation with RFF and RFC also led to certain differences in the rumen microbial community up to weaning (13). Thus, it was hypothesized that inoculation with a more diverse bacterial inocula adapted to the forage digestion (RFF) could be beneficial for an efficient forage utilization in adult life. Unfortunately, our results suggest that animals inoculated with RFF or RFC had similar rumen microbiota. This microbial convergence could be a partially explained by an adaptation to the diet given the high plasticity of the rumen microbiota (39). This observation suggests that the long-germ effects of using inocula with different microbial composition are negligible since only those microbes able to survive in the rumen environment will ultimately flourish independently of their abundance in the initial inocula (40).

Rumen microbial fermentation

Fiber fermentation is a complex process which requires the combined action of multiple microbial groups (41), therefore it may be expected that a greater rumen microbial diversity would favor the adaptation and utilization of fibrous diets by the host. Our study showed that all animals were ultimately adapted to the forage diet without experiencing stress or digestive disorders based on the similar levels of hair cortisol, blood metabolites and absence of diarrhea across treatments (42). However, this adaptation process was substantially faster for RFF and RFC given their higher DMI and rumen VFA concentration than reported for CTL animals at 4 d after the dietary shift, possibly as a result of a higher forage degradation by the rumen microbes (43).

The presence of a complex rumen microbial community in RFF and RFC animals also promoted higher butyrate (+36%) and propionate (+10%) molar proportions in detriment to acetate (−6.1%) after the 21 days of adaptation to the diet, mostly as a result of a complex rumen protozoal community (44). The ability of protozoa to engulf carbohydrates and exogenous fatty acids may divert more carbon toward VFA production, butyrate being the main fermentation product derived from the protozoal activity (45). This observation was confirmed by positive correlation observed between the butyrate proportion and several protozoal variables including concentration, richness and abundance of holotrich protozoa (*Isotricha* and *Dasytricha*). Holotrich protozoa, which represented 20% of the protozoa in RFF and RFC animals, have a limited ability to digest fiber (46) and to predate bacteria in the rumen (47). However, they exhibit a chemotaxis to simple sugars (48) which are fermented into butyrate, CO_2 and H_2 as the main fermentation products. This H_2 production favors the inter-species H_2 transfer toward protozoal epi- and endo-symbiotic methanogens (49, 50), and it could explain the high butyrate and CH_4 production observed in holotrich-monofaunated sheep (51) as well as in the RFF and RFC animals (+26%) in comparison to the CTL animals.

Over the last decade, important research efforts have been focussed on studying rumen microbiota and their correlations with the feed efficiency and the overall biology of the host. After analyzing the rumen microbiota of 146 dairy cows fed concentrate feeds, Shabat et al. (52) concluded that cows that feed efficient cows (in terms of feed conversion ratio) had higher propionate, butyrate, valerate and isovalerate molar proportions, increased rumen abundances of *Megasphaera elsdenii* and *Coprococcus catus*, as well as lower bacterial diversity and CH_4 emissions than less efficient cows. On the contrary, Myer et al. (53) reported no differences in rumen bacterial diversity between steers differing in feed conversion ratio. Likewise, Lopes et al. (54) observed similar rumen bacterial and fungal diversities, but higher archaeal diversity and Bacteroidetes to Firmicutes ratio in feed efficient Nellore steers. The discrepancy between studies seems to rely on type of diets consumed by the ruminants indicating that it is unlikely to find a one-size-fits-all approach to optimize the rumen function across the different ruminant production systems. Our correlation analysis based on animals fed forage identified two distinctive types of rumen microbes according to their activity: (i) butyrate producers that positively correlated with butyrate and negatively with acetate molar proportions such as, *Ophryoscolex*, *Isotricha intestinalis*, *Anaeroplasma*, *Lachnospiraceae_ND3007_group*, *Veillonellaceae_UCG-001*, *Fretibacterium* and protozoal richness, and (ii) acetate producers that positively correlated with acetate and negatively with butyrate including *Desulfobacterota*, *Coprococcus, Oscillospira* and *CAG-352*. On the contrary, the increased propionate molar proportion observed in RFF and RFC animals (+10%) was not associated with rumen microbiological changes suggesting that it may be a beneficial but indirect effect derived from compositional changes in other VFA proportions. This indirect effect was exemplified with

Coprococcus, that is one of the few rumen bacteria able to degrade phloroglucinol into acetate as unique fermentation product (55), and in our study it had a strong positive correlation with acetate but also an indirect negative correlations with propionate and butyrate molar proportions. Rumen lactate concentration was unaffected by the treatments, however up to 10 bacterial taxa had strong negatively correlations with lactate concentration, including the starch utilizer *Succinivibrionaceae_UCG-002* and Oscillospiraceae. This observation indicates that lactate may have been transformed into propionate in RFF and RFC as a result of a higher fiber digestion and availability of simple sugars (56). Similarly, the increased valerate molar proportion in RFF and RFC animals (+20%) could be linked to a higher digestion of glucose, starch and cellulose (57). Increased Firmicutes to Bacteroidota ratio, as noted in RFF and RFC animals, has been correlated to higher DMI, BW gain and feed efficiency in beef cattle (58), but not in dairy cows (54) possibly as a result of differences in the type of diets. These findings suggest that inoculation with fresh rumen fluid led to a shift toward an energetically efficient carbohydrate fermentation. This shift is attributed to the increased production of propionate and butyrate, which offer higher energy yields, release less H_2, and enhance the host's energy utilization efficiency compared to acetate production (59). However, this nutritional approach faces significant limitations. In practice, inoculating rumen fluid is not feasible under on-farm conditions due to management and health issues such as the presence of potential pathogens; moreover, gaining access to fistulated animals poses its own set of challenges. Therefore, it becomes imperative to explore alternative nutritional and management strategies to enhance feed utilization in ruminants fed forage diets. These may include the use of probiotics, dietary diversification, or the introduction of adult companions to foster a more diverse rumen microbial community (8).

Rumen microbiota and feed digestion

Most of forage sources used in the Mediterranean basin, as the one used in this study (oats hay) are preserved as hay due to the low precipitation and high seasonality resulting on low quality and highly fibrous forages. These peculiarities make that increasing forage digestibility represent a highly desirable attribute to increase productivity is such conditions. It has been demonstrated that forage digestion is linked with the forage microbial colonization, which is a three-steps process and of greater complexity for preserved than for fresh forages (60). The primary feed colonization is initiated by the rumen microbes associated with the liquid phase. Due to their high motility, protozoa are the microbes that most rapidly colonize forage during the primary colonization (60). Thus, the presence of a complex protozoal community in RFF and RFC animals, along with higher bacterial and methanogens diversities, could accelerate this primary colonization favoring the DM (+8.9%) and OM (+10%) digestibility. A meta-analysis reported similar increases in OM digestibility (+5%), total VFA (+5%) and CH_4 emissions (+13%) in presence of rumen protozoa (44), as noted in our study.

Once feed is colonized by protozoa, the soluble plant components act as chemo-attractants, allowing secondary colonizers such as fungal zoospores and bacteria to further colonize feed particles (61). In this sense, inoculation with fresh rumen fluid led to lower abundance of *Anaeromyces* and *Caecomyces* which have a preference for glucose and fructose (62) but higher of *Neocallimastix* which is a monocentric fungus able to utilize a wider spectrum of substrates including cellulose, xylose, glucose, starch, grass and straw (63). The ability of anaerobic fungi to form resistant spores that allow them to retain viability in dung, soil and feed, may explain the similar anaerobic fugal concentration and diversity observed across treatments (64). Moreover this anaerobic fungal concentration was higher (2 logs) than we previously reported in ruminants fed concentrate diets suggesting an active role in fiber degradation (65). Anaerobic fungi, due to their long life cycle and they ability to digest recalcitrant lignocellulosic substrates, are efficient feed degraders (66) and able to penetrate the plant cuticle providing additional sites for bacteria to attach to protected plant tissues (60). The positive correlation observed between the fungal community structure in RFF and RFC and the bacterial richness seems to support this microbial symbiosis. This cooperation between different microbial groups could explain the higher NDF (+22%) and ADF digestibility (+37%) observed in RFF and RFC than in CTL animals. In particular, the correlation analysis identified the rumen protozoa as the key microbes for fiber digestion. Williams and Coleman (67) described high endoglucanase and xylanase activity for large *Ophryoscolecide* such as *Epidinium, Ophryoscolex*, *Enoploplastron*, *Polyplastron* and *Eudiplodinium*, weak activity for *Entodinium* and negligible fibrolytic activity for Isotrichidae. These carbohydrate-active enzymes (CAZymes) activities have recently been confirmed based on the study of the protozoal proteome and metagenome (68, 69), and here they are supported by our correlation analysis. A recent meta-analysis indicated that the effects of the absence of rumen protozoa tend to decrease as time progress after the defaunation as a result of a partial compensation by other microbial groups (70).

Methanogens are able to utilize H_2 and prevent its accumulation during the fiber degradation process (71). Considering that between 9 and 25% of rumen methanogens are protozoal epi- and endo-symbiotic microbes (72), the presence of rumen protozoa also increased the methanogens concentration and diversity as previously shown (8, 73). This intimate association made challenging to elucidate the specific effects of each microbial group and to link changes in the methanogens community with rumen fermentation and *in vitro* CH_4 production. Direct *in vivo* measurements of CH_4 emissions, as well as a more detailed analysis of the H_2 fluxes in the rumen, would be needed to better explore the impact of methanogens on the energy metabolism.

Rumen microbiota and N metabolism

The availability of dietary protein is often a limiting factor for productivity when ruminants are fed low quality forages (74). The dietary shift from concentrate to forage feeding represented a substantial N shortage, derived from the lower CP content (from 19.9 to 7.9%) and N digestibility, which required an adaptation process. As a result, a decrease in the rumen CP degradation characterized by lower levels of protein breakdown products (i.e., N-NH_3, iso-butyrate and iso-valerate) was noted from day 4 to day 21 across treatments. Moreover, the N-NH_3 values were always below the theoretical threshold (50 mg/L) which can limit the microbial protein synthesis in the rumen (75), indicating that the availability of rumen degradable N was a limiting factor in our experimental conditions. Despite this limitation, the RFF and RFC animals had 2.8 and 6.6 times higher rumen N-NH_3 concentrations

than CTL kids at 4 and 21 days after the dietary shift, respectively, implied a higher N availability for the microbes which could ease the transition to the forage diet. It is known that rumen protozoa can influence the rumen N metabolism at a number of different levels with conflicting effects. Their intense proteolytic activity can explain the positive correlations between rumen N-NH_3 concentration and the protozoal concentration and richness. On one hand, rumen entodiniomorphids possess a vestibulum surrounded by cilia which make them particularly efficient in taking feed particles and proteins suspended in the rumen, being more active in degrading insoluble than soluble N (76). As a result, up to three entodinimorphid genera (i.e., *Entodinium*, *Diplodinium* and *Polyplastron*) were positively correlated with N digestibility leading to higher values (+16.6%) than in the CTL animals. Similar increments in N digestibility were reported when heifers were repeatedly inoculated with bison rumen fluid containing 2.5 times higher protozoal concentration (77). Increased N digestibility represents a positive digestive adaptation when animals are fed low N diets, and could partially explain the higher rumen N-NH_3 concentration. Newbold et al. (44) found that increased N digestibility in presence of protozoa is often accompanied by a shift in the N partition, leading to higher urinary N excretion. This phenomenon was not observed in our study, possibly because the urea-N recycling through the saliva was enhanced as a compensatory mechanism (78). On the other hand, rumen bacteria represent the main N source for rumen protozoa (67), this process being associated with increased rumen N-NH_3 levels and lower microbial protein flow to the intestine (−23%) and the EMPS (−21%), making the rumen less efficient (44). Our findings showed that RFF and RFC animals had approximately half the microbial protein flow than the CTL animals, being this difference greater than observed between faunated and defaunated lambs with higher N intake (7, 46). Low microbial protein flow can represent a relevant handicap for growing, pregnant of lactating ruminants given their high N requirements. Bacterial predation by rumen protozoa is proportional to the protozoal concentration and size, moreover entodiniomorphids had higher predatory activity than holotrich protozoa (47) and ultimately higher impact on microbial protein flow (46, 79). Considering the rumen protozoal concentration and the rates of bacterial CP breakdown activity described for the different protozoal groups (47), it was estimated that 7.7 and 25.3% of the CP intake was broken-down by rumen protozoa in animals inoculated with autoclaved or fresh rumen fluid, respectively. Moreover, considering the proportions of each protozoal taxa it was concluded that most of the bacterial CP breakdown was due to *Entodinium* (83–96%), followed by Diplodiniinae (3.5–6.7%), *Dasytricha* (0.4–5.3%), *Isotricha* (0.1–4.6%) and Ophyoscolecinae (0.01–0.5%). These estimations could explain the positive correlation observed between rumen N-NH_3 concentration and the abundance of most protozoal taxa including entodiniomorphids (*Entodium_sp_LDK-2011, Enoploplastron, Polyplastron* and *Ohryoscolex*) and hototrich (*Isotricha* and *Dasiytricha*). This suggests that all protozoal groups had, to some extent, bacterial breakdown activity. Interestingly, *Entodinium* was the only protozoal taxa with a negative correlation with urinary PD excretion and EMPS, possibly because they represented the most abundant genus in the rumen. These findings suggest that nutritional strategies based on the use of anti-protozoal feed additives should be re-considered to improve N use efficiency in ruminants (80), especially when they are fed low-N diets.

Our study demonstrated that, in addition to protozoa, the bacterial community was also associated with the rumen N metabolism profile and feed utilization, whereas the impact of methanogens and anaerobic fungi was negligible. In particular, the rumen N-NH_3 concentration was positively correlated with several taxa including Verrucomicrobiota and Erysipelatoclostridiaceae, *Selenomonas*, *Veillonellaceae_UCG-001* and *vadinBE97*, and negative correlated with *Desulfobacterota* and *Christensenellaceae_R-7*. Although the impact of these bacterial taxa on the rumen N metabolism is largely unknown, it has been described that *Selenomonas ruminantium* can utilize nitrate, urea and amino acids resulting on N-NH_3 as the main fermentation product (81), as noted in RFF and RFC animals. The *Christensenellaceae*, a recently described family in the phylum *Firmicutes*, is emerging as an important player in human gut health given its inverse correlation to host body mass index and inflammatory bowel disease (82), this microbe being also more abundant in CTL animals. Moreover, our study identified several bacterial taxa such as *Prevotella_ruminicola, Prevotellaceae_Ga6A1_group, Coprococcus, Oscillospira_guilliermondii* and *CAG-352* that had positive correlations with the urinary PD excretion and EMPS, making them candidates for being indicators of efficient N utilization. *Prevotella ruminicola* is one of the few rumen microbes with dipeptidyl peptidase activity facilitating the feed proteolysis and N incorporation into the microbial protein (83). As a result, *Prevotella* and *Oscillospira* have been postulated as indicators of feed efficiency in steers and beef cattle, respectively (53, 58). Similar increases in the levels of *Selenomonas*, *Prevotella* and *Oscillospira* have been described in response to a shift from concentrate feed to grazing diets in sheep (41, 84). A recent study (85) noted that high N efficiency was associated with less diverse rumen bacterial community whereas low N utilization was associated with high abundance of bacteria taxa that promoted greater N excretion through protein degradation in beef cattle. These findings suggest that having a complex rumen microbial community characterized by a high bacterial diversity and presence of rumen protozoa can favor N digestibility but also can limit the microbial protein flow to the intestine (86). Since the low CP content in the forage was a clear dietary limiting factor in this study, the positive effects of having a complex rumen microbiota on the energy metabolism were partially compensated by the negative effects on the N metabolism, resulting on similar animal performances across treatments. These findings underscore the importance of ensuring a sufficient supply of rumen-degradable N when feeding low-quality forages because it promotes a more active and complex rumen microbiota, a phenomenon previously observed in dairy cows (87). This enhanced microbial diversity and activity play ultimately a pivotal role in favoring the degradation of fibrous materials by the rumen microbes.

Conclusion

This study demonstrated that inoculation of young ruminants with fresh rumen fluid from adult ruminants promoted a greater rumen microbial complexity characterized by higher bacterial and methanogens diversity, as well as the presence of a complex protozoal community, which persisted later in life. This increased rumen microbial complexity represented a competitive advantage when adult animals were fed forage allowing a faster adaptation to the diet and optimized energy metabolism (higher DMI, fiber digestion and VFA production). On the contrary, high rumen microbial complexity had contrasting effects on the N metabolism because it favored the N

digestibility but also had a negative impact on the microbial protein flow to the host as a result of increased microbial protein breakdown by the rumen protozoa. These results, suggest that promoting greater rumen microbial diversity is a desirable attribute when animals are fed forages in which the N supply does not represent a limiting factor.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found at: https://www.ebi.ac.uk/ena, PRJEB63607.

Ethics statement

The animal study was approved by animal procedures were conducted in accordance to the National guidelines (RD53/2013) and were approved by the Ethical Committee for Animal Research (EEZ-CSIC, 09/03/2017). The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

AB: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Resources, Software, Validation, Writing – original draft. JP-H: Methodology, Resources, Writing – review & editing. EJ: Methodology, Resources, Writing – review & editing. DY-R: Conceptualization, Funding acquisition, Supervision, Validation, Writing – review & editing.

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Conflict of interest

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

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

Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2023.1272835/full#supplementary-material

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Effect of feeding hydroponic barley seedlings to lactating ewes on blood biochemical indexes and growth performance of lambs

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The aim of this study was to investigate the effects of replacing different ratios of basal diets with hydroponically barley seedlings (HBS) on the serum biochemical indexes and growth performance of lambs. It provides a theoretical basis for the use of HBS in ruminant health and scientific feeding management. In total, 30 ewes were randomly categorized into six groups (two control groups, 4 treatment groups, and 8 replicates in each group). All experiments were conducted under the same feeding and management conditions, on this basis the control group was CK1 and CK2 groups, which CK1 group added 15% corn silage, the treatment groups replacing 5% (group A), 10% (group B), 15% (group C) and 20% (group D) of the basal diet (dry matter basis) with HBS, and the experimental period lasted for 36 days, and the lambs were lactating with their mothers throughout the experimental period. Key results. The contents of total protein (TP), albumin (ALB), milk fat percentage and total solids (TS) in group C were significantly higher than CK1 and CK2 groups ($p < 0.05$) in milk samples; malondialdehyde (MDA) content in groups A and C was significantly lower than groups CK1 and CK2 ($p < 0.05$), alanine aminotransferase (ALT) and azelaic transaminase (AST) contents in groups A and B were significantly higher than CK1 group ($p < 0.05$), TC content in groups A and D was significantly higher than CK1 and CK2 groups ($p < 0.05$), high-density lipoprotein (HDL-c) content in group D was significantly higher than CK1 and CK2 groups ($p < 0.01$) in blood samples; Body height in C group was significantly higher than CK2 group ($p < 0.05$), ear width in group B was significantly higher than CK1 group ($p < 0.05$). In conclusion, under the conditions of this experiment, HBS instead of 5–15% of the basal diet could improve the milk quality of lactating Hu ewes and alleviate the oxidative stress of the body.

KEYWORDS

hydroponic barley seedlings, lactating Hu ewes, lambs, milk quality, antioxidants

1 Introduction

With the continuous expansion of animal husbandry breeding scale, feed shortage has become one of the restrictive factors for the healthy development of the industry. Therefore, it is urgent to accelerate the development and use of unconventional feeds to alleviate the shortage of feed resources. The dry matter content (DM) of hydroponic barley grass is 10.17%, the crude protein (CP) content is 14.98%, the crude fat (EE) content is 3.1%, the calcium (Ca) content is 0.24%, and the phosphorus (P) content is 0.40%. The crude protein, crude fat, and phosphorus content are all higher than that of barley hay (1). Moreover, during the growth period of hydroponic barley grass, the dry matter and starch content decreases with the culture time, and starch is metabolized into soluble polysaccharides to support metabolic and energy needs. The total content of mineral elements, vitamins, and amino acids increases with the increasing culture time (2). As the germination process of barley grains progresses, the content of water-soluble proteins increases, and the total amino acid content also increases significantly (3, 4). The protein and starch involved in the decomposition process involve multiple digestive hydrolytic enzymes, which can enhance the body's digestive function and improve appetite problems (5). By measuring the nutritional value of barley seedlings under different hydroponic time, it was found that its dry matter and starch content decreased with the increase of hydroponic time, and the content of mineral elements such as calcium (Ca), phosphorus (P), potassium (K), magnesium (Mg), crude protein content and crude fat content increased. Magnesium ions mainly act as enzyme activation, regulate calcium and potassium ion channels and promote bone growth (6). Calcium ions are mainly involved in the construction of bones, and potassium ions are mainly involved in maintaining the osmotic pressure (7). Acid–base balance and glycometabolism of the intracellular fluid in animal cells (8).

Barley seedling cultivated by hydroponic technology can be used as a green feed to alleviate the shortage of animal feed resources, grassland degradation and water shortage in pastoral areas. Research has shown that hydroponic barley sprouts have a high vitamin content, which can improve the lactation ability of herbivorous livestock. Harvested hydroponic barley sprouts can provide vitamin E to herbivorous livestock, which has a positive effect on their cell antioxidant capacity and reproductive performance (9). Devendar et al. (10) found that replacing 50% of mixed concentrate with hydroponic barley sprouts in lamb diets can improve their growth performance. Replacing 30% of barley grains can also improve certain rumen characteristics, and increase the digestibility and feed conversion rate of most nutrients. Fazaeli et al. (11) found that replacing 20, 40, and 60% of corn silage with hydroponic barley sprouts in dairy cow diets had no significant effect on their average daily milk production or lactation performance. In conclusion, hydroponic barley sprouts can meet the nutritional needs of herbivorous animals and have the potential to be developed into high-quality forages (12, 13), but the available research on using hydroponic barley sprouts as livestock feed, especially during lactation in sheep, is limited. Therefore, this study selected lactating Hu lambs as the experimental subjects to investigate the effects of different proportions of hydroponic barley sprouts replacing basic feed on serum biochemical indexes and growth performance, providing a reference for further research on the feeding value of hydroponic barley sprouts in sheep production.

2 Materials and methods

2.1 Test animals and materials

Hu ewes were used as test animals in this experiment, which were provided by the Hu ewes breeding base of Xinjiang Mai teng Herding Science and Technology Development Co. HBS were cultivated by Xinjiang Green Chuangfeng Agricultural Development Co. and the growing period was 7 days.

2.2 Experimental design and diet composition

Forty-eight healthy lactating Hu ewes with similar age (2–3 years), litter size (2–3), body weight (45 ± 5 kg), and two lambing number, were selected for the experiment, and were divided into six groups by a one-way completely randomized experimental design, with two control groups of eight replicates each and four treatment groups of eight replicates each. The experimental diets were based on the NRC (2007) 45 kg with double lamb ewes lactation nutritional requirements, All experiments were conducted under the same feeding and management conditions, the control groups was CK1 and CK2 groups, which CK1 group added 15% corn silage, and the treatment group diets were used in an equal replacement method by replacing 5% (group A), 10% (group B), 15% (group C), and 20% (group D) of the basal diet (dry matter basis) with hydroponically grown barley seedlings, respectively (Table 1).

2.3 Feeding management

Before the beginning of the test, the sheep pens were sterilized and disinfected, and all the test lake sheep (big sheep) were orally dewormed with Ivermectin before the test, and ear numbers were marked. The whole animal feeding test was carried out in the same environment by house feeding, and the diets of each group were accurately weighed and evenly mixed according to the proportion of the formula, and then fed regularly at 9:00 and 17:00 every day, and the test period was 36 days, of which the pre-feeding period was 6 days, and the official period was 30 days, and the lambs nursed with the ewes throughout the whole period of the test, and they could freely feed on ewes' materials and drink water freely.

2.4 Sample collection and indicator measurement

2.4.1 Sample collection and processing

(1) At the end of the 31 days feeding period of the formal test, collect the blood of lambs through the jugular vein into sodium heparin anticoagulant tubes, centrifuge at 3,000 rpm/min for 15 min at 4°C, take the supernatant, and separate it into 1.5 mL centrifuge tubes (3 tubes), and store it at −20°C, which is mainly used for the analysis of the immune indexes in the blood.

(2) On the 28th to 30th day of the official test, milk samples were collected from test ewes by hand milking, and the ewes milk were collected three times in the morning at 9:00 a.m., in the middle at

TABLE 1 Experimental diet composition and nutritional level (in dry matter basis).

Items	Groups					
	CK[1]	CK[2]	A	B	C	D
Composition of raw materials/%						
Corn silage	15.00	–	–	–	–	–
Hydroponic barley seedlings	0.00	0.00	5.00	10.00	15.00	20.00
Halm	21.60	26.20	24.89	23.58	22.27	20.80
Alfalfa 17% CP	10.10	15.00	14.25	13.50	12.75	12.00
Mixed concentrate	52.80	58.30	55.36	52.42	49.48	46.70
Premix1	0.50	0.50	0.50	0.50	0.50	0.50
Total	100.00	100.00	100.00	100.00	100.00	100.00
Nutritional level						
ME (MJ/kg)	10.09	10.09	10.13	10.17	10.21	10.25
CP (g/kg)	128.45	128.40	122.93	117.45	111.98	106.50
NDF (%)	39.93	33.26	33.02	32.77	32.53	32.29
Ca (g/kg)	4.68	4.56	4.36	4.15	3.95	3.75
P (g/kg)	3.14	2.90	2.79	2.69	2.58	2.48

[1]The premix provides per kg of concentrate supplement: vitamin A 4200 IU, vitamin B1 0.4 mg, vitamin B22 mg, vitamin B6 1.2 mg, vitamin C 20 mg, vitamin D3 880 IU, vitamin E 500 IU, pantothenic acid 10 mg, niacinamide 100 mg, copper 25 mg, iron 107 mg, manganese 81 mg, zinc 74 mg, and Iodine 6 mg, Selenium 14 mg, Cobalt 3 mg, Choline Chloride 120 mg. [2]Nutritional levels are measured.

14:00 a.m. and in the evening at 20:30 p.m. in the ratio of 4:3:3, and then packed in 50 mL ewes milk bottles, immediately send to the testing company to determine the relevant indexes.

(3) All test lambs were weighed and recorded on a hanging scale before morning feeding on the 1st and 31st days of the test to calculate the average total weight gain and average daily weight gain of the lambs; the body size of the lambs was measured on the 0 and 30th days of the positive test period before morning feeding. During the measurement, the lambs were pulled to the flat ground in a natural standing position, and the body height and body length of the lambs were measured with a measuring stick, and the chest circumference, chest depth, chest width, tube circumference, ear length, ear width, and cross section height were measured with a tape measure and recorded.

2.4.2 Measurement of sample indexes

Ewes milk composition indexes: total protein (TP), albumin (ALB), β-globulin (β-AFP), lactoferrin (LTF), lysozyme (LZM), lactose content, milk fat rate, total solids (TS).

Antioxidant indexes of ewes milk: antioxidant indexes: malondialdehyde (MDA), total superoxide dismutase (T-SOD), glutathione peroxidase (GSH-Px), total antioxidant power (T-AOC), which were determined by Nanjing Aoqing Biotechnology Co.

Plasma biochemical indexes: glucose (GLU), creatine kinase (CK), lactate dehydrogenase (LDH), alanine aminotransferase (ALT), azelaic transaminase (AST), cholesterol (TC), triglyceride (TG), low-density lipoprotein (LDL), high-density lipoprotein (HDL), total protein (TP), urea nitrogen (BUN).

Immune indexes: interleukin-1β (IL-1β), interleukin-2β (IL-2β), interferon gamma (IFN-γ), CD4-T lymphocytes (CD4); antioxidant indicators: malondialdehyde (MDA), total superoxide dismutase (T-SOD), and glutathione peroxidase (GSH-Px), as determined by Nanjing Jianjian Bioengineering Institute.

2.5 Data processing

The data of blood samples, milk samples, body weight and body measurements were preliminarily organized by Excel 2010, and the ANOVA procedure of SPSS 24.0 statistical software was used for one-way ANOVA, while significant differences were used for multiple comparisons by Duncan's method, and the results of the test were expressed as the mean (MEAN) and standard error of the mean (SEM), and $p < 0.05$ as significant level of difference, and $p < 0.01$ as highly significant level of difference.

3 Results and analysis

3.1 Effect of HBS substituting different proportion of basal diet on protein content of ewes milk

As shown in Table 2, the content of TP in group C was significantly or highly significantly higher than groups CK1 and CK2 increased by 18.97 and 15.42%, respectively ($p < 0.01$ or $p < 0.05$), and group D was highly significantly higher than group CK1 increased by 18.97% ($p < 0.01$); ALB in groups C and D was significantly higher than groups CK1 and CK2 ($p < 0.05$); There was no significant differences in β-AFP, LTF, and LZM contents among groups ($p > 0.05$).

3.2 Effect of HBS substituting different proportion of basal diet on antioxidant capacity of ewes milk

As shown in Table 3, the content of GSH-PX in group B was significantly or highly significantly higher than group CK1 increased

TABLE 2 Effect of HBS substituting different proportion of basal diet on protein content of ewes milk.

Items	Groups							
	CK1	CK2	A	B	C	D	SEM	*P*
TP g/L	40.27^{Cc}	41.51^{BCbc}	44.87^{ACBcba}	45.70^{ABCab}	49.84^{Aa}	47.91^{ABa}	0.904	0.004
ALB g/L	21.00^{b}	22.44^{b}	33.41^{ab}	28.41^{ab}	35.60^{a}	39.74^{a}	2.009	0.020
β-AFP g/L	4.05	5.31	5.35	5.70	5.09	5.31	2.016	0.925
LTF g/L	171.87	163.31	178.14	186.66	218.17	177.32	34.725	0.897
Lysyme μg/mL	132.90	210.88	185.00	158.33	232.06	203.46	25.314	0.911

Different lowercase letters show significant differences after peer data ($P < 0.05$), and different uppercase letters show significant differences after peer data ($P < 0.01$). The same as Tables 3–10.

TABLE 3 Effect of HBS substituting different proportion of basal diet on antioxidant capacity of ewes milk.

Items	Groups							
	CK1	CK2	A	B	C	D	SEM	*P*
T-AOC U/mL	7.23	6.26	7.59	5.48	8.42	7.79	0.602	0.791
SOD U/mL	482.47	522.41	584.58	546.26	640.08	613.04	35.011	0.835
GSH-px U/mL	170.32^{Bd}	184.85^{ABcd}	213.74^{Aab}	217.75^{Aa}	208.08^{Aabc}	190.84^{ABbcd}	4.545	0.003
MAD nmol/mL	1.96	1.94	2.15	2.98	2.03	2.09	0.176	0.560

by 27.85% ($p < 0.01$ or $p < 0.05$); There was no significant differences in T-AOC, SOD and MAD contents among groups ($p > 0.05$).

3.3 Effect of HBS substituting different proportion of basal diet on milk composition content

As shown in Table 4, the content of Lactose in group A was extremely significantly higher than groups CK1 and CK2 increased by 34.55 and 27.54%, respectively ($p < 0.01$); Milk fat percent in Groups A, C, and D were significantly higher than group CK2 increased by 28.25, 32.69, and 25.71%, respectively ($p < 0.05$); TS in groups A and C were significantly higher than group CK1, increased by 12.65 and 16.36%, respectively ($p < 0.05$).

3.4 Effects of HBS substituting different proportion of basal diet on plasma antioxidant capacity of lambs

As shown in Table 5, the content of MAD in groups A and C was significantly lower than groups CK1 ($p < 0.05$); and there was no significant effect on the SOD and GSH-px contents among the groups ($p > 0.05$).

3.5 Effects of HBS substituting different proportion of basal diet on plasma glucose and enzyme-related indexes of lambs

As shown in Table 6, the content of CK in group B was significantly higher than group CK1 increased by 92.86% ($p < 0.05$); AST in groups A and B was significantly higher than group CK1 increased by 59.03%,56.43%, respectively ($p < 0.05$); ALT groups A and B was significantly higher than group CK1 and other experimental groups increased by 69.74, 73.03%, respectively ($p < 0.05$); there was no significant effect on the Glu and LDH contents among the groups ($p > 0.05$).

3.6 Effects of HBS substituting different proportion of basal diet on relevant indexes of plasma lipid metabolism in lambs

As shown in Table 7, the content of TC in groups A and C was significantly lower than groups CK1 and CK2 ($p < 0.05$); HDL-c in group D was extremely significantly higher than groups CK1 and CK2 increased by 36.11, 40.00%, respectively ($p < 0.01$); and there was no significant effect on the LDL-c and TG contents among the groups ($p > 0.05$).

3.7 Effects of HBS substituting different proportion of basal diet on plasma immune-related indexes of lambs

As shown in Table 8, the content of IFN-γ in group C was significantly lower than groups CK1 and CK2 decrease by 20.09, 24.95%, respectively ($p < 0.05$); there was no significant effect on the content of IL-1β, IL-2β, and CD4 among the groups ($p > 0.05$).

3.8 Effects of HBS substituting different proportion of basal diet on plasma nitrogen metabolism of lambs

As shown in Table 9, the content of TP in groups A and C was significantly lower than group CK1 ($p < 0.05$); there was no significant effect on BUN content between the groups ($p > 0.05$).

TABLE 4 Effect of HBS substituting different proportion of basal diet on milk composition content.

Items	Groups							
	CK1	CK2	A	B	C	D	SEM	*P*
Lactose g/100 mL	3.82^{Cd}	4.03^{BCcd}	5.14^{Aa}	4.62^{ABCabc}	4.86^{ABab}	4.35^{ABCbcd}	0.120	0.002
Milk fat percent %	3.43^{ab}	3.15^{b}	4.04^{a}	3.59^{ab}	4.18^{a}	3.96^{a}	0.114	0.045
Total solids %	16.20^{Cc}	16.93^{BCc}	18.25^{ABab}	$17.10B^{Cbc}$	18.85^{ABa}	16.88^{Cc}	0.238	0.002

TABLE 5 Effects of hydroponic barley seedling substituting different proportion of basal diet on plasma antioxidant capacity of lambs.

Items	Groups							
	CK1	CK2	A	B	C	D	SEM	*P*
SOD U/mL	171.98	167.73	165.17	170.21	154.45	165.13	2.898	0.608
GSH-px U/mL	27.49	23.20	29.22	28.88	20.87	22.64	1.069	0.079
MAD nmol/mL	7.32^{Aa}	6.21^{ABab}	4.50^{Bc}	5.56^{ABbc}	4.81^{Bc}	5.79^{ABbc}	0.228	0.009

TABLE 6 Effects of HBS substituting different proportion of basal diet on plasma glucose and enzyme-related indexes of lambs.

Items	Groups							
	CK1	CK2	A	B	C	D	SEM	*P*
Glu mmol/L	5.88	6.16	5.26	6.47	6.38	6.19	0.127	0.061
CK U/L	0.14^{b}	0.18^{ab}	0.24^{ab}	0.27^{a}	0.15^{b}	0.15^{b}	0.084	0.020
LDH U/L	4011.56	3953.76	4269.75	4269.75	3680.15	3868.97	107.299	0.597
AST U/L	10.35^{c}	12.37^{abc}	16.46^{a}	16.19^{ab}	10.53^{c}	11.22^{bc}	0.756	0.032
ALT U/L	3.04^{b}	3.71^{ab}	5.16^{a}	5.26^{a}	2.59^{b}	2.55^{b}	0.456	0.007

3.9 Effects of HBS substituting different proportion of basal diet on growth performance of lambs

As shown in Table 10, average daily weight gain, body length, chest circumference, chest depth, chest width, ear length, tube circumference, cross section in each group did not have a group C height was significantly higher than group CK2 ($p < 0.05$); group B ear width was significantly higher than groups CK1, C and D ($p < 0.05$).

4 Discussion

Milk components are divided into two parts, water and solids, solids include a variety of substances such as fat, protein, lactose, minerals and vitamins, and the amount of various components of solids affects the milk quality (14). Lactose, milk fat percentage, total protein, albumin etc. as regular nutrients in sheep milk play a vital role in the growth and development of lambs. Saidi and Omar (15) and Badran et al. (16) replaced regular wheat hay with hydroponically grown barley seedling in the diets of lactating ewes and found that there was no effect on the feed intake, body weight, milk yield and milk composition of the ewes. Samir et al. (12) replace the basal diet with 25% hydroponic feed in the diet of Holstein dairy cows, which can improve the dry matter intake, daily gain and body condition score of dairy cows, as well as milk yield, milk fat percentage and TS content in milk, which is consistent with the results of this study. In this experiment, addition of different proportions of HBS significantly increased lactose, milk fat percentage, TS, TP and ALB contents in sheep milk, which may be related to the fact that the present experiment was conducted by substituting hydroponically grown barley seedlings for different proportions of the basal diet, under the same dry matter conditions, hydroponic feed contains a large amount of protein and amino acid gas, which can improve the digestion and absorption of ruminants, and contains more bioactive substances such as vitamins and polyphenols (17), thus having a positive impact on milk composition. Studies have shown that wheat seedling flour contains a large number of flavonoids and related compounds with strong antioxidant activity (18). In this experiment, the addition of different ratios of hydroponically grown barley seedlings significantly provided GSH-px content in sheep milk, which may be attributed to the antioxidant effect of hydroponically grown barley seedlings containing polyphenolic actives that can scavenge free radicals in the organism and prevent them from damaging the organism (19).

Most of the nutrients required by lambs in the pre-development period come from breast milk, which has the advantages of high elimination and comprehensive nutrition, and is an important source of nutrients during the period from birth to weaning. Blood biochemical level reflects the nutrition and organ metabolism of protein, amino acid, sugar and lipid in the animal body, and blood biochemical indexes are affected by the health condition of the body on one hand, and the nutritional status of the body as well as the

TABLE 7 Effects of HBS substituting different proportion of basal diet on relevant indexes of plasma lipid metabolism in lambs.

Items	Groups							
	CK1	CK2	A	B	C	D	SEM	*P*
TC mmol/L	2.38^{a}	2.28^{a}	1.67^{b}	2.05^{ab}	1.76^{b}	2.14^{ab}	0.074	0.023
HDL-c mmol/L	0.72^{Bb}	0.70^{Bb}	0.69^{Bb}	0.87^{ABa}	0.87^{ABa}	0.98^{Aa}	0.059	<0.001
LDL-c mmol/L	0.88	0.85	0.79	0.74	0.54	0.68	0.036	0.059
TG mmol/L	0.74	0.66	0.45	0.66	0.59	0.55	0.028	0.054

TABLE 8 Effects of HBS substituting different proportion of basal diet on plasma immune-related indexes of lambs.

Items	Groups							
	CK1	CK2	A	B	C	D	SEM	*P*
βIL-1β ng/L	29.05	28.59	24.35	27.06	23.35	27.24	0.648	0.050
βIL-2β ng/L	34.77	37.40	35.34	37.33	31.12	34.82	0.852	0.309
IFN-γ ng/mL	86.24^{a}	82.89^{ab}	74.77^{bc}	82.83^{ab}	69.02^{c}	79.23^{abc}	1.621	0.015
CD4 ng/mL	259.43	250.50	217.10	244.04	190.09	227.64	7.330	0.060

TABLE 9 Effects of HBS substituting different proportion of basal diet on plasma nitrogen metabolism of lambs.

Items	Groups							
	CK1	CK2	A	B	C	D	SEM	*P*
TP g/L	66.69^{a}	62.48^{abc}	58.54^{bc}	61.59^{abc}	56.32^{c}	63.27^{ab}	0.948	0.018
BUN mmol/L	6.86	8.11	6.99	7.55	7.75	8.52	0.184	0.060

nutritional level of the diet on the other hand. In this experiment, with the increase of hydroponic barley seedling substitution, the contents of CK, AST, and ALT had a tendency to increase in all groups, among which, the contents of AST and ALT in group A were significantly higher than those in group CK1; and the contents of CK in group B were significantly higher than group CK1, which means that the substitution of different ratios of basal diets by hydroponic barley seedling had a positive influence on the metabolism of the liver. Plasma cholesterol content can reflect the lipid metabolism of the body (20) and cholesterol can be divided into LDL cholesterol and HDL cholesterol (21). Studies have shown that hydroponically grown barley seedlings are a good source of vitamins, with each 1 kg of hydroponically grown barley seedlings containing 62.4 mg of vitamin A and 1.05 mg of free folic acid (22, 23). Raeisi et al. (24) used 7, 14, and 21% of the body lipids in the diets of sheep with hydroponically grown barley seedlings (fresh weight) in equal proportions to replace some of the barley kernels, and the differences in BUN content between the groups were not significant as the amount of hydroponically grown barley seedlings replaced increased. Studies in diabetic patients and mouse animal models have found that the nutrients in barley seedling can reduce blood lipids, alleviate liver function damage and improve oxidative stress levels. Jiaqiang et al. (25). found that supplementation of hydroponic barley seedlings with restricted basal diet can significantly reduce the blood total cholesterol content and abdominal fat percentage of seeded geese, and hydroponic barley seedlings may regulate the body's fat deposition capacity by reducing blood lipid, which is consistent with the results of blood biochemical indexes in this study. In this experiment, with the increase of hydroponic barley seedling substitution, HDL-c content significantly increased, TP and TC content decreased, and the difference of BUN content was not significant among groups, which may be related to the vitamins contained in hydroponic barley seedling, and folic acid has an important role in regulating the metabolism of lipids and other metabolisms, whereas the content of folic acid in breast milk may be insufficient to promote the protein synthesis and the metabolism of sugar and lipids in lambs (26, 27), and this may be the reason for the decrease of TP and TC content. The reason for the decrease in TP and TC content. A complex network of antioxidant enzymes and non-enzymatic antioxidants that effectively scavenge reactive oxygen species exist in mammalian cells (28). In the present experiment, hydroponically grown barley seedlings in place of different proportions of basal diets increased plasma GSH-px levels and decreased serum MDA levels in lambs. The results of the experiment may be attributed to the fact that the polyphenol content contained in hydroponically grown barley seedlings indirectly increased the content of relevant antioxidant enzymes in the ewe's organism, thus improving the antioxidant capacity of the organism (29).

Growth and development of young lactating animals are influenced by various factors, including breed (genetic factors), maternal effects (newborn weight), and feeding management (breeding environment, diet composition, and dietary nutrition level). The addition of 23% hydroponic barley seedlings to the diet of laying hens breeders can increase the growth rate of breeders, promote the growth and egg production rate of laying hens, and enhance the overall economic efficiency of laying hens (30). The addition of 5% barley malt meal to the diet of lactating piglets increased feed utilization by 7.1% (31).In this experiment, the optimal daily weight gain of lambs in group CK2 may be due to the reduction in the number of lambs, which resulted in an increase in the feed intake of

TABLE 10 Effects of HBS substituting different proportion of basal diet on growth performance of lambs.

Items	Groups							
	CK1	CK2	A	B	C	D	SEM	*P*
Average daily weight gain g/d	92.00	134.22	112.44	104.89	114.89	101.89	8.97	0.84
Dead weight loss %	20.00	20.00	6.67	13.33	6.67	13.33		
Height cm	4.93^{ab}	2.2^{b}	5.33^{ab}	4.47^{ab}	5.93^{a}	5.2^{ab}	0.47	0.27
Body length cm	3.07	3.73	4.73	4.13	4.93	4.20	0.42	0.84
Chest circumference cm	11.00	14.40	10.53	9.27	14.93	11.20	0.83	0.29
Chest depth cm	2.73	2.20	2.87	2.40	3.20	3.07	0.28	0.90
Chest width cm	0.47	0.40	1.33	1.00	0.73	1.26	0.22	0.75
Perimeter cm	0.40	0.33	0.40	0.53	0.47	0.27	0.07	0.89
Ear length cm	1.93	1.60	1.80	2.27	1.67	1.53	0.15	0.76
Ear width cm	0.33^{b}	0.8^{ab}	0.8^{ab}	1.2^{a}	0.4^{b}	0.4^{b}	0.09	0.04
Crucifixion cm	4.93	4.20	5.07	4.80	5.13	3.67	0.46	0.94

individual lambs and an increase in the weight of lambs in comparison to the replacement group. However, in terms of overall substitution level, group C had the best average daily weight gain, which may be due to the low dry matter and starch content of hydroponically grown barley seedling, in which starch can be mostly decomposed and metabolized to soluble polysaccharides, and the addition of 15% hydroponically grown barley seedling can be used to meet the metabolic and energy needs of the lambs' organisms. From the analysis of the mortality rate, the mortality rate of each experimental group was lower than that of the control group, and the mortality rate of groups A and C was the lowest. The mortality rate was significantly lower in all the test groups than in the control group, and the lowest mortality rate was achieved with the use of 5 and 15% hydroponically grown barley seedlings in place of the basal ration. Since the mortality rates were within the permissible normal range, there was no mortality due to external factors.

5 Conclusion

Under the conditions of this test, HBS instead of 5–15% of the basal diet were able to improve milk quality and alleviate oxidative stress in the body of lactating Hu ewes under the conditions of this experiment.

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 the Feed Research Institute, Xinjiang Academy of Animal Sciences. The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

YM: Writing – original draft, Writing – review & editing. TG: Conceptualization, Data curation, Investigation, Writing – original draft. ZZ: Methodology, Software, Writing – review & editing. GA: Formal analysis, Project administration, Writing – review & editing. YJ: Formal analysis, Project administration, Writing – review & editing. YT: Funding acquisition, Resources, Writing – review & editing. LH: Methodology, Project administration, Writing – review & editing.

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Conflict of interest

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

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Rumen-protected methionine supplementation alters lipid profile of preimplantation embryo and endometrial tissue of Holstein cows

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Our objective is to evaluate the effects of feeding rumen-protected Met (RPM) throughout the transition period and early lactation on the lipid profile of the preimplantation embryos and the endometrial tissue of Holstein cows. Treatments consisted of feeding a total mixed ration with top-dressed RPM (Smartamine® M, Adisseo, Alpharetta, GA, United States; MET; $n = 11$; RPM at a rate of 0.08% of DM: Lys:Met = 2.8:1) or not (CON; $n = 9$, Lys:Met = 3.5:1). Endometrial biopsies were performed at 15, 30, and 73 days in milk (DIM). Prior to the endometrial biopsy at 73 DIM, preimplantation embryos were harvested via flushing. Endometrial lipid profiles were analyzed using multiple reaction monitoring-profiling and lipid profiles of embryos were acquired using matrix assisted laser desorption/ionization mass spectrometry. Relative intensities levels were used for principal component analysis. Embryos from cows in MET had greater concentration of polyunsaturated lipids than embryos from cows in CON. The endometrial tissue samples from cows in MET had lesser concentrations of unsaturated and monounsaturated lipids at 15 DIM, and greater concentration of saturated, unsaturated (specifically diacylglycerol), and monounsaturated (primarily ceramides) lipids at 30 DIM than the endometrial tissue samples from cows in CON. In conclusion, feeding RPM during the transition period and early lactation altered specific lipid classes and lipid unsaturation level of preimplantation embryos and endometrial tissue.

KEYWORDS

amino acids, lipid profiling, embryo, endometrium, dairy cow

Introduction

Fertility is the main physiological process that is affected by dietary imbalances (1). During the transition period, high-producing dairy cows face increased nutritional requirements to support colostrum and milk production while voluntary feed intake is limited, thus experiencing at least some degree of negative energy and protein balance (2–4). This nutritional

imbalance during the transition period increases the risk for metabolic disorders, including reproductive tract inflammatory disorders, which can impair the fertility of dairy cows (5, 6). Multiple studies have established the importance of implementing nutritional strategies to improve fertility in dairy cows (5, 7–9). Unbalanced maternal nutrition also impacts embryonic development (10), especially during early stages of development, when embryos require several indispensable nutrients to support the rapid cell division (11). In addition, low-intake of Met, choline, or folate can decrease the concentration of S-adenosylmethionine (SAM), potentially leading to hypomethylation of the DNA (12), and thus altering gene expression and phenotype (13). For instance, reductions in Met and B vitamins resulted in altered epigenetics and DNA methylation of offspring, leading to changes in health-related phenotypes in sheep (14). Therefore, Met assumes particular significance during the developmental stage of the embryo. Methionine is an important methyl donor (15), and is a key player in the one-carbon catabolism, as noted in studies by Locasale (16) and Shiraki et al. (17). This process not only supplies the essential building blocks required for biosynthesis but also generates antioxidant components through the transsulfuration pathway and intermediate metabolites that serve as substrates or cofactors for various enzymatic activities, as elucidated by Xu and Sinclair (18) and summarized in Coleman et al. (19). Supplementation of rumen-protected Met (RPM) guarantees the intestinal delivery of this limiting amino acid (20), increasing circulating plasma Met for utilization (21, 22).

In vitro studies report that the amino acid availability in culture media affects the embryo's development (23–25). Bonilla et al. (23) reported that the Met requirements for the preimplantation embryo is between 14 and 21 μM/L. Additionally, there was a lower percentage of oocytes that developed to blastocyst in cultures without Met at day 7 and 8, and the proportion of expanded, hatching, or hatched blastocysts on day 7 was reduced at lower concentrations of Met (23). Although less available, *in vivo* studies reported that increased supply of intestinally available Met to dairy cows during the transition period alters the transcriptome of bovine blastocyst, which could impact embryonic and adult functions (26, 27). Additionally, supplementation of RPM increased the expression of 3β-HSD in follicular cells, which aids in synthesis of all classes of steroid hormones that are involved in pregnancy maintenance processes (28). In the same study, Acosta et al. (28) also reported increased concentration of Met in the follicular fluid harvested from cows that were supplemented with RPM. While the direct effect of Met on the development of the preimplantation bovine embryo has not yet been investigated, the addition of 20 amino acids in media resulted in an increased number of cells in the blastocyst, suggesting improved growth (29).

Despite some differences in the response according to the organ of interest, Met restriction has been associated with altered lipid metabolism, particularly in monogastric studies (30). Thus, an increase in Met availability could also impact lipid metabolism. For instance, Obeid et al. (15) reported that Met aids in the synthesis of phospholipids. Additionally, Met can act as a lipotropic agent, assisting with lipid removal from the liver through stimulation of very low-density lipid formation (27). Furthermore, Acosta et al. (31) reported an increased lipid accumulation in preimplantation embryos from cows fed RPM during the transition period, suggestive of a response to increased fatty acid concentration in blood because Met stimulates triacylglycerol (TG) clearance from the liver (31). Therefore, dietary supplementation of Met could possibly increase the lipid availability in the embryo.

Waladkhani et al. (32) reported an increase of arachidonic acid and docosahexaenoic acid in rats upon supplementation with Met, suggesting that Met could be increasing the activity of desaturases. In support, the enzyme stearoyl-CoA desaturase-1 appears to be directly related to the metabolism of sulfur amino acids, particularly cysteine (33). Cysteine is a product of the Met metabolism (34). Additionally, Met-restricted diets resulted in a decrease in SCD-1 expression by the liver in mice (35). Thus, we hypothesize that increased availability of Met during the transition period and early lactation would affect lipid diversity and accumulation in the bovine uterus and embryo, possibly because of an increase in desaturases. Additionally, there is limited research regarding the effects of RPM on the lipid profile of bovine embryos and on the extent of lipid classification in the bovine uterus. Therefore, we aim to investigate the lipid profile in the preimplantation embryo and in the endometrial tissue of Holstein cows fed RPM during the transition period and early lactation.

Materials and methods

Experimental design and dietary treatments

All experimental procedures were approved by the University of Illinois (Urbana-Champaign) Institutional Animal Care and Use Committee (no. 13023). Detailed procedure is described elsewhere (31). Briefly, 20 Holstein cows entering their second or greater lactation (parity 3.0 ± 1.2) enrolled in the experiment at 21 ± 1 days before expected calving date. Cows were randomly assigned to one of two treatments, which consisted either of a TMR top-dressed with RPM [MET; $n = 11$; RPM at a rate of 0.08% of dry matter (DM): Lys:Met 2.8:1 (Smartamine® M, Adisseo, Alpharetta, GA, United States)] or without (CON, $n = 9$; Lys:Met 3.5:1), which were fed until 73 ± 1 days in milk (DIM). Cows were blocked according to the expected calving date, and within each block, cows were balanced for parity, previous lactation milk yield, and BCS prior the close-up. We included this information in the text now. All cows were milked three times a day and received a complete total mixed ration (TMR) diet that met or exceeded their energy requirements according to the NRC (20). Supplementation of RPM (0.08% of DM) was established based on Zhou et al. (22) and calculated using data of TMR offered on a DM basis.

Estrus synchronization, superstimulation, and embryo flushing protocol

Protocols for synchronization, super stimulation, and embryo flushing were performed according to Acosta et al. (31). Pre-synchronization protocol consisted of with one injection of prostaglandin F2α (25 mg, intramuscular of dinoprost tromethamine; 5 mL of Lutalyse, Zoetis Animal Health, NJ, United States), at 30 ± 1 DIM, an injection of GnRH (100 μg, intramuscular, of gonadorelin hydrochloride; 2 mL of Factrel, Zoetis Animal Health) at 44 DIM immediately followed by the insertion of a controlled internal drug release (CIDR; Eazi-Breed CIDR, Zoetis Animal Health) containing 1.38 g of progesterone. At 51 Dim, the CIDR was removed and an

injection of prostaglandin F2α was administered. At 53 DIM, cows received the second injection of GnRH. At 60 DIM, all follicles greater than 5 mm were aspirated using an ultrasound-guided transvaginal approach, with an 18-gauge × 55 cm aspiration needle. After follicular aspiration, a CIDR device was inserted in all cows. One day and a half later, superstimulation protocol was initiated, with intramuscular administration of FSH equivalent NIHFSH-P1 (400 mg, Folltropin-V, Bioniche, Life Science, Belleville, ON, Canada). Eight subsequent decreasing dosages of FSH were administered at 12-h interval over a period of 4 days. Additionally, all cows received an injection of PGF2α at 63 and 64 DIM, and the CIDR was removed at 65 DIM. Ovulation was induced with administration of GnRH 24 h after CIDR removal. Artificial insemination was performed by the same technician at 12 and 24 h after administration of GnRH, with semen from a commercially available Holstein sire (014HO05388; two doses per insemination). Superstimulation response was assessed by counting the number of CL in each ovary through ultrasound imaging (Ibex Pro portable ultrasound, E. I. Medical Imaging, Loveland, Colorado, United States) and was reported by Acosta et al. (31). The number of CL for cows that received MET (11.2 ± 0.9) was greater ($p = 0.01$) than for cows in CON (9.2 ± 0.9). Finally, embryos were flushed 6.5 days after the first timed artificial insemination and evaluated according to the manual of the International Embryo Transfer Society (36). Embryos were exposed to 1.5 M EG cryoprotectant solution (Vigro Ethylene Gycol®, Bioniche Animal Health, Pullman, WA, United States) for 5 min and loaded into 0.25 mL plastic straws before being placed into a temperature controller (Freeze Control®, Margaret River, Western, Australia) at − 6.5°C. Afterwards, the straws were seeded, equilibrated for 10 min at − 6.5°C, and cooled at − 0.6°C/min until the temperature reached − 35°C. Recovery rate, number of embryos recovered, embryo quality, and embryo stage were not different between treatments [$p = 0.22$; (31)]. Embryos were stored in liquid nitrogen until analysis.

Lipid analysis of embryos by MALDI-TOF MS

Embryos from three cows (MET = 1 cow yielding 5 embryos, CON = 2 cows yielding 3 embryos total) that were quality = 1 and were at stage = 4 or greater were used for lipid analysis (36). Each embryo was washed in phosphate buffer solution (PBS) three times to remove media and 25% methanol two times to remove salts that could disturb ionization. Using a mouth pipette, the embryos were placed onto an Opti-TOF 384 well plate (Ab SCiex, Concord, Ontario, CA), and allowed to dry before being stored at − 80°C. Individual embryos had their lipid profile acquired via matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) at the Bindley Bioscience Center at the Discovery Park of Purdue University (West Lafayette, IN). Prior to analysis, a matrix was administered to the sample plate, which consisted of 8 mL of TA30 solvent (30:70 [v/v] acetonitrile:0.1% TFA in water) to 160 mg of 2,5-DHB (2,5–Dihydroxybenzoic acid) for a total volume of 8 mL of matrix solvent that was loaded and administered 16 times in a cross section pattern at 45°C by a matrix sprayer (HTX TM-Sprayer, HTX Technologies, Chapel Hill, NC, United States). From there, images and mass spectra were obtained in a MALDI 4800 TOF instrument (AB SCiex, Concord, Ontario, CA) while utilizing the 4,000 Series Explorer Software (v3.7, AB SCIEX) in reflectron at the positive ion mode. All images were acquired with the 4,800 Imaging Tool Software[1] (M. Stoeckli, Novartis Pharma, Basel, Switzerland) with an imaging raster of 100 µm at 50 shots per subspectrum in the m/z range of 700 to 1,200.

Uterine biopsy collection

Uterine biopsy was performed at 15, 30, and 73 DIM from all eligible cows. Exclusion criteria for the performance of uterine biopsy were presence of vaginal discharge (Metricheck) score plus smell >3 on the biopsy day or a drop in DMI greater than 20 kg from the day before. Uterine biopsies were not performed on 10 cows (CON = 4, MET = 6) on d 15; on 11 cows (CON = 5, MET = 6) on d 30; and on 7 cows on d 73 (CON = 4, MET = 3). Endometrial tissue samples were collected transcervically from the body of the uterus using a biopsy forceps (48 cm in length, 2 cm diameter; Aries Surgical, Davis, CA, United States). Endometrial tissue was collected at approximated 2 cm beyond the end of the cervix. Endometrial samples were then placed into sterile DNA/RNA-free cryotubes (Simport, Beloeil, Quebec, Canada), and flash-frozen in liquid nitrogen.

Lipid analysis by MRM-profiling

Lipid analysis on uterine tissue samples were performed at the Bindley Bioscience Center using Multiple Reaction Monitoring profiling (MRM-profiling) mass spectrometry. The uterine samples were homogenized, and the lipids were extracted before the discovery phase of analysis using the Bligh and Dyer method (37). The discovery phase consisted of pooling the lipid extracts from each sample, resulting in two pooled samples that were representative of each treatment, to identify the groups of lipids that were present in the samples. Methods used for monitoring the lipids found in the discovery phase are in Table 1. From there, the samples were delivered through a micro-autosampler (G1377A) into a triple quadrupole (QqQ) mass spectrometer (6,460, Agilent Technologies, San Jose, CA). Selection of the parent ion occurred at the first quadrupole, collision induced dissociation caused fragmentation at the second quadrupole, and finally, the fragment was monitored at the third quadrupole. Each sample was run through five different methods (4 methods for positive and 1 for negative ion mode) during the screening step to ensure accurate screening of the targeted lipids found during the discovery phase. Solvent (ACN + 0.1% formic acid) was pumped through the mass spectrometer between each injection of the sample. Further details regarding the MRM-profiling method have been described (38, 39).

Statistical analyses

Statistical analyses were performed using Metaboanalyst 3.0[2] and SAS 9.4 (SAS Institute Inc. Cary, NC, United States). Lipid profiles were

1 http://www.maldi-msi.org

2 www.metaboanalyst.ca

TABLE 1 Discovery phase methods and respective scan modes for individual screening of uterine biopsy samples for 15, 30 and 73 DIM.

Method	Ion mode	Number of transitions	Scan mode used for the discovery step
1	Positive	178	Phosphatidylcholine (PC), Sphyngomyelin (SM), Glycerolipids containing palmitoleic acid (16:1), palmitic acid (16:0), DHA (22:6), tetracosahexaenoic acid (24:6) and dotriacontanoic acid (32:0).
2	Positive	178	Glycerolipids containing palmitic acid (16:0), linolenic acid (18:3), linoleic acid (18:2), oleic acid (18:1), stearic acid (18:0), eicosatetraenoic acid (20:4), eicosatrienoic acid (20:3), eicosadienoic acid (20:2), arachidic acid (20:0), docosatetraenoic acid (22:4), docosahexaenoic acid (22:6).
3	Positive	178	Glycerolipids containing 22:6, 22:4, docosadienoic acid (22:2), docosenoic acid (22:1), behenic acid (22:0), lignoceric acid (24:0), Phosphatidylcholine (PC) and Sphyngomyelin (SM).
4	Positive	181	Phosphatidylcholine (PC) and Sphyngomyelin (SM), Ceramides, Phosphatidylinositol (PI), Acylcarnitines, nervonic acid (24:1), montanic acid (28:0).
5	Negative	64	Glycerolipids containing 14:1 residue, 16:0, 18:0, Phosphatidylinositol (PI).

Less than 200 MRMs were used per method due to limited time for data collection.

processed as relative ion count intensities (each ion in the mass spectrum had the ion counts divided by the total ion count of all ions in the mass spectrum) and 10% of background noise in relation to the highest peak value was removed for the embryo data to reduce false positives. Principal component analysis (PCA) was performed by replacing values below the 10% threshold with means and auto scaling according to the Metaboanalyst (see Footnote 2). Only auto scaling was applied for the uterine biopsy data due to background noise being removed during the discovery phase of analysis. All PC with eigenvalues (λ) greater than or equal to 0.9 were extracted, and only loadings greater than 0.49 were discussed. Lipids were tentatively attributed based on the LipidMaps database[3] considering a mass tolerance appropriated for the instruments used (0.4 Da for MALDI and up to unitary resolution for the QqQ). For uterine biopsies, lipid attribution was also pursued using METLIN.[4] For MALDI-MS data, a standard deviation of 0.4 Da was used for the theoretical masses m/z 708.90 and 732.90 due to limited attributions from the database. Additionally, the theoretical mass of m/z 710.90 was used to attribute the lipids for m/z 708.90 due to one unsaturation loss equaling 2 mass units for the embryo data. Based on previous experience using MALDI and 2,5-dihydroxybenzoic acid as a matrix, as well as MS/MS of lipids that are present in oocytes, we only considered phosphatidylcholine (PC), sphingomyelin (SM), and triacylglycerol (TG) as possible lipids for the tentative attributions in the embryos. Finally, variables that were considered to be important according to the PCA (i.e., loadings in the top 5%) were analyzed using the MIXED procedure of SAS. The model for the previously stated response variables contained the fixed effect of treatment. Cow was considered the experimental unit and treated as a random effect. Residual distribution was evaluated for normality and homoscedasticity of variance in all analyses. Statistical significance was declared as $p \leq 0.05$ and tendency at $0.05 > p \leq 0.15$.

Results

Productive responses and uterine health status data are reported in Zhou et al. (22) and (61), respectively. Briefly, cows fed RPM had greater DMI during close-up and first 30 DIM, greater milk yield, energy-corrected milk, and fat-corrected milk (22). In addition, the incidence of ketosis and retained placenta tended to be lower in cows that received RPM (22). Body weight and energy balance did not differ between cows that were fed RPM or not (22). Cows fed RPM had a greater percentage of uterine polymorphonuclear (PMN) cells at 15 DIM, but lower at 30 DIM and at 73 DIM, than cows in CON (61).

Embryo lipid analysis

To better explain which lipids were detected in the embryos, four PCs were extracted from the PCA. These four PCs accounted for 85% of the total variability (Table 2). Principal component 2 represents embryos that mainly have phosphatidylcholine (PC) lipids. Principal component 3 represents embryos that mainly have TG lipids, whereas PC4 is an even mixture of TG, sphingomyelin (SM), and PC lipids. Analysis of the relative amounts of lipids that were of interest based on PCA for embryos retrieved at 73 DIM is in Table 3. The embryos from cows in MET tended ($p = 0.10$) to have lower monounsaturated lipid amounts than cows in CON. Alternatively, the embryos from cows in MET had greater ($p < 0.01$) polyunsaturated lipid amounts, specifically TG and PC lipids, compared to the embryos from cows in CON. Tentative attributions for lipids of interest for embryos based on PCA are presented in Supplementary Table S1. The extracted loadings (PC1: loadings ≥0.15459; PC2: loadings ≥0.20502; PC3: loadings ≥0.19442; PC4: loadings ≥0.23844) indicate that PC1 represents animals that have triglycerides lipids present. Scores plots for lipid markers in embryos retrieved at 73 DIM are in Supplementary Figure S1.

Endometrial biopsy lipid analysis at 15 DIM

To better explain which lipids were found in the uterine endometrial biopsies obtained at 15 DIM, method 1 had PCs extracted accounting for 93.4% variability, and methods 2–5 had 2 PCs extracted each accounting for 79.6, 83.9, 85.3, and 91.9% variability, respectively (Table 4). Grouping identification, while an added benefit, was not revealed for PC1 for methods 1, 2, 4, or 5. Analysis of lipid markers that

3 http://www.lipidmaps.org

4 https://metlin.scripps.edu/index.php

TABLE 2 Principal components (PC), proportion (p), and cumulative proportion (Cp) of the principal component analysis for embryos retrieved at 73 DIM.

PC	p	Cp
PC1*	37.9	37.9
PC2*	19.8	57.6
PC3*	15.4	73.1
PC4*	11.9	85.0 93.2 97.8
PC5	8.2	93.2
PC6	4.6	97.8

*Extracted principal components.

TABLE 3 Least squares means and associated standard errors of normalized lipid marker intensity ion counts[1] for embryos from Holstein cows supplemented with rumen-protected Met (MET) or not (CON) from 21 days before calving until 73 DIM.

Variable	Treatment			*p* value
	CON[2]	MET[3]	SEM[4]	
Lipid attribution, *m/z*				
[PC(P-30:2) + Na]$^+$, 708.90	2.25	1.79	0.17	0.09
[SM(d34:1) + Na]$^+$, 725.51	5.44	2.13	1.30	0.11
[TG(50:4) + H]$^+$; [TG(48:1) + Na]$^+$, 827.50	1.41	2.78	0.37	0.06
[PC(42:1) + H]$^+$; [PC(P-42:4) + Na]$^+$, 872.90	1.48	2.10	0.16	0.03
[PC(42:0) + H]$^+$; [PC(O-42:4) + Na]$^+$, 874.90	5.67	8.30	0.78	0.04
[TG(54:8) + H]$^+$; [TG(52:5) + Na]$^+$, 875.90	2.07	3.01	0.31	0.05
[TG(54:6) + H]$^+$; [TG(52:3) + Na]$^+$, 879.50	1.56	4.81	1.26	0.11
non-attributed, 906.70	1.34	2.92	0.63	0.12
[TG(66:12) + H]$^+$; [TG(64:9) + Na]$^+$; [TG(64:17) + K]$^+$; [TG(62:3) + K]$^+$, 1,036	1.29	3.27	0.66	0.07
Group				
Unsaturated	21.16	24.25	1.98	0.26
Monounsaturated	8.32	5.05	1.32	0.10
Polyunsaturated	17.07	25.90	1.42	0.003

[1]Intensity ion counts multiplied by 1000. [2]CON, control, no rumen protected Met, $n = 3$. [3]MET, rumen protected Met, $n = 5$. [4]SEM multiplied by 1000.

were of interest according to the PCA for 15 DIM are in Table 5. Cows in CON tended ($p = 0.14$) to have higher amounts of saturated lipids compared to cows in MET. Similarly, cows in CON tended ($p = 0.13$) to have higher amounts of unsaturated/monounsaturated lipids, specifically PC lipids, than cows in MET. Tentative attributions for lipids of interest for uterine endometrial biopsies obtained at 15 DIM based on PCA are in Supplementary Table S3. The extracted loadings (Method 1: PC1 loadings ≥0.077435; Method 2: PC1 loadings ≥0.093893 and PC2 loadings ≥0.17326; Method 3: PC1 loadings ≥0.095559 and PC2 loadings ≥0.14156; Method 4: PC1 loadings ≥0.087559 and PC2 loadings ≥0.15073; Method 5: PC1 loadings ≥0.14552 and PC2 loadings ≥0.27031) are in Supplementary Table S4. Scores plots for methods 1–5 for lipid markers in uterine biopsies retrieved at 15 DIM are found in Supplementary Figures S2–S6.

TABLE 4 Principal components (PC) of each method, proportion (p), and cumulative proportion (Cp) of the principal component analysis for uterine biopsies retrieved at 15 DIM.

Variable	p	Cp
Method 1		
PC1*	93.4	93.4
PC2	3.3	96.8
PC3	1.9	98.6
PC4	0.6	99.2
Method 2		
PC1*	63.5	63.5
PC2*	16.1	79.6
PC3	8.9	88.5
PC4	4.8	93.3
Method 3		
PC1*	61.2	61.2
PC2*	22.6	83.9
PC3	5.3	89.1
PC4	4.6	93.8
Method 4		
PC1*	71.4	71.4
PC2*	14.0	85.3
PC3	5.6	91.0
PC4	5.2	96.2
Method 5		
PC1*	71.2	71.2
PC2*	20.7	91.9
PC3	3.6	95.6
PC4	1.9	97.5

*Extracted principal components.

Endometrial biopsy lipid analysis at 30 DIM

To better explain which lipid markers are present in the endometrial biopsies obtained at 30 DIM, two PCs were extracted for methods 1–5 with the following variability: 97.5, 92.2, 85.5, 85.4, and 85.8%, respectively (Table 6). Analysis of lipids of interest according to the PCA for 30 DIM are in Table 7. Cows in MET tended ($p = 0.10$) to have increased saturated lipid amounts compared to cows in CON. Cows in MET had increased ($p \leq 0.05$) amounts of unsaturated and monounsaturated lipids, specifically diacylglycerol (DG) and ceramides (CER), in comparison with cows in CON. Tentative attributions for lipids of interest for

TABLE 5 Least squares means and associated standard errors of normalized lipid marker intensity ion counts[1] for uterine endometrial biopsies obtained at 15 DIM from Holstein cows supplemented with rumen-protected Met (MET) or not (CON) 21 days before calving until 73 DIM.

	Treatment			*p* value
Variable	CON[2]	MET[3]	SEM[4]	
Method 3, *m/z*				
PC (O-34:1); PC (P-34:0), 746.4→184.2	6.05	4.89	0.52	0.15
PC (O-34:1); PC (P-34:0), 746.5→184.2	4.46	3.61	0.35	0.13
PC (34:1), 760.7→184.2	2.66	2.13	0.23	0.14
Group				
Saturated	10.51	8.50	0.86	0.14
Unsaturated/ Monounsaturated	13.16	10.62	1.07	0.13

[1]Intensity ion counts multiplied by 1000. [2]CON, control, no rumen protected Met, $n = 5$. [3]MET, rumen protected Met, $n = 5$. [4]SEM multiplied by 1000. *Variables for Methods 1, 2, 4, and 5 were not statistically different.

TABLE 6 Principal components (PC) of each method, proportion (p), and cumulative proportion (Cp) of the principal component analysis for uterine biopsies retrieved at 30 DIM.

PC	p	Cp
Method 1		
PC1*	81.2	81.2
PC2*	16.3	97.5
PC3	1.3	98.8
PC4	0.7	99.5
Method 2		
PC1*	66.5	66.5
PC2*	25.7	92.2
PC3	4.0	96.2
PC4	2.3	98.5
Method 3		
PC1*	71.8	71.8
PC2*	13.7	85.5
PC3	7.0	92.4
PC4	3.0	95.4
Method 4		
PC1*	50.8	50.8
PC2*	34.6	85.4
PC3	6.8	92.2
PC4	2.5	94.8
Method 5		
PC1*	71.4	71.4
PC2*	14.4	85.8
PC3	4.6	90.4
PC4	3.9	94.3

*Extracted principal components.

uterine endometrial biopsies obtained at 30 DIM based on PCA are in Supplementary Table S5. The extracted loadings (Method 1: PC1 loadings ≥0.082637 and PC2 loadings ≥0.15433; Method 2: PC1 loadings ≥0.092384 and PC2 loadings ≥0.14449; Method 3: PC1 loadings ≥0.0884 and PC2 loadings ≥0.15648; Method 4: PC1 loadings ≥0.098898 and PC2 loadings ≥0.12144; Method 5: PC1 loadings ≥0.14766 and PC2 loadings ≥0.27869) are in Supplementary Table S6. Identification of groups based on extracted loadings for methods 1, 2, and 5 did not reveal valuable information due to the vast possibilities of tentative lipids. Scores plots for methods 1–5 for lipid markers in uterine biopsies retrieved at 30 DIM are found in Supplementary Figures S2–S6.

Endometrial biopsy lipid analysis at 73 DIM

One PC was extracted for method 1 accounting for 89.7% of the total variability. Three PCs were extracted for methods 2 and 3 accounting for 83.4 and 82.6%, respectively, of the total variability. Finally, two PCs were extracted for methods 4 and 5 accounting for 82.0 and 83.9%, respectively, of the total variability (Table 8). Principal component one for method 1 at 73 DIM represents animals whose uterine biopsies contain mainly DG lipids. While group identification could not be accomplished for PC1 of method 2, PC2 represents animals that have higher abundances of TG lipid amounts. Moreover, PC3 represents animals that have higher DG lipid amounts. Finally, no groups were discovered for PC1 of method 5 at 73 DIM, however, PC2 represents animals that have higher DG lipid amounts. Analysis of lipids of interest according to the PCA for 73 DIM are in Table 9. Relative amounts of saturated lipids were higher ($p = 0.06$) for cows in MET compared to cows in CON. Likewise, cows in MET tended ($p = 0.10$) to have increased amounts of unsaturated lipids, specifically DG lipids, than cows in CON. Cows in MET had increased ($p \leq 0.05$) monounsaturated lipid amounts compared to cows in CON. Tentative attributions for lipids of interest for endometrial biopsies obtained at 73 DIM based on PCA are in Supplementary Table S7. The extracted loadings (Method 1: PC1 loading ≥0.078854; Method 2: PC1 loadings ≥0.10085, PC2 loadings ≥0.1695, and PC3 loadings ≥0.21204; Method 3: PC1 loadings ≥0.099032, PC2 loadings ≥0.14595, and PC3 loadings ≥0.16354; Method 4: PC1 loadings ≥0.090057 and PC2 loadings ≥0.13231; Method 5: PC1 loadings ≥0.14999 and PC2 loadings ≥0.30029) are in Supplementary Table S8. Scores plots for methods 1–5 for lipid markers in uterine biopsies retrieved at 73 DIM are found in the Supplementary Figures S2–S6.

Discussion

We aimed to assess the effects of supply of RPM during the transition period until around peak of lactation on lipid profiles of the preimplantation embryo and postpartum endometrial tissue of Holstein cows. We postulated that the lipid profiles, as well as unsaturation level, would be different in embryos and uterine tissue from cows fed RPM when compared with cows in CON. Our hypothesis was corroborated through the demonstration of increased PC and TG content, and polyunsaturated fatty acids in embryos from cows fed RPM in comparison with embryos from cows in CON. Additionally, feeding RPM also increased unsaturated and

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TABLE 7 Least squares means and associated standard errors of normalized lipid marker intensity ion counts[1] for uterine endometrial biopsies obtained at 30 DIM from Holstein cows supplemented with rumen-protected Met (MET) or not (CON) 21 days before calving until 73 DIM.

	Treatment			*p* value
Lipid, *m/z*	CON[2]	MET[3]	SEM[4]	
Method 1, *m/z*				
LysoPE (24:6); PS (20:0); CER (d34:1), 554.1→280.8	4.65	10.77	2.70	0.14
DG (36:7), 611.7→354.4	0.59	1.49	0.42	0.15
PI (P-20:0), 612→354.7	1.41	19.81	7.86	0.12
non-attributed, 627.9→354.6	4.08	10.14	2.38	0.10
non-attributed, 627.9→370.6	1.87	3.95	0.94	0.14
non-attributed, 630→356.7	2.58	5.66	1.22	0.10
DG (44:4); PA (36:2), 701.9→428.6	1.82	3.76	0.88	0.14
Method 2, *m/z*				
non-attributed, 432.1→106.8	14.35	8.85	2.07	0.09
non-attributed, 432.2→110.9	16.44	10.48	2.65	0.14
DG (36:8), 609.8→280.5	2.00	7.76	2.13	0.08
DG (36:7); PG (24:0), 611.9→282.6	2.29	6.73	1.70	0.09
DG (36:6); PA (30:4), 613→283.7	1.99	3.85	0.70	0.09
Method 4, *m/z*				
CER (d34:1), 538.3→282.2	2.83	6.67	0.97	0.02
CER (d34:1), 538.9→282.2	2.30	8.99	1.87	0.03
CER (d34:1), 539→282.2	2.82	12.77	2.14	0.01
non-attributed, 539.2→282.2	2.69	12.26	2.78	0.04
CER (d34:0), 540.2→282.2	1.14	2.90	0.58	0.06
SM (d42:2), 811.4→184.2	26.18	16.55	3.78	0.10
SM (d42:2), 811.5→184.2	17.54	10.93	2.43	0.08
Group				
Saturated	9.48	40.21	12.19	0.10
Unsaturated	64.99	90.26	7.82	0.05
Monounsaturated	12.59	39.20	6.51	0.02
Polyunsaturated	57.05	61.83	6.65	0.61

[1]Intensity ion counts multiplied by 1000. [2]CON, control, no rumen protected Met, $n = 4$. [3]MET, rumen protected Met, $n = 5$. [4]SEM multiplied by 1000. *Variables for Methods 3 and 5 were not statistically different.

TABLE 8 Principal components (PC) of each method, proportion (p), and cumulative proportion (Cp) of the principal component analysis for uterine biopsies retrieved at 73 DIM.

Method	p	Cp
Method 1		
PC1*	89.7	89.7
PC2	5.4	95.0
PC3	2.4	97.4
PC4	1.2	98.6
Method 2		
PC1*	55.5	55.5
PC2*	16.6	72.1
PC3*	11.3	83.4
PC4	7.1	90.5
Method 3		
PC1*	55.9	55.9
PC2*	14.7	70.6
PC3*	11.9	82.6
PC4	7.1	89.6
Method 4		
PC1*	65.2	65.2
PC2*	16.8	82.0
PC3	6.4	88.5
PC4	5.4	93.9
Method 5		
PC1*	67.6	67.6
PC2*	16.3	83.9
PC3	4.3	88.2
PC4	2.7	90.8

*Extracted principal components.

monounsaturated fatty acids in the endometrium at 30 DIM and at 73 DIM.

Phosphatidylcholine and sphingolipids are common lipids present in mammalian membranes, while TG are commonly stored in the cell cytoplasm (40). Unfortunately, the extent of research regarding the specific lipid classes present in the bovine embryo is limited. In the current study, embryos from cows supplemented with RPM showed an increase in TG and PC lipid content with an overall increase in polyunsaturated lipids. Ferreira et al. (41) reported similar results regarding an increase in PC lipids that contained a high degree of unsaturation for bovine embryos. Likewise, an evaluation of the fatty acid composition of lipids in cattle, sheep, and pig oocytes revealed that TG lipids contained the greatest fatty acid-rich fraction by mass (42). A possible explanation for the increased TG concentrations in the embryos may be a result of the RPM supplementation enabling cows to efficiently export TG from the liver, possibly increasing fatty acid concentration in blood (22). Embryos, like any other cell type, can generate lipid droplets in response to increase fatty acid availability (43). Increased embryonic lipid content, although detrimental to cryopreservation, indicates that these embryos have surplus energy substrates that could be utilized for other physiological processes (44). A recent study observed higher cleavage and blastocyst rates in embryos from large follicles that also had increased cytoplasmic lipid content as compared to small follicles, further denoting the importance of lipids during embryonic development (45). Additionally, lipid profiles in *Bos indicus* and *Bos taurus* suggest contributions toward embryonic development and changes in pre-ovulation (44).

Previous studies have demonstrated the importance of lipids in the uterine environment as well as the influence on embryo implantation to improve fertility (46, 47). Lysophosphatidic acid (LPA), a phospholipid derivative, may be an important factor in embryo implantation: LPA deficient mice had smaller embryos with

TABLE 9 Least squares means and associated standard errors of normalized lipid marker intensity ion counts[1] for uterine endometrial biopsies obtained at 73 DIM from Holstein cows supplemented with rumen-protected Met (MET) or not (CON) 21 days before calving until 73 DIM.

	Treatment			*p* value
Variable	CON[2]	MET[3]	SEM[4]	
Method 2, *m/z*				
DG (30:3); PC (18:1); PE (22:1); LysoPE (22:1), 535.9→206.6	2.75	4.82	0.61	0.02
DG (30:3); PC (18:1); PE (22:1); LysoPE (22:1), 536→206.7	4.51	10.72	1.93	0.03
PC (P-20:0); PE (22:1); LysoPE (22:1), 536.1→206.8	7.00	19.76	3.75	0.02
DG (30:2); PG (20:2); PA (24:2), 537→207.7	2.26	3.61	0.47	0.04
DG (36:8), 609.8→280.5	2.24	3.56	0.45	0.04
DG (36:7); PG (24:0), 611→282.2	4.42	10.49	2.75	0.11
DG (36:7); PG (24:0), 611.9→282.6	2.23	3.12	0.45	0.15
DG (36:7); PG (24:0), 612→283.2	2.95	4.76	0.87	0.13
DG (36:7); PG (24:0), 612.1→283.2	3.66	7.23	1.65	0.12
Method 3, *m/z*				
PC (34:2), 758.2→184.2	23.77	19.96	1.80	0.12
Method 4, *m/z*				
non-attributed, 537→282.2	18.23	57.55	11.49	0.02
non-attributed, 537.1→282.2	19.73	63.16	12.15	0.02
non-attributed, 537.2→282.2	17.44	55.78	10.82	0.02
PC (38:5); PI-CER (d36:1), 808.7→184.2	1.33	1.18	0.08	0.14
SM (d42:3), 811.7→184.2	2.77	2.16	0.21	0.05
PC (38:3), 812.8→184.2	2.07	1.78	0.11	0.06
PC (38:1), 816.5→184.2	8.79	7.40	0.71	0.15
Group				
Saturated	20.25	45.36	9.37	0.06
Unsaturated	70.74	100.50	12.95	0.10
Monounsaturated	24.38	43.88	5.89	0.02
Polyunsaturated	50.54	62.89	7.02	0.19

[1]Intensity ion counts multiplied by 1000. [2]CON, control, no rumen protected Met, $n = 5$. [3]MET, rumen protected Met, $n = 8$. [4]SEM multiplied by 1000. *Variables for Methods 1 and 5 were not statistically different.

decreased implantation and lower prostaglandin components in the uterus, all factors that contribute to reduced embryo vitality (47). The metabolism of Met results in S-adenosylmethionine, which is a major methylation agent, leading to methylation of phospholipids into phosphatidylcholine, which may explain the increase in PC lipids found in embryos from RPM supplemented cows (48, 49).

Ruminant reproduction requires a substantial amount of energy, as stressed by Mattos et al. (50), with the most important sources of energy being derived from the fatty acids in lipids. The energy usage by the ruminant reproductive tract occurs mainly through providing precursors for steroids, ultimately impacting pregnancy rates (50). In the endometrium, the platelet-activation factor rapidly hydrolyzes PC, resulting in the formation of DG (51), which serves as signal messengers and activate cellular events during implantation (52). For the current study, there was decreased PC lipids at 15 DIM with a notable increase in DG lipids in the endometrial tissue at 30 and 73 DIM with few to no PC lipids that were detected. It is possible that the PC lipids were hydrolyzed more efficiently in the cows supplemented with RPM, which could lead to increased signaling and improved implantation. Additionally, DG can further be metabolized by diacylglycerol/monoacylglycerol lipase, resulting in the formation of non-esterified fatty acids (53), such as arachidonic acid, leading to prostaglandin production. Weems et al. (54) described how prostaglandin E2 stimulated the secretion of progesterone in the bovine corpus luteum *in vitro* by day 200, showing that the luteotropic support of the hormone. Protein kinase C has been observed in gonadal tissue and can aid in secretory functions (55). Diacylglycerol increases protein kinase C enzymatic sensitivity 16-fold in pig ovarian cytosol, possibly resulting in increased endocrine secretions (55). Likewise, as previously stated, LPA is an important component for embryo implantation in mice. Ye et al. (56) modeled the importance of diacylglycerol for the formation of LPA which can also aid in uterine receptivity, oocyte maturation, and angiogenesis of the placenta and uterus. Based on these studies, DG can be considered an important component for all aspects of reproduction. Additionally, feeding dairy cattle with monounsaturated fatty acids has been reported to result in increased number of oocytes collected with a greater recovery rate (57).

Supplementation of RPM to multiparous Holstein cows resulted in increased ability to maintain pregnancy and overall embryonic size, leading to reduced embryo mortality (58). Thatcher et al. (59) hypothesized that the endometrial lipid status may act synergistically with bovine IFNτ, which is actively being produced by the conceptus, aiding in corpus luteum maintenance and embryonic survival through antagonizing the synthesis of prostaglandin F2α during the early stages of pregnancy. The typical voluntary waiting period for dairy cows to be bred is between 50 and 60 days after calving (60). The observed increase in lipids around this time further confirms the importance of lipids in the bovine uterus and embryos to maintain a viable pregnancy. Additionally, the increase in monounsaturated fatty acids in the endometrium at 30 DIM and at 73 DIM as a result of supplementation with RPM further corroborates the theory of SCD-1 being the link between lipid metabolism and sulfur amino acids. Furthermore, this is the first time this link is demonstrated to happen in the bovine endometrium. The enzyme SCD-1 is involved in the synthesis of monounsaturated fatty acids, and it was previously reported to increase the availability of sulfur amino acids (33). Thus, it is likely that the increase in monounsaturated fatty acids in the endometrial tissue is a response to increase SCD-1 due to the increased availability of Met.

The current study provides a comprehensive analysis of lipid profiles found in uterine endometrial tissue and preimplantation embryos from cows supplemented with RPM. Lipid profiling of the preimplantation embryos revealed an increase of TG, mainly polyunsaturated, which could be utilized for energy by the developing embryo. Moreover, the notable increase in DG and monounsaturated lipids signifies the possibility of the uterine endometrium in cows supplemented with RPM to assist with signaling, embryo implantation, and embryo growth. Therefore, providing RPM to Holstein cows

during the transition period and early lactation may alter the lipid profiles of preimplantation embryos and the uterine endometrium, possibly leading to improved embryonic development and survival.

Data availability statement

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

Ethics statement

The animal study was approved by University of Illinois (Urbana-Champaign) Institutional Animal Care and Use Committee (no. 13023). The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

SS: Data curation, Investigation, Project administration, Writing – original draft. AG: Validation, Visualization, Writing – review & editing. DV-A: Data curation, Formal analysis, Investigation, Writing – review & editing. CF: Formal analysis, Investigation, Writing – review & editing. MR: Investigation, Writing – review & editing. MW: Resources, Validation, Writing – review & editing. DL: Resources, Visualization, Writing – review & editing. FC: Conceptualization, Funding acquisition, Project administration, Resources, Supervision, Writing – review & editing.

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Conflict of interest

DL from Adisseo had input in the experimental design and had no influence in performing the experiment and analyzing the data.

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.2023.1301986/full#supplementary-material

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Effects of dietary addition of ellagic acid on rumen metabolism, nutrient apparent digestibility, and growth performance in Kazakh sheep

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Plant extracts have shown promise as natural feed additives to improve animal health and growth. Ellagic acid (EA), widely present in various plant tissues, offers diverse biological benefits. However, limited research has explored its effects on ruminants. This study aimed to investigate the effects of dietary addition EA on rumen metabolism, apparent digestibility of nutrients, and growth performance in Kazakh sheep. Ten 5-month-old Kazakh sheep with similar body weight (BW), fitted with rumen fistulas, were randomly assigned to two groups: the CON group (basal diet) and the EA group (basal diet + 30 mg/kg BW EA). The experiment lasted 30 days, and individual growth performance was assessed under identical feeding and management conditions. During the experimental period, rumen fluid, fecal, and blood samples were collected for analysis. The results indicated a trend toward increased average daily gain in the EA group compared to the CON group ($p = 0.094$). Compared with the CON group, the rumen contents of acetic acid and propionic acid were significantly increased in the EA group and reached the highest value at 2 h to 4 h after feeding ($p < 0.05$). Moreover, the relative abundances of specific rumen microbiota (Ruminococcaceae, *uncultured_rumen_bacterium*, *unclassified_Prevotella*, Bacteroidales, *Bacteroidota*, Bacteroidia, *unclassified_Rikenellaceae,* and *Prevotella_spBP1_145*) at the family and genus levels were significantly higher in the EA group ($p < 0.05$) compared to the CON group. The EA group exhibited significantly higher dry matter intake ($p < 0.05$) and increased the digestibility of neutral detergent fiber and ether extract when compared with the CON group ($p < 0.05$). Additionally, the plasma activities of total antioxidant capacity (T-AOC), superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GSH-Px) were significantly higher, while malondialdehyde (MDA) concentration was significantly lower in the EA group compared to the CON group ($p < 0.05$). In conclusion, dietary supplementation with 30 mg/kg BW EA in 5-month-old Kazakh sheep increased the dry matter intakQ16e, apparent digestibility of neutral detergent fiber, and ether extract, as well as the contents of acetic acid and propionic acid in rumen fluid. Moreover, EA supplementation regulated the ruminal microbiota, enhanced antioxidant capacity, and improved daily weight gain.

KEYWORDS

growth performance, apparent nutrient digestibility, ellagic acid, Kazakh sheep, rumen fermentation, bacterial diversity, antioxidant capacity

1 Introduction

In response to the antibiotic ban policy in animal husbandry, the quest for antibiotic alternatives in animal feed has gained momentum. Plant extracts stand out as vital options due to their medicinal attributes in enhancing animal health and productivity. Compared to conventional chemical drugs, plant extracts offer unique advantages in controlling inflammation and oxidative stress. In addition, they remarkably enhance animal health by bolstering immunity, satisfying healthy breeding standards to avert animal diseases, and improving animal production performance and product quality (1).

Ellagic acid (EA), a dimerized derivative of gallic acid widespread in various plants (2), has emerged as a potent alternative to antibiotics and possesses a variety of biological functions, such as antimutagenicity, antibacterial, anti-inflammatory, and antioxidant properties (3, 4). Xu et al. (5) evaluated the effect of gallic acid on calves, observing improved growth performance metrics, such as average daily gain, rumen fermentation parameters (total volatile fatty acids, propionate, and butyrate), and antioxidant levels (catalase and T-AOC). Notably, gallic acid reduced malondialdehyde and tumor necrosis factor-α levels in preweaning dairy calves while influencing ruminal microbial abundances of *Prevotellaceae_UCG-001*, Saccharofermentans, and *Prevotella_1*, while reducing the abundance of *Prevotella_7*. Gallic acid, a phenolic compound in plant extracts, demonstrates robust antioxidant capabilities, scavenging hydroxyl radicals, and exhibiting potent reducing power (6). Previous research has found that the addition of concentrated pomegranate peel extract (containing EA) to the diet of lactating dairy cows can prolong daily ruminating time and enhance the digestibility of dry matter, crude protein, neutral detergent fiber, cellulose, and hemicellulose (7). Moreover, concentrated pomegranate peel extract influences the relative abundance of methanogenic archaea, which are rumen-specific bacteria responsible for cellulose decomposition and lactic acid fermentation, and significantly improves the milk yield and growth performance of cattle (7). Studies involving monogastric animals have shown that dietary supplementation with EA can improve animal growth performance, intestinal health, and antioxidant capacity. Lu et al. found that feeding 500 g/t of EA to 30-day-old weaned piglets for 40 days increased the average daily gain and reduced diarrhea (8). Moreover, Qin et al. (9) demonstrated that the supplement of 0.1% EA to the basal diet of weaned piglets increased average daily feed intake and daily gain while reducing fecal score, thus suggesting an effect on intestinal bacteria. Additionally, it could alleviate oxidative stress and intestinal injury in weaned piglets (9). However, few studies have explored the effects of EA supplementation in ruminants.

EA primarily exists in the tannin form in nature (10). While traditionally considered antinutrients, low doses of plant-derived tannins enhance ruminant protein utilization (11). Condensed tannins bind to dietary crude protein, inhibiting its ruminal degradation. Consequently, this elevates rumen protein concentration, enhances amino acid utilization, and augments digestible protein throughout the digestive tract, ultimately improving production performance (12). Notably, limited studies have explored the effects of dietary supplementation of EA in sheep. In this study, 5-month-old Kazakh sheep received an EA-supplemented diet to investigate the effects of EA on growth performance, rumen metabolism, and apparent nutrient digestibility.

2 Materials and methods

2.1 Ethical considerations

All animal care and handling procedures in this study were conducted under the Guidance of the Care and Use of Laboratory Animals in China and were approved by the Animal Care Committee of Xinjiang Agricultural University of China (protocol permit number: 2020024).

2.2 Animal and experimental design

Ten 5-month-old Kazakh sheep (35.61 ± 2.32 kg, rams) of similar body weight (BW), well cared for, healthy, and equipped with rumen fistulas, were randomly assigned to two groups: the CON group ($n = 5$) and the EA group ($n = 5$) using a random number generator (http://www.r-project.org/). These sheep were housed in individual feeding pens (1.20 × 1.50 m) within a naturally ventilated barn structure. The ten pens were located inside a barn open on two sides and arranged in two rows of five, separated by the central feeding lane. The pens are enclosed by horizontal metal rail bars, which also delimit the pens at the feeding lane. The floor had a concrete base covered with barley straw bedding, of which one fresh flake (around 1.5 kg) per pen was added over the permanent bedding once a day. The sheep were untethered and did not have any access to a paddock area. The sheep in the CON group received a basal diet devoid of EA, whereas the diet for the EA group included EA supplementation at 30 mg/kg BW. The quality of the hay was checked according to guidelines in Cavallini et al. (13), ensuring the absence of molds and spores. The corn aflatoxin levels were assessed according to the procedure described in Girolami et al. (14) and were found to be below the maximum tolerable threshold recommended by the EU. Health checks, including fecal consistency and pH, as described below, were completed twice a week. The EA (≥90.00%) was purchased from Wufeng Chicheng Biotechnology Co., Ltd. (Hubei, China). The experimental sheep were raised at the 103 regiment experimental base of the 6th division of the Xinjiang Production and Construction Corps in China. The feed was provided twice a day at 8:00 am and 8:00 pm, and the EA was weighed and mixed into the concentrate before feeding. The composition and ingredients of the basic diet are presented in Table 1. All sheep had *ad libitum* access to feed and clean water during the experiment. The experimental period lasted 30 days, preceded by a 5-day adaption stage.

2.3 Sample collection and analysis

The dry matter intake (DMI) of sheep was recorded daily. On the 21st to 25th day of the experiment, self-made fecal collection bags were used to collect fecal samples for 5 consecutive days.

TABLE 1 Composition and nutrient levels of the diet (DM basis).

Items	Content
Ingredients	
Yellow corn	35.00, %
Wheat bran	6.40, %
Soybean meal	10.00, %
Cottonseed meal	5.60, %
Premix[a]	3.00, %
Mixed hay (Alfalfa hay: Corn straw = 1:1)	40.00, %
Total	100.00
Nutrient levels[b]	
Dry matter	87.56, %
Organic matter	95.25, %
Metabolic energy	11.26, MJ
Crude protein	16.78, %
Ether extract	2.86, %
Neutral detergent fiber	37.58, %
Acid detergent fiber	20.45, %
Calcium	1.02, %
Phosphorus	0.36, %

[a] The premix provided the following per kg of the concentrate supplement: VA 10,000.00 IU, VD_3 2,550.00 IU, VE 20.00 IU, niacin 20.00 mg, biotin 0.06 mg, Cu (as copper sulfate) 22.00 mg, Fe (as ferrous sulfate) 94.00 mg, Mn (as manganese sulfate) 80.00 mg, Zn (as zinc sulfate) 88.00 mg, I (as potassium iodide) 0.75 mg, Se (as sodium selenite) 0.50 mg, Co (as cobalt chloride) 0.33 mg, Ca (as calcium carbonate and calcium hydrogen phosphate) 0.35%, P (calcium hydrogen phosphate) 0.125%, NaCl 0.80%; [b] Nutritional levels were measured.

Specifically, the fecal samples were collected four times daily at fixed time intervals, meticulously documented, and pooled across each consecutive 5-day period. Subsequently, 10% of the total collected fecal amount was randomly selected, weighed, and dried for analysis. Feed and feces samples were sent to the Animal Nutrition Laboratory, College of Animal Science, Xinjiang Agricultural University, for dry matter (DM) and chemical analysis. To determine the DM content, the samples were dried in a forced air oven at 65°C until a constant weight was achieved. Upon drying, the samples were ground to pass through a 1 mm screen (Cyclotec Mill, model 1093; Foss Tecator, Höganäs, Sweden). The ash content of the ground samples was analyzed after 4 h of combustion in a muffle furnace at 550°C (Vulcan 3-550, Dentsply Ney-tech, Burlington, NJ, USA) ash-corrected α-amylase–treated neutral detergent fiber (NDF) with the addition of sodium sulfite (aNDFom) and acid detergent fiber (ADF) (15); Crude protein (CP) using a Kjeldahl nitrogen analyzer (Gerhardt Vapodest 50, Gerhardt GmbH, Königswinter, Germany); Starch (16), method 996.11; and Ether extract (EE; according to EC Regulation No. 152/2009). Further, the calcium (Ca) and phosphorus (P) contents of feed and feces were analyzed by atomic absorption spectrophotometer (17). Average daily gain (ADG) was calculated as the difference between final and initial body weight divided by the number of days of feeding. Feed/Gain (F/G) was calculated as the ratio of average daily feed intake to daily weight change (g/g).

Rumen fluid samples were collected on the 15th and 30th day of the trial period, both before feeding (0 h) and at 2, 4, 6, and 8 h after feeding from the same position of the fistula with a self-made rumen fluid collector. Fluid sampling devices consisted of Tygon tubing terminating in a pot scrubber, weighted with several steel nuts installed in each animal, and were threaded through holes in the cannula plug to maintain the anaerobic rumen environment. One pot scrubber was placed in the cranial portion of the rumen, and one was placed in the caudal portion. The ends of the Tygon tubing were scored to allow a lure lock syringe to be screwed directly onto each tube. For each rumen fluid sample collected, a 50 mL syringe was then used to sample equal volumes of fluid from each sampling line. Samples were mixed in the syringe, and the bulk sample was aliquoted into 2 glass vials (18), filtered with a 60-mesh nylon bag, and packed into frozen tubes immediately after pH was measured by a portable pH meter (PB-21, Sartorius, Germany), and preserved in liquid nitrogen for the subsequent rumen fermentation parameter analysis including the volatile fatty acids (VFAs) and ammonia nitrogen (NH_3-N) (Agilent Cary 60UV-Vis Spectrophotometer, USA) by gas chromatography (colorimetry).

Blood samples were collected from the jugular vein on the 30th day of the experiment at 07:30 am into tubes containing an anticoagulant (heparin lithium). The samples were centrifuged at 3,500 × *g* for 15 min to collect plasma and stored at −20°C. The Catalase (CAT; #A005), superoxide dismutase (SOD; #A001-1), glutathione peroxidase (GSH-PX, #A007-2), total antioxidant capacity (T-AOC, #A015), and malondialdehyde (MDA, #A003-1) were determined with the commercial test kit procured from Nanjing Jiancheng Biotechnology Research Institute (Nanjing, China).

2.4 16S rDNA sequencing and bioinformatics analysis of the rumen bacteria

Total DNA extraction and PCR amplification of rumen fluid samples followed the methodology outlined in Ma et al.'s study (19). Briefly, total DNA extraction involved the use of cetyltrimethylammonium bromide (CTAB), with subsequent assessment of DNA purity and concentration via 1% agarose gel electrophoresis and spectrophotometry. Universal prokaryotic primers 341F (5′-CCTACGGGNGGCWGCAG-3′) and 806R (5′-GGACTACHVGGGTATCTAAT-3′) were used to amplify the V3-V4 variable region of bacterial 16S rRNA gene. The amplifiers were sequenced on the Miseq PE300 platform (Illumina, USA). The 16S rRNA gene sequencing raw reads were qualitatively filtered using Flash (version 1.20) and QIIME (Quantitative Insight into Microbial Ecology, version 1.8.0) (20). The filtered sequences were compared using homologous clusters to obtain the operational taxonomic units (OTUs). The α and β diversity indices were measured and analyzed. Linear discriminant analysis (LEfSe) of effect size was used to identify differential microflora, and PICRUSt analysis was performed to predict microbial function.

TABLE 2 Effect of the dietary addition of ellagic acid on growth performance of Kazakh sheep.

Item	CON group	EA group	p-value
Initial body weight, kg	35.33 ± 2.83	35.88 ± 2.29	0.744
Final body weight, kg	41.61 ± 2.33	43.14 ± 2.52	0.347
Average daily gain, g/sheep·d	209.33 ± 21.43	242.00 ± 31.98	0.094
Feed:Gain, g/g	4.82 ± 0.55	4.30 ± 0.51	0.159

CON group, fed a basal diet; EA group, fed a basal diet with EA (30 mg/kg BW).

TABLE 3 Effect of the dietary addition of ellagic acid on rumen fermentation parameters of Kazakh sheep.

Item	CON group	EA Group	p-value
Day 15			
pH	6.67 ± 1.16	6.68 ± 0.30	0.946
Acetic acid, mmol/L	74.88 ± 0.88[b]	77.56 ± 1.12[a]	0.003
Propionic acid, mmol/L	18.08 ± 0.51[b]	21.37 ± 1.71[a]	0.003
Isobutyric acid, mmol/L	0.38 ± 0.05	0.37 ± 0.04	0.567
Butyric acid, mmol/L	13.20 ± 0.89	13.10 ± 0.91	0.863
Isovaleric acid, mmol/L	0.63 ± 0.06	0.58 ± 0.07	0.314
Valeric acid, mmol/L	0.75 ± 0.05	0.74 ± 0.05	0.966
Ammonia nitrogen, mmol/L	14.87 ± 2.38	14.28 ± 2.16	0.689
Lactic acid, mmol/L	2.92 ± 0.27	3.43 ± 0.43	0.086
Day 30			
pH	6.33 ± 0.17	6.52 ± 0.36	0.320
Acetic acid, mmol/L	79.24 ± 6.13	87.42 ± 5.42	0.056
Propionic acid, mmol/L	24.37 ± 4.04	26.70 ± 4.12	0.337
Isobutyric acid, mmol/L	0.54 ± 0.02	0.57 ± 0.05	0.249
Butyric acid, mmol/L	15.19 ± 1.95	17.87 ± 3.15	0.145
Isovaleric acid, mmol/L	1.01 ± 0.05	1.03 ± 0.25	0.836
Valeric acid, mmol/L	1.02 ± 0.19	1.34 ± 0.40	0.147
Ammonia nitrogen, mmol/L	17.49 ± 2.08	19.99 ± 4.10	0.258
Lactic acid, mmol/L	4.94 ± 0.29	5.20 ± 0.22	0.151

[a,b] Values within a row without common superscripts differ significantly ($p < 0.05$); CON group, fed a basal diet; EA group, fed a basal diet with EA (30 mg/kg BW).

2.5 Statistical analysis

Preliminary analysis of the experimental data was conducted using Excel 2010. The normality (Shapiro-Wilk) test was performed prior to the statistical analysis. The data of growth performance, rumen fermentation, nutrient apparent digestibility, and plasma antioxidant were analyzed for normality using the Shapiro–Wilk test, and further statistical analysis was carried out using SPSS 20.0 (SPSS Statistics 20, IBM Japan, Ltd., Tokyo, Japan) software using independent sample *t*-tests. The 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. Pearson's correlation analysis was performed to evaluate the correlation between rumen differential bacteria and rumen fermentation parameters and apparent digestibility of nutrients, and graphs were rendered using Orgin 8.0 (OriginLab Co., Northampton, MA, USA).

3 Results

3.1 Growth performance

Table 2 demonstrates that the EA group sheep exhibited increased final body weight, average daily gain, and feed conversion efficiency compared to sheep in the CON group. However, no statistically significant differences were observed between the CON and EA groups ($p > 0.05$).

3.2 Rumen fermentation parameters

Rumen fermentation parameters on Day 15 and Day 30 are presented in Table 3. No significant differences were observed in rumen pH value and isobutyric acid, butyric acid, isovaleric acid, valeric acid, and ammonia nitrogen contents between the EA and CON groups ($p > 0.05$). Nevertheless, the contents of acetic acid ($p = 0.003$) and propionic acid ($p = 0.003$) exhibited significant increases, while lactic acid ($p = 0.086$) contents tended to be higher in the EA group compared to the CON group. Specifically, on the 30th day, acetic acid content in the EA group increased by 10.32% compared to the CON group ($p = 0.056$), although other parameters showed no significant differences.

As shown in Figure 1, on the 15th day of the experiment, at 1 h after morning feeding, the rumen fluid contents of acetic acid (Figure 1A), propionic acid (Figure 1B), and lactic acid (Figure 1H) in the EA group reached the maximum value, which was higher ($p < 0.05$) than that of the CON group, and then began to decrease. The contents of butyric acid (Figure 1D) and valeric acid (Figure 1F) showed the same trend. However, no significant difference was observed between the EA and CON groups ($p > 0.05$). The contents of isobutyric acid (Figure 1C) and isovaleric acid (Figure 1E) in the rumen fluid of the two groups began to decrease after feeding.

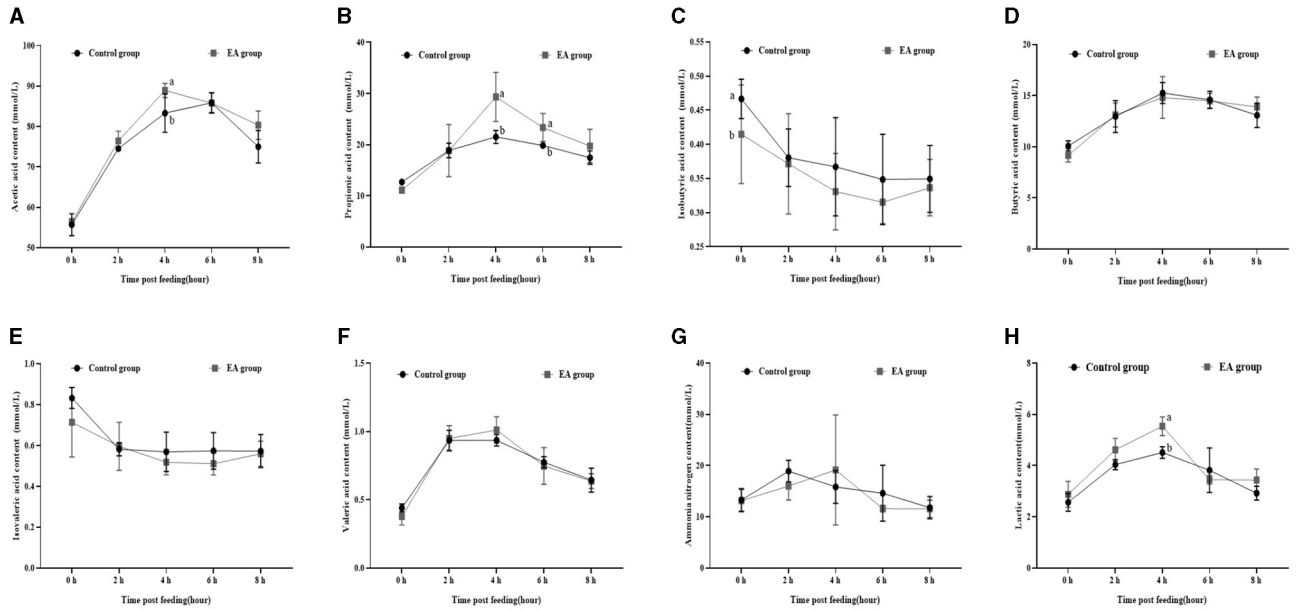

FIGURE 1
Effect of the dietary addition of ellagic acid on the rumen fermentation dynamics of Kazakh sheep (on day 15). [a,b] Values within a row without common superscripts differ significantly ($p < 0.05$); CON group, fed a basal diet; EA group, fed a basal diet with EA (30 mg/kg BW). **(A)** Acetic acid, **(B)** Propionic acid, **(C)** Isobutyric acid, **(D)** Butyric acid, **(E)** Isovaleric acid, **(F)** Valeric acid, **(G)** Ammonia nitrogen, and **(H)** Lactic acid.

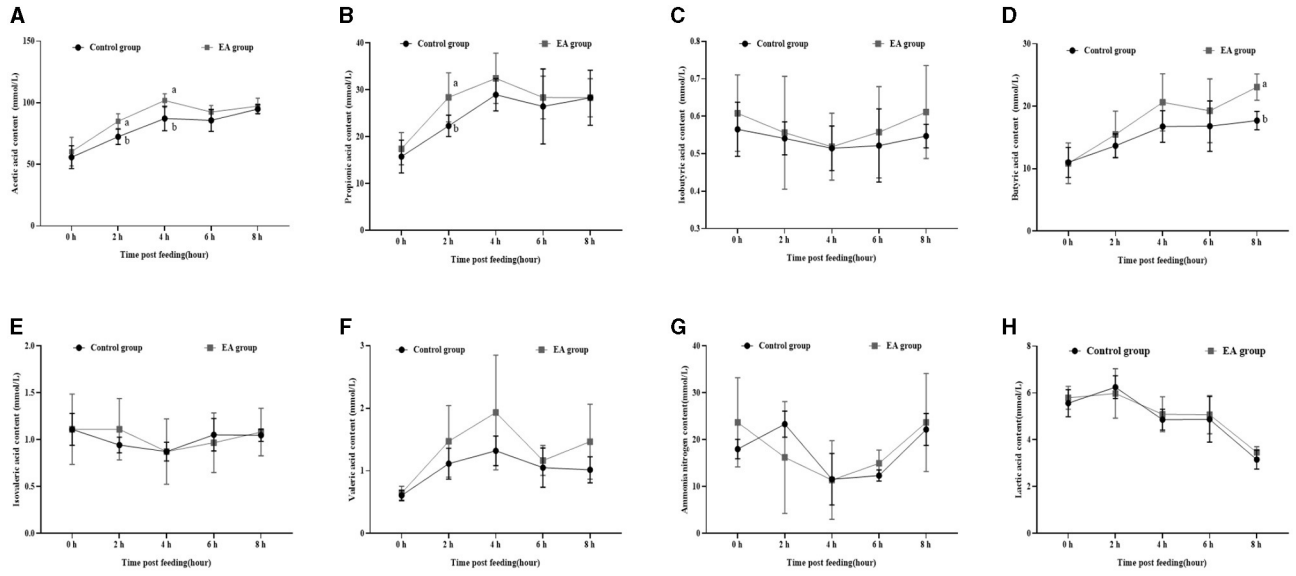

FIGURE 2
Effect of the dietary addition of ellagic acid on the rumen fermentation dynamics of Kazakh sheep (on day 30). [a,b] Values within a row without common superscripts differ significantly ($p < 0.05$); CON group, fed a basal diet; EA group, fed a basal diet with EA (30 mg/kg BW). **(A)** Acetic acid, **(B)** Propionic acid, **(C)** Isobutyric acid, **(D)** Butyric acid, **(E)** Isovaleric acid, **(F)** Valeric acid, **(G)** Ammonia nitrogen, and **(H)** Lactic acid.

As shown in Figure 2, on the 30th day of the experiment, the changing trend of acetic acid (Figure 2A), propionic acid (Figure 2B), isobutyric acid (Figure 2C), butyric acid (Figure 2D), isovaleric acid (Figure 2E), valeric acid (Figure 2F), and ammonia nitrogen (Figure 2G) in rumen fluid of the two groups was similar to that on the 15th day, whereas lactic acid content showed the opposite trend (Figure 2H). Moreover, 2 h after morning feeding, the contents of acetic acid and propionic acid in the rumen fluid of the EA group were significantly higher than those of the CON group ($p < 0.05$).

3.3 Rumen bacterial diversity

An average of 65,767 effective labels per sample were obtained on the 15th day. By the 30th day, an average of 678 OTUs per sample

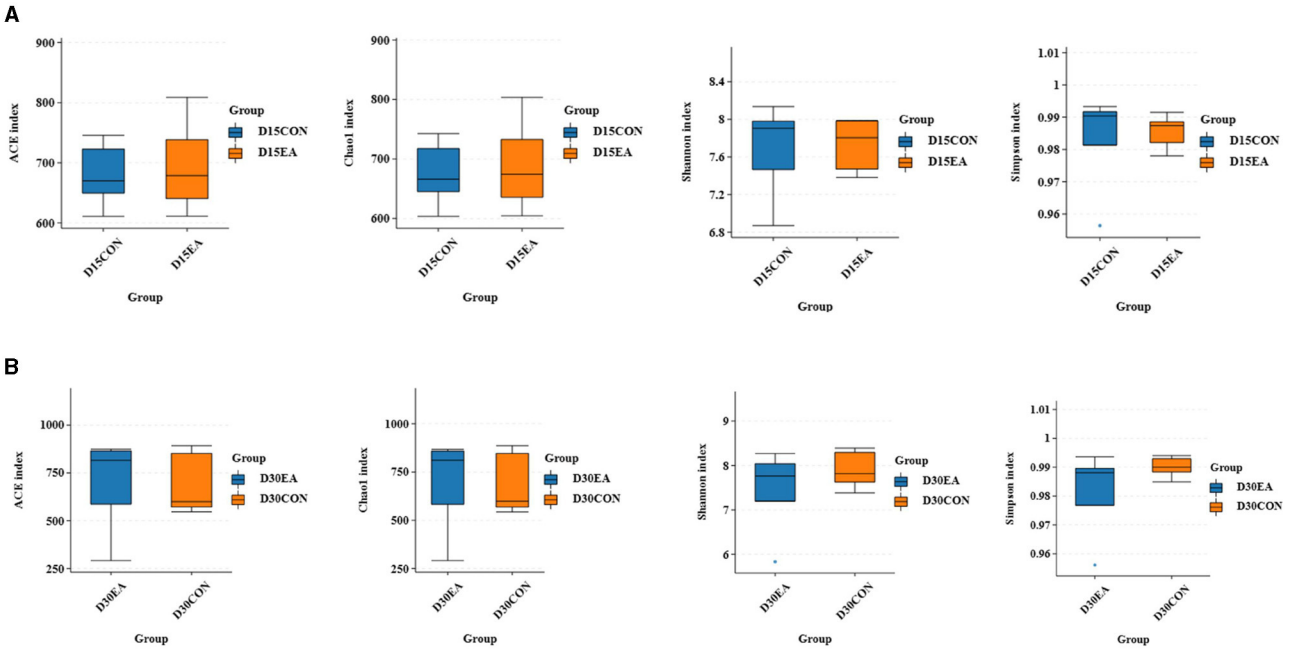

FIGURE 3
The results of α diversity analysis. **(A)** The ACE, Chao1, Shannon, and Simpson indices of rumen microorganisms on the 15th day of the experiment. **(B)** The ACE, Chao1, Shannon, and Simpson indexes of rumen microorganisms on the 30th day of the experiment.

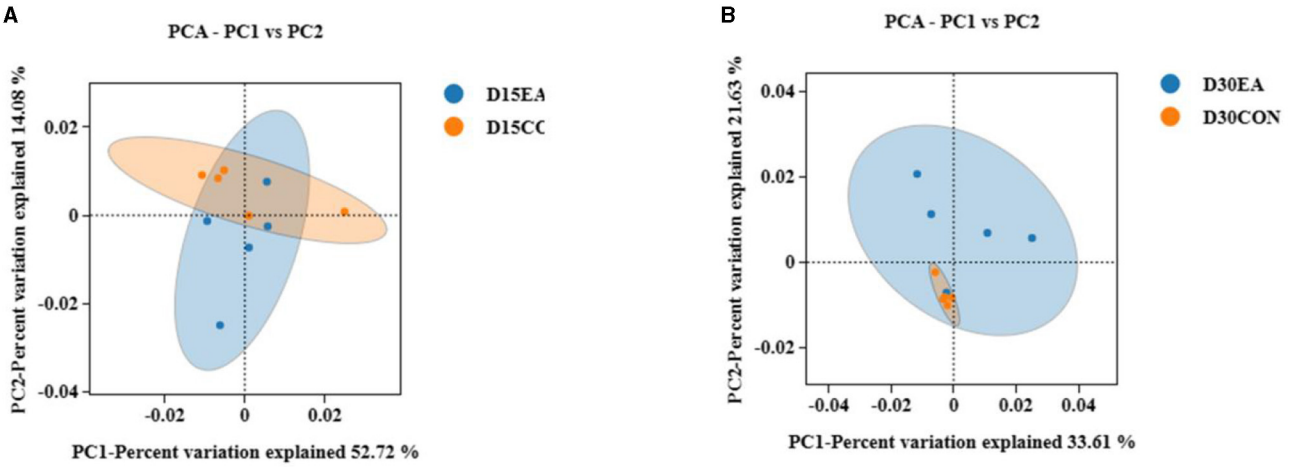

FIGURE 4
The results of β diversity analysis. **(A)** Weighted Unifrac PCoA scatter plot on the 15th day of the experiment. **(B)** Weighted Unifrac PCoA scatter plot on the 30th day of the experiment.

were acquired with 97% paired sequence identity. Additionally, an average of 66,514 effective tags per sample was obtained, resulting in an average of 692 OTUs per sample with 97% paired sequence identity. ACE, Chao1, Shannon, and Simpson indices exhibited no significant differences between the CON and EA groups (Figures 3A, B). The intestinal microbiomes of both groups exhibited wide distribution and effective isolation, suggesting EA's impact on rumen microflora composition (Figure 4).

The results of the microbiome composition analysis are presented in Figure 5. On the 15th day, Bacteroidetes and Firmicutes were the dominant phyla in the rumen of the two groups, accounting for over 85% ($p < 0.05$) of microflora. At the family level, Prevotellaceae and Rikenellaceae were the dominant families in the rumen of the two groups, while at the genus level, the abundance of *Prevotella*, *uncultured_rumen_bacterium,* and other bacteria in the rumen of sheep in the EA group was marginally

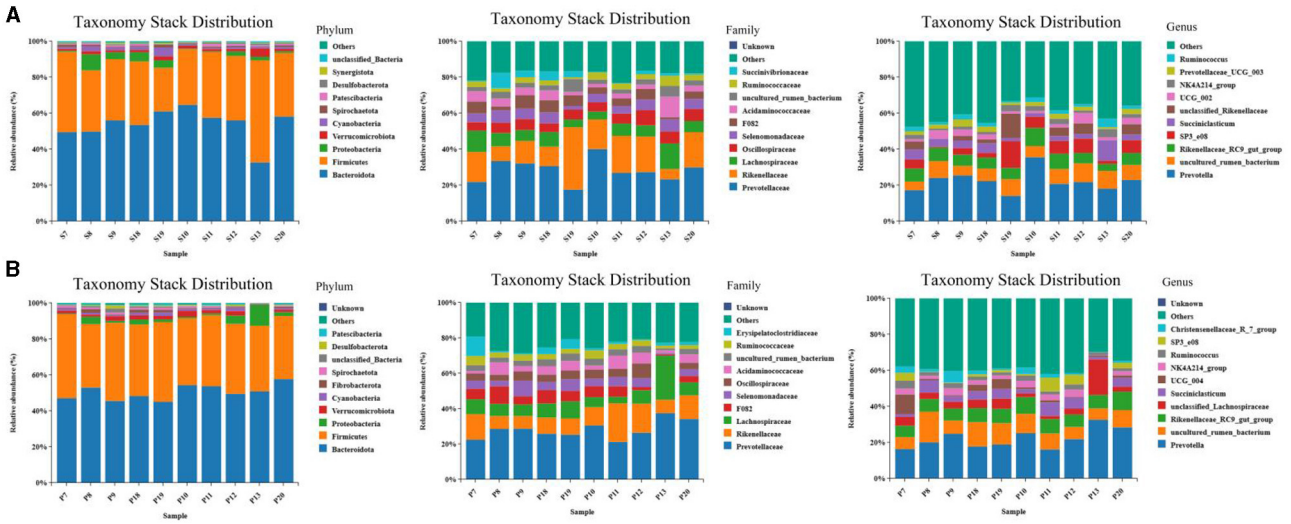

FIGURE 5
Taxonomic and stack distribution of different species. **(A)** Taxonomic stack distribution at phylum, family, and genus levels on the 15th day of the experiment. **(B)** Taxonomic stack distribution at gate, family, and genus levels on the 30th day of the experiment.

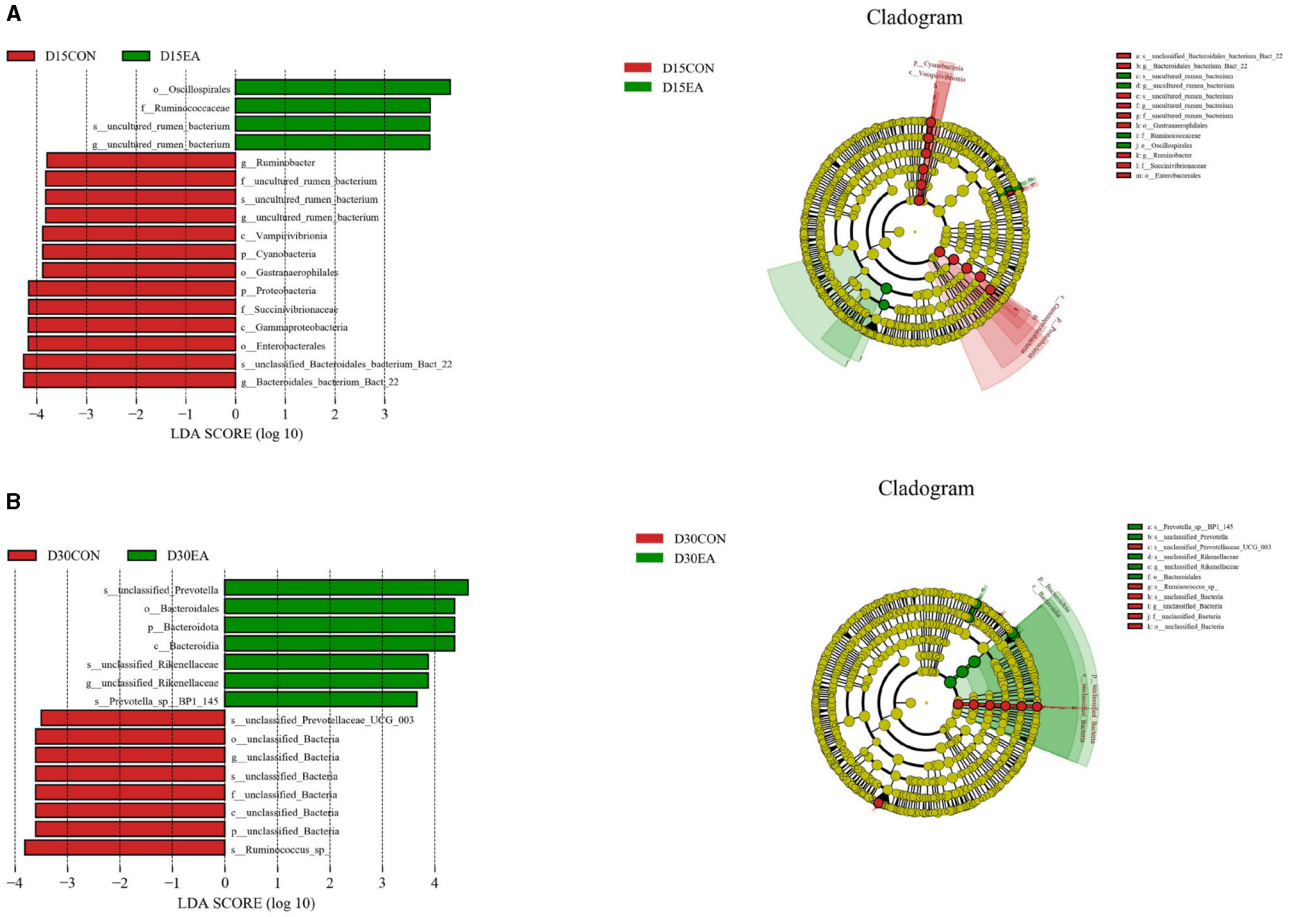

FIGURE 6
LEfSe analysis of rumen microflora. **(A)** LEfSe analysis results on the 15th day of the experiment. **(B)** LEfSe analysis results on the 30th day of the experiment. D15CON, D30CON, fed a basal diet; E15 EA, E30 EA, fed a basal diet with EA (30 mg/kg BW).

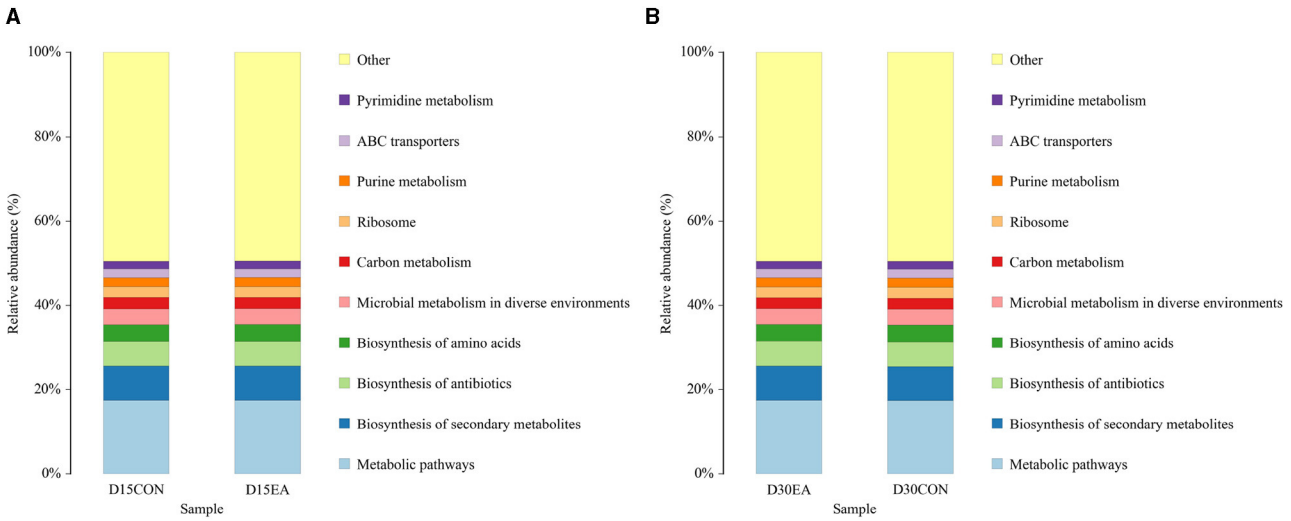

FIGURE 7
PICRUSt function prediction chart. **(A)** The 15th day of the experiment. **(B)** The 30th day of the experiment. D15CON, D30CON, fed a basal diet; E15 EA, E30 EA, fed a basal diet with EA (30 mg/kg BW).

but insignificantly higher than that in the CON group ($p > 0.05$) (Figure 5A). On the 30th day, at the phylum level, Bacteroides and Streptomyces were the most predominant phyla of the two groups of the rumen, accounting for more than 90% of all microorganisms; at the family level, the abundance of Erysipelatoclostridiaceae in the rumen of the EA group was significantly higher than that of the CON group ($p < 0.05$); at the genus level, the abundance of *SP3_e08* in the rumen of EA group was higher than that of CON group, while the abundance of *UCG_004* was significantly lower than that in the CON group ($p < 0.05$) (Figure 5B).

LEfSe analysis was used to compare the microbiota in the rumen contents of the two groups. On the 15th day of the experiment, the abundances of Oscillospirales, Ruminococcaceae, uncultured_rumen_bacterium, and *uncultured rumen bacterium* in the rumen of the EA group were higher than those in the CON group ($p < 0.05$) (Figure 6A). On the 30th day of the experiment, the rumen abundances of unclassified_Prevotella, Bacteroidales, *Bacteroidota*, Bacteroidia, *Unclassified_Rikenellaceae*, *unclassified_Rikenellaceae*, and *Prevotella_spBP1_145* in the EA group were higher than those in the CON group ($p < 0.05$) (Figure 6B).

PICRUSt analysis revealed comparable functions of rumen microbiota in both groups, primarily associated with metabolic pathways, biosynthesis of secondary metabolites, antibiotics, amino acids, and microbial metabolism (Figure 7).

3.4 Apparent nutrient digestibility

As shown in Table 4, compared with the CON group, the intake of DM and OM of sheep in the EA group increased significantly ($p < 0.05$). Additionally, the apparent digestibility of NDF and EE were increased significantly by 6.12% and 3.17% ($p < 0.05$).

3.5 Plasma antioxidant capacity

As shown in Table 5, the activities of T-AOC, SOD, CAT, and GSH-Px in the plasma of the EA group were significantly higher than those in the CON group ($p < 0.05$), and the content of MDA decreased significantly ($p < 0.05$).

3.6 Correlation analysis between rumen differential bacteria and rumen fermentation parameters, apparent digestibility of nutrients, and plasma antioxidant capacity

The correlation between rumen differential bacteria and rumen fermentation parameters, apparent digestibility of nutrients, and plasma antioxidant capacity was explored. Acetic acid content in the rumen was positively correlated with uncultured_rumen_bacterium and *Bacteroidota* abundance, and significantly negatively correlated with *Bacteroidales_bacterium_Bact_22*. Isobutyric acid content was positively correlated with Ruminococcaceae abundance, and Butyric acid content was negatively correlated with *Bacteroidales_bacterium_Bact_22* (Figure 8A). The apparent digestibility of NDF was positively correlated with rumen uncultured_rumen_bacterium abundance and negatively correlated with *Bacteroidales_bacterium_Bact_22* (Figure 8B). *Bacteroidales_bacterium_Bact_22* abundance was negatively correlated with SOD, GSH-Px, and CAT activity and positively correlated with MDA content; uncultured_rumen_bacterium abundance was negatively correlated with MDA content and positively correlated with GSH-Px; unclassified_Bacteria abundance was negatively correlated with GSH-Px, CAT activity,

and T-AOC; Bacteroidota was negatively correlated with MDA content (Figure 8C).

4 Discussion

EA exerts various biological functions, including anti-oxidative, anti-cancer, and anti-inflammatory properties, which have spurred substantial research interest in its practical applications (21). At present, there are few reports on the effect of EA on ruminants. To better understand the effects of dietary EA supplementation in sheep, in the present experiment, we used 10 ruminally cannulated Kazakh sheep to assess the effects of dietary addition of EA on growth performance, rumen metabolism, and apparent nutrient digestibility. The DMI required for ruminants to maintain their life activities determines the quality of their growth, development, and reproduction (22). In this study, we found that dietary supplementation with EA had a tendency to increase ADG ($p = 0.094$), indicating its beneficial effect on growth performance in sheep. The underlying cause may be the higher DMI intake of sheep in the EA group. This suggests that the inclusion of EA in the diet could have positive effects on palatability, and consequently, on feed intake. Orzuna-Orzuna et al. evaluated the effects of dietary tannin supplementation on performance, carcass characteristics, meat quality, oxidative stability, and serum antioxidant capacity of sheep by meta-analysis. The results showed that dietary tannin supplementation did not affect the dry matter intake of sheep but increased the daily gain (23). These findings are similar to those of this study. It could be presumed that tannins reduce the consumption of microbial protein, improve the efficiency of microbial protein synthesis, promote protein flow to the duodenum, and thus improve the production performance of ruminants by inhibiting ciliated protozoa in the rumen (24). Unfortunately, rumen protozoa were not studied in the current research. We plan to investigate this in future studies.

Many plants rich in secondary metabolites or bioactive compounds can affect the growth or activity of rumen microorganisms through different mechanisms to regulate rumen fermentation characteristics (25). The change in pH of rumen fluid is a comprehensive reflection and intuitive manifestation of the change in the internal environment of rumen fermentation. Extreme pH values adversely affect the growth and reproduction of rumen microorganisms and feed substrate fermentation (26). In this study, the rumen fluid pH values of the two groups were in the normal range (6.33–6.68) without significant differences, which is consistent with the previous studies (27). Rumen NH_3-N concentration is not only one of the main internal environmental indicators of rumen fermentation but also the most important N source of microbial protein synthesis in rumen (28). The increase of rumen VFAs and NH_3-N production generally indicates the improvement of rumen microbial metabolic activity, nitrogen use efficiency, and overall productivity of ruminants (29, 30). In this study, after the dietary supplementation of EA, we found no significant increase in rumen ammonia nitrogen concentration, except for improved rumen VFAs (acetic acid and propionic acid significantly increased). Manoni et al. used a short-term *in vitro* rumen fermentation model to better understand the effects of EA and gallic acid on rumen fermentation and discovered that EA had a significant effect on reducing CH_4 emission and ammonia formation as well as affecting rumen degradability and total SCFA yield (31), which is consistent with the results of our study. Regrettably, we did not measure the CH_4 of the rumen, limiting the scope of inquiry. Xu et al. (5) evaluated the effect of Gallic acid on rumen fermentation of pre-weaning calves. The results showed that the concentrations of total volatile fatty acids, propionate, butyrate, and valerate in the rumen fluid of calves increased linearly with the addition of gallic acid, resulting in a linear decrease in pH (5). While EA might have a dose-dependent effect in this experiment, since only a single EA concentration was used in this study, follow-up research is required to investigate this aspect. Bhatta et al. studied the effect of tannin on rumen fermentation *in vitro* and found that different addition of tannin could reduce the average NH_3-N concentration (32). However, the addition of tannin to the diet of dairy cows did not affect the

TABLE 4 Effect of the dietary addition of ellagic acid on apparent nutrient digestibility of Kazakh sheep.

Item	CON group	EA Group	p-value
Intake, g/day			
Dry matter	1,253.68 ± 52.31^b	1,321.81.67 ± 30.37^a	0.036
Organic matter	1,194.18 ± 49.83^b	1,259.07 ± 28.93^a	0.036
Apparent digestibility, %			
Dry matter	67.58 ± 1.31	68.11 ± 1.00	0.487
Organic matter	68.91 ± 1.38	69.99 ± 0.94	0.188
Crude protein	64.98 ± 3.88	66.99 ± 1.01	0.294
Neutral detergent fiber	60.02 ± 1.96^b	63.44 ± 1.34^a	0.020
Acid detergent fiber	60.97 ± 1.35	61.84 ± 4.95	0.713
Ether extract	68.71 ± 2.01^b	70.89 ± 1.38^a	0.044
Calcium	47.80 ± 0.53	48.50 ± 2.05	0.483
Phosphorus	44.63 ± 5.70	45.04 ± 3.01	0.888

^{a,b} Values within a row without common superscripts differ significantly ($p < 0.05$); CON group, fed a basal diet; EA group, fed a basal diet with EA (30 mg/kg BW).

TABLE 5 Effect of the dietary addition of ellagic acid on plasma antioxidant capacity of Kazakh sheep.

Item	CON group	EA Group	P-value
T-AOC, U/mL	0.69 ± 0.01^b	0.74 ± 0.02^a	0.007
SOD, U/mL	123.48 ± 4.99^b	134.79 ± 2.70^a	0.002
CAT, U/mL	0.54 ± 0.11^b	0.88 ± 0.10^a	0.001
GSH-Px, U/mL	75.95 ± 5.61^b	87.95 ± 7.41^a	0.020
MDA, nmol/mL	3.36 ± 0.20^a	3.01 ± 0.17^b	0.018

^{a,b} Values within a row without common superscripts differ significantly ($p < 0.05$); CON group, fed a basal diet; EA group, fed a basal diet with EA (30 mg/kg BW).

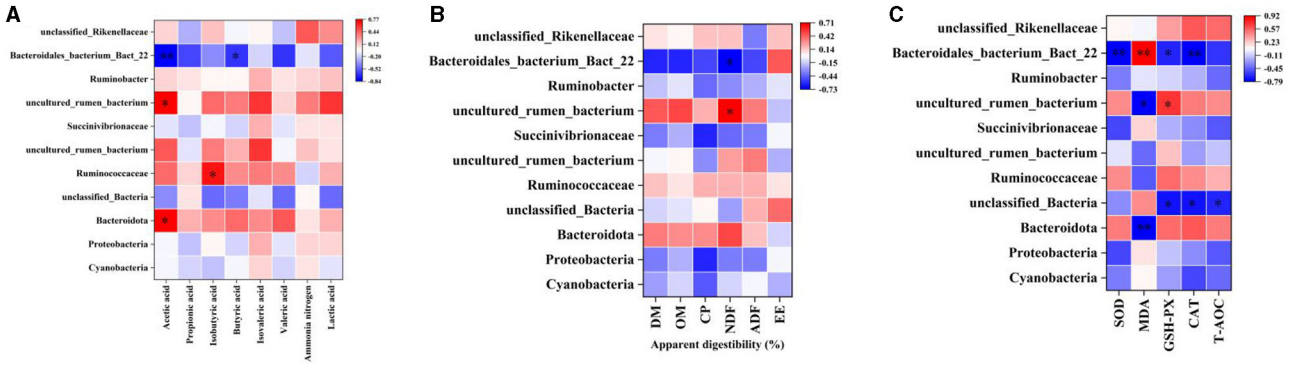

FIGURE 8
Correlation analysis. **(A)** Rumen differential bacteria and Rumen fermentation parameters. **(B)** Rumen differential bacteria and apparent digestibility of nutrients. **(C)** Rumen differential bacteria and plasma Antioxidant capacity. "*" indicates a significant difference between groups where $p < 0.05$. "**" indicates a significant difference between groups where $p < 0.01$.

concentration of NH_3-N (33). The differences in the above results may be related to the source, type, and molecular weight of tannins. Furthermore, our assessment of rumen fermentation dynamics in Kazakh sheep revealed a pattern: initially, the levels of acetic acid, propionic acid, butyric acid, pentanoic acid, and lactic acid increased before gradually declining, reaching their peak at the 4-hour mark post-feeding (refer to Figures 1, 2).

Large and diverse rumen microflora play a key role in the growth and health of ruminants. There is increasing evidence that concentrated tannins from various plants or plant extracts have a significant effect on the rumen microflora of ruminants and can selectively change specific rumen bacteria, thus altering the metabolism of volatile fatty acids (VFAs) in the rumen (34). The effect of EA on rumen microorganisms has not been reported. Herein, we performed 16S rDNA high-throughput sequencing to detect the effect of dietary EA on the composition of rumen microflora of sheep and found that the trend of microbial diversity was consistent between the two groups of sheep rumen samples (Figure 3). Secondly, whether in the CON group or EA group, Bacteroides and Streptomyces were the dominant phyla in the rumen of Kazakh sheep, which was consistent with the findings of other ruminants (35, 36), and there was no significant change in the composition of the top 10 dominant families and genera between the two groups (Figure 5). However, dietary EA could regulate the abundance of rumen microorganisms. For example, after the addition of EA to the diet, the abundance of Cyanobacteria and Synergistota in the rumen of sheep in the EA group decreased. Synergistota has been found in a wide range of anaerobic environments, and some members are associated with amino acid transport (37). Cyanobacteria is a common rumen bacterial phylum, which plays a vital role in hemicellulose and pectin degradation and methane production reduction (38). The change in the relative abundance of Cyanobacteria in the rumen may be driven by the change in feed quality and their ability to degrade plant hemicellulose and pectin. The deficiency of our study is also the lack of determination of amino acid content and methane production. Therefore, the effects of dietary EA on energy utilization and amino acid fermentation in sheep need to be further studied. LEfSe was used to classify the characteristics of microflora with rich differences among animal subgroups. In this experiment, two groups of sheep rumen differential bacteria were compared. Results showed that the abundances of Ruminococcaceae, *uncultured_rumen_bacterium*, *Prevotella,* and *SP3_e08* in the EA group were significantly higher than those in the CON group (Figure 6). The increase in Ruminococcaceae abundance could be attributed to the inclusion of EA in the diet, potentially enhancing the cellulose and hemicellulose degradation capabilities in Kazakh sheep. This family possesses a significant quantity of hemicellulase and oligosaccharide degradase enzymes, which might explain this observed change (39). Subsequently, we analyzed the correlation between rumen differential bacteria and rumen fermentation parameters and found that dietary supplementation with EA could significantly increase the rumen Ruminococcaceae abundance in sheep. Moreover, we observed a significant positive correlation between the bacteria and isobutyric acid content. Therefore, the increase of rumen cocci abundance in sheep may be responsible for the increase in TVFA production in our study. In addition, we found that the significantly upregulated bacterial *Bacteroidales_bacterium_Bact_22* in the rumen of sheep in the CON group had a significant negative correlation with acetic acid and butyric acid content. In the EA group, the significantly upregulated bacterial *uncultured_rumen_bacterium* was positively correlated with acetic acid content, and *Bacteroidota* content was positively correlated with acetic acid content. Our findings suggest that dietary supplementation of ellagic acid can improve the rumen fermentation of Kazakh sheep by regulating the abundance of rumen microorganisms, which has a beneficial effect on growth performance.

Dietary nutrient digestibility is another important parameter for evaluating dietary utilization rate (40). The improvement of animal growth performance is related to the high digestibility of diet. After adding EA to the diet, we observed that the digestibility of NDF increased significantly, and the digestibility of dry matter and crude protein showed a noticeable but insignificant increase (Table 3). In a previous study, digestibility was improved with the addition of 30 mg/kg BW/d EA to equine diets for various components including DM, OM, gross energy, NDF, ADF, and Ca (41). Hence, based on the results of the current

study, sheep supplemented with EA had a high potential to improve the digestibility of DM and nutrients. Additionally, we analyzed the correlation between rumen differential bacteria and apparent nutrient digestibility. The results showed that the apparent digestibility of NDF was positively correlated with the upregulated bacterial *uncultured_rumen_bacterium* in the rumen of sheep in the EA group and negatively correlated with the digestibility of NDF in the rumen of sheep in the CON group. In terms of apparent digestibility, the apparent digestibility of NDF in the EA group was significantly higher than that in the CON group (Figure 8B). It can be concluded that the dietary supplementation of EA can improve the apparent digestibility of Kazakh sheep, which may be related to the upregulation of rumen *uncultured_rumen_bacterium* bacteria.

EA can inhibit oxidative stress by directly scavenging free radicals, inhibiting lipid peroxidation, increasing the activity of antioxidant enzymes and gene expression, maintaining cell stability, and reducing DNA damage by regulating SOD, MDA, CAT, and GSH-Px levels in the blood (42). We evaluated the effect of dietary EA on the plasma antioxidant indices of Kazakh sheep. The results showed that dietary EA could significantly increase the activities and T-AOC of SOD, CAT, GSH-Px, and other enzymes, and decrease the MDA content (Table 4). Previous studies in piglets (9), mice (43), and broilers (44) have highlighted the antioxidant effect of EA, which is consistent with the results of our experimental study. Moreover, changes in the microbiota are linked to alterations in the redox state (45). A previous study (46) found that antioxidants can regulate the dynamic balance of intestinal microbiota by scavenging excessive free radicals and strengthening organic immunity. Furthermore, some studies have indicated that *Lactobacillus* and *Bifidobacterium* possess excellent antioxidant capacity by scavenging free radicals (47, 48). In our experiment, the dietary supplementation of ellagic acid improved the antioxidant capacity of sheep and regulated the ruminal microbiota. Hence, we analyzed the correlation between rumen differential bacteria at the phylum, family, and genus level, and plasma antioxidant capacity. The analysis unveiled significant associations, such as the positive correlation of *uncultured_rumen_bacterium* abundance in the EA group with GSH-Px activity and its negative correlation with MDA content. Conversely, the upregulated *Bacteroidales_bacterium_Bact_22* in the CON group showed negative correlations with SOD, CAT, and GSH-Px activities and a positive correlation with MDA content. The findings suggest that dietary EA may enhance Kazakh sheep's antioxidant capacity, partly influenced by rumen microorganisms like *Bacteroidota*, *Bacteroidales_bacterium_Bact_22*, and *uncultured_rumen_bacterium*.

5 Conclusions

In conclusion, the present study demonstrated that the dietary supplementation of 30 mg/kg BW (sheep/day) EA for 5-month-old Kazakh sheep improves the dry matter intake and apparent digestibility of NDF and EE, increases the acetic acid and propionic acid contents in the rumen fluid, regulates the ruminal microbiota, enhances antioxidant capacity, and improves daily weight gain. These findings offer valuable insights into EA supplementation's potential benefits.

Data availability statement

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

Ethics statement

The animal study was approved by Animal Experiment Ethics Committee of Xin-jiang Agricultural University (permit number: 2020024). The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

WZ: Investigation, Visualization, Writing – original draft, Writing – review & editing, Data curation. FR: Investigation, Writing – original draft, Writing – review & editing, Supervision. CZ: Supervision, Writing – original draft, Writing – review & editing. FY: Investigation, Writing – original draft, Writing – review & editing. XuL: Investigation, Writing – original draft, Writing – review & editing. XH: Investigation, Writing – original draft, Writing – review & editing. XiL: Conceptualization, Data curation, Visualization, Writing – original draft, Writing – review & editing. KC: Conceptualization, Funding acquisition, Project administration, Supervision, Writing – original draft, Writing – review & editing.

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Conflict of interest

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

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Multi-omics analysis on the mechanism of the effect of Isatis leaf on the growth performance of fattening sheep

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Introduction: This study evaluated the effects of Isatis Leaf (ISL) on the growth performance, gastrointestinal tissue morphology, rumen and intestinal microbiota, rumen, serum and urine metabolites, and rumen epithelial tissue transcriptome of fattening sheep.

Methods: Twelve 3.5-month-old healthy fattening sheep were randomly divided into two groups, each with 6 replicates, and fed with basal diet (CON) and basal diet supplemented with 80 g/kg ISL for 2.5 months. Gastrointestinal tract was collected for histological analysis, rumen fluid and feces were subjected to metagenomic analysis, rumen fluid, serum, and urine for metabolomics analysis, and rumen epithelial tissue for transcriptomics analysis.

Results: The results showed that in the ISL group, the average daily gain and average daily feed intake of fattening sheep were significantly lower than those of the CON group ($P < 0.05$), and the rumen ammonia nitrogen level was significantly higher than that of the CON group ($P < 0.01$). The thickness of the reticulum and abomasum muscle layer was significantly increased ($P < 0.05$). At the genus level, the addition of ISL modified the composition of rumen and fecal microorganisms, and the relative abundance of Methanobrevibacter and Centipeda was significantly upregulated in rumen microorganisms, The relative abundance of Butyrivibrio, Saccharofermentans, Mogibacterium, and Pirellula was significantly downregulated ($P < 0.05$). In fecal microorganisms, the relative abundance of Papillibacter, Pseudoflavonifractor, Butyricicoccus, Anaerovorax, and Methanocorpusculum was significantly upregulated, while the relative abundance of Roseburia, Coprococcus, Clostridium XVIII, Butyrivibrio, Parasutterella, Macellibacteroides, and Porphyromonas was significantly downregulated ($P < 0.05$). There were 164, 107, and 77 different metabolites in the rumen, serum, and urine between the ISL and CON groups ($P < 0.05$). The differential metabolic pathways mainly included thiamine metabolism, niacin and nicotinamide metabolism, vitamin B6 metabolism, taurine and taurine metabolism, beta-Alanine metabolism and riboflavin metabolism. These metabolic pathways were mainly involved in the regulation of energy metabolism and immune function in fattening sheep. Transcriptome sequencing showed that differentially expressed genes were mainly enriched in cellular physiological processes, development, and immune regulation.

Conclusion: In summary, the addition of ISL to the diet had the effect of increasing rumen ammonia nitrogen levels, regulating gastrointestinal microbiota, promoting body fat metabolism, and enhancing immunity in fattening sheep.

KEYWORDS

Isatis leaf (ISL), fattening sheep, growth performance, multi-omics, immunity

1 Introduction

The animal husbandry industry has entered a new era of healthy farming, and in order to promote the development of healthy livestock and poultry farming, finding natural feed additives to replace antibiotics has become a research hotspot. Plant additives contain bioactive substances such as alkaloids, saponins, volatile oils, tannins, and polysaccharides, which have various functions such as sterilization, growth promotion, and oxidation resistance. They are considered one of the natural feed additives as substitutes for feed antibiotics (1–4). In recent years, research on the nutritional regulation and production application of Chinese herbal medicine in livestock and poultry has received widespread attention (5, 6). Research has found that Chinese herbal feed additives can improve the growth performance and carcass quality of livestock and poultry (7–9). Different Chinese herbal medicines have a certain promoting effect on the growth performance of livestock, and the main effect on livestock is to improve the structure of beneficial bacteria in the gastrointestinal tract, enhance nutrient absorption levels and immune performance in the gastrointestinal tract (10–12). As one of the most common herbaceous plants, ISL is widely distributed around the world. As a high-yield, efficient, high-quality, and high-economic crop, it has rich nutritional value and is widely used in industries such as food, traditional Chinese medicine, dietary therapy, and health products (13).

The main active substances contained in ISL include three types of compounds: indoles (indigo, indirubin), quinazolones (tryptamines), and glucosinolates. These active substances have antibacterial, anti-inflammatory, antiviral, and immune regulating effects (14–16). In addition, ISL is rich in cellulose, which helps gastrointestinal peristalsis, promotes food digestion, increases satiety, and lowers cholesterol, reduces the accumulation of fat in the body. Research has found that the extract of ISL has multiple effects on inhibiting skin fibroblast aging by regulating mTOR-NF-κB-SASP signaling (17), reduces stress-induced behavior and cellular disorders in mice through antioxidant and anti-inflammatory effects (18), and the extract also has anti-wrinkle effects (19). ISL is a common herbaceous plant and Chinese herbal medicine, but there is currently no in-depth study on the growth performance of fattening sheep fed with ISL. This experiment studied the effect of 8% ISL diet on the growth performance of fattening sheep, and further explored the mechanism of ISL on the dynamic changes of rumen and intestinal microbiota, serum antioxidant capacity, metabolome (rumen fluid, serum and urine), and transcriptome in fattening sheep.

2 Material and methods

2.1 Experimental animals and experimental design

The experiment selected 12 healthy 3.5-month-old Hu sheep (Chinese native sheep breeds) with an average weight of 22.42 ± 2.15 kg and randomly divided into two groups, with 6 sheep in each group. The feeding experiment was conducted at Tianchang Zhoushi Sheep Industry Co., Ltd. (Chuzhou, China). The CON was fed a basic diet, while the ISL was fed a basic diet replaced by 8% ISL. The ingredient composition is shown in Table 1. The experimental period was 2.5 months, and the pre-feeding period was 0.5 months. Before the experiment, the enclosure was disinfected and ventilated, routine epidemic prevention measures such as insect repellent were carried out on the experimental sheep, and fattening sheep were fed at fixed times in the morning and evening every day, with free feeding and drinking water. The experimental plan was approved by the Animal Protection Committee of Anhui Agricultural University (NO: SYDW–P20190600601), and the experimental design and workflow are shown in the Figure 1.

2.2 Experimental samples and data collection

Three feed samples were collected per week (Monday, Wednesday, and Friday), and each sheep feed sample was mixed and sampled twice. The samples were dried at 85°C for 2,880 min and filtered through a 1 mm sieve to analyze dry matter (DM), crude protein (CP) and ash (20), as well as neutral derivative fiber (NDF) and acid derivative fiber (ADF) (21). On the first day of the formal experiment of fattening sheep, the body weight before morning feeding was the initial body weight. On the last day of the formal experiment, the test sheep were weighed before morning feeding, and the average daily gain of each stage was calculated. The daily feeding amount and leftover amount were accurately recorded, and the average daily feed intake was calculated. The

Abbreviations: CON, basal diet; ISL, isatis leaf; DM, dry matter; CP, crude protein; NDF, neutral detergent fiber; ADF, acid detergent fiber; VFA, volatile fatty acid; UPLC-Q-TOF-MS, ultra-high-performance liquid chromatography-tandem quadrupole time-of-flight mass spectrometry; ADG, average daily gain; ADFI, average daily feed intake; VIP, variable projection importance; DEG, differentially expressed gene; GO, Gene ontology; circRNA, circular RNA; lncRNA, long stranded non coding RNA; miRNA, MicroRNA; LPS, lipopolysaccharides.

TABLE 1 Main components of the diet for fattening sheep during the experimental period.

Item	CON	ISL
Ingredient (%)		
Ground corn grain	28.00	25.76
Soybean meal	15.00	13.80
Rapeseed meal	9.00	8.28
Wheat bran	4.00	3.68
Sodium bicarbonate	1.00	0.92
Salt	1.00	0.92
Dicalcium phosphate	0.50	0.46
Calcium carbonate	0.50	0.46
Premix[a]	1.00	0.92
Isatis tinctoria L. leaf	0.00	8.00
Peanut straw	15.00	13.80
Soybean straw	25.00	23.00
Chemical composition (%, DM)		
Organic matter	91.30	91.56
CP	15.10	15.28
NDF	38.70	39.88
ADF	23.20	25.61
Ether extract	3.10	2.91
Calcium	0.75	0.75
Phosphorus	0.43	0.43
Metabolizable energy[b], MJ/Kg	9.83	9.77

CON, control diet; ISL, isatis leaf.
[a]Formulated to provide (per kilogram of premix) 600 KIU of Vitamin A, 80 KIU of vitamin D3, 5,000 IU of Vitamin E, 8,000 mg of Zn, 60 mg of Se, 200 mg of I, 9,400 mg of Fe, 72 mg of Co, 10,400 mg of Mn, and 1,600 mg of Cu.
[b]Calculated according to Ministry of Agriculture of P.R. China, 2004.

feed conversion rate was calculated based on the average daily gain/average daily feed intake.

On the last morning of the feeding experiment, samples of rumen fluid, feces, serum, and urine were collected from fattening sheep. A total of 12 fattening sheep in the ISL and CON groups were collected with a rumen fluid sampler. The collected rumen fluid was filtered with 4 layers of gauze, and 1 mL of each sample was analyzed for volatile fatty acids (VFAs) (22). Two mL was used for colorimetric analysis of ammonia nitrogen content (23). The remaining liquid nitrogen was immediately stored in a liquid nitrogen tank for future use after being transferred to a frozen storage tube. Blood was collected from the jugular vein of the blood collection vessel on an empty stomach and allowed to stand at room temperature for 120 min. The serum was centrifuged at 3500 r/min for 15 min. After being drawn, it was divided into 1.5 mL centrifuge tubes and stored at −20°C. Fresh urine from 12 fattening sheep was collected and divided into 2 mL cryotubes, which were stored in liquid nitrogen. The fresh feces of the fattening sheep were collected using the rectal fecal collection method. The feces were immediately placed in a 15 mL centrifuge tube and stored in liquid nitrogen for storage.

After the feeding experiment, the fasted fattening sheep were slaughtered, and the rumen epithelial tissue was collected. After being washed with physiological saline, it was placed in an EP tube and quickly stored in liquid nitrogen for transcriptome sequencing. The collected gastrointestinal tissue was fixed with 4% paraformaldehyde, and histological sections were made using HE staining following the steps of fixation, dehydration, transparency, wax immersion, embedding, sectioning, staining, and sealing. After neutral gum sealing, the tissue sections were observed under a microscope, and disposable sterile gloves and masks were worn during sample collection to avoid contamination.

2.3 16S rRNA sequencing of rumen and fecal microorganisms

The rumen fluid and fecal samples were thawed at 4°C, and the total DNA was extracted with E.Z.N.A. fecal DNA kit (D4015, Omega Bio tek, Norcross, GA, United States). The concentration and purity of DNA samples were determined under the condition of OD260/OD280 absorbance, and DNA integrity was detected by 1% agarose gel electrophoresis. PCR amplification was performed on the V3-V4 variable region of the bacterial 16S rRNA gene, with primer sequences 515F (5′- CCTACACGACGTCTTCCGATCTN-3′) and 806R (5′- GACTGGATCCCTTGACCCGATTCCA-3′). A total of 30 μL PCR amplification reaction system: 15 μL 2 × Phanta Master Mix; 1 μL Bar PCR prime F (10 μM), 1 μL prime R (10 μM), 10 ng Genomic DNA, supplemented with ddH_2O to 30 μL. Amplification conditions: pre-denaturation at 95°C for 5 min; 95°C denaturation for 0.5 min, 55°C annealing for 0.5 min, 72°C extension for 0.75 min, 27 cycles; After extending at 72°C for 10 min, the PCR products were collected for detection and purification, and sent to Sangon (Shanghai, China) for sequencing based on the Illumina Miseq platform. After quality control and processing of the original sequencing data, the differences in microbial diversity and community structure in the rumen and feces of fattening sheep in the CON and ISL groups were analyzed.

2.4 Metabolomics detection of rumen fluid, serum, and urine

Frozen rumen fluid, serum, and urine samples were sent to Shanghai Baiqu Biotechnology Co., Ltd. for metabolomics testing and analysis, including sample processing, non-targeted serum metabolomics testing, and data processing analysis. The rumen fluid, serum, and urine samples were thawed, and 50 μL of the sample was mixed with 150 μL of pre-cooled ice methanol (containing 1 μg/mL of 2-Chlorophenylalanine). The supernatant was centrifuged at 4°C at 12,000 r/min for 10 min, and metabolomics analysis was performed using ultra-high-performance liquid chromatography-tandem quadrupole time-of-flight mass spectrometry (UPLC-Q-TOF-MS). Chromatographic

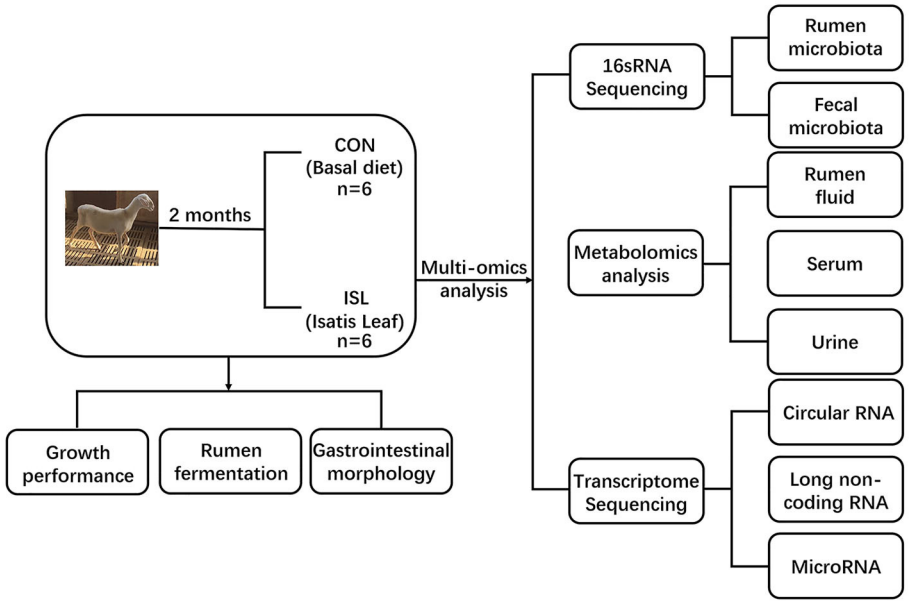

FIGURE 1
Experimental design and workflow of feeding fattening sheep with Isatis leaf diet. Including rumen and fecal microbiome, rumen fluid, serum and urine metabolome, and transcriptome sequencing of rumen epithelial cells. Twelve Hu sheep were randomly assigned to a basal diet (CON) or a basal diet supplemented with 80 g/kg Isatis leaf DM (ISL).

column: ACQUITY UPLC HSS T3 column (100 mm × 2.1 mm, 1.8 μm); The mobile phase A is ultrapure water (0.1% formic acid), and the B phase is acetonitrile (0.1% formic acid); Elution gradient: 0–1 min (2% B), 1–2 min (2%–5% B), 2–5 min (5%–12% B), 5–10 min (12%–20% B), 10–12 min (20%–30% B), 12–13 min (30%–50% B), 13–15 min (50%–100% B). Flow rate of 0.5 mL/min, column temperature of 40°C, injection volume of 5 μL. The ionization source is an electric spray ionization source (ESI) in positive and negative ion scanning mode. The raw data obtained from UPLC-Q-TOF-MS sequencing was processed and differential metabolites were screened under the conditions that the *p*-value of Student's *t*-test was <0.05, and the Variable Importance in the Projection (VIP) of the first principal component in the OPLS-DA model was >1. Metabolic pathway enrichment analysis was performed on the screened differential metabolites.

2.5 Transcriptomics analysis

The rumen epithelial tissue samples preserved in liquid nitrogen were sent to Shanghai Shenggong Biotechnology Co., Ltd. in China for transcriptome sequencing. The total RNA of rumen epithelial tissue was extracted with Trizol reagent, the concentration and purity of total RNA (OD 260 nm/OD 280 nm and OD 260 nm/OD 230 nm) were detected with Nanodrop 2000 ultra micro spectrophotometer, the RNA integrity was detected with 1% agarose gel electrophoresis, and the qualified RNA was stored in an ultralow temperature refrigerator at −80°C for standby.

High throughput sequencing was performed using the Illumina Hiseq™ sequencing platform, and the raw data obtained from sequencing was evaluated and controlled for quality. FastQC was used for quality evaluation, Trimmatic was used for quality pruning, and DESeq2 was used for significance analysis of samples. The screening conditions were set to $P < 0.05$ and |fold change|>2 and the differentially expressed genes (DEGs) obtained were subjected to Gene ontology (GO) enrichment analysis using ClusterProfiler software.

2.6 Statistical analysis

We used Excel to organize the data, and SPSSAU data analysis platform (https://spssau.com/) was used for data normal distribution testing. SPSS software independent sample *T*-test was used for statistical analysis, with $P < 0.05$ indicating significant differences and $P < 0.01$ indicating extremely significant differences. The test sample consists of 6 replicates.

3 Results

3.1 Growth performance

5Before the formal experiment, we measured the weight of fattening sheep in the CON and ISL groups. The initial average weight of the ISL group was 23.31 ± 1.98 kg, while the CON group was 23.25 ± 1.54 kg, with no significant difference. After 2 months of feeding, the average daily gain of fattening sheep in the ISL group and CON group was 0.18 ± 0.02 kg and 0.21 ± 0.01 kg. The average daily gain (ADG) of fattening sheep in the ISL group was significantly lower than that in

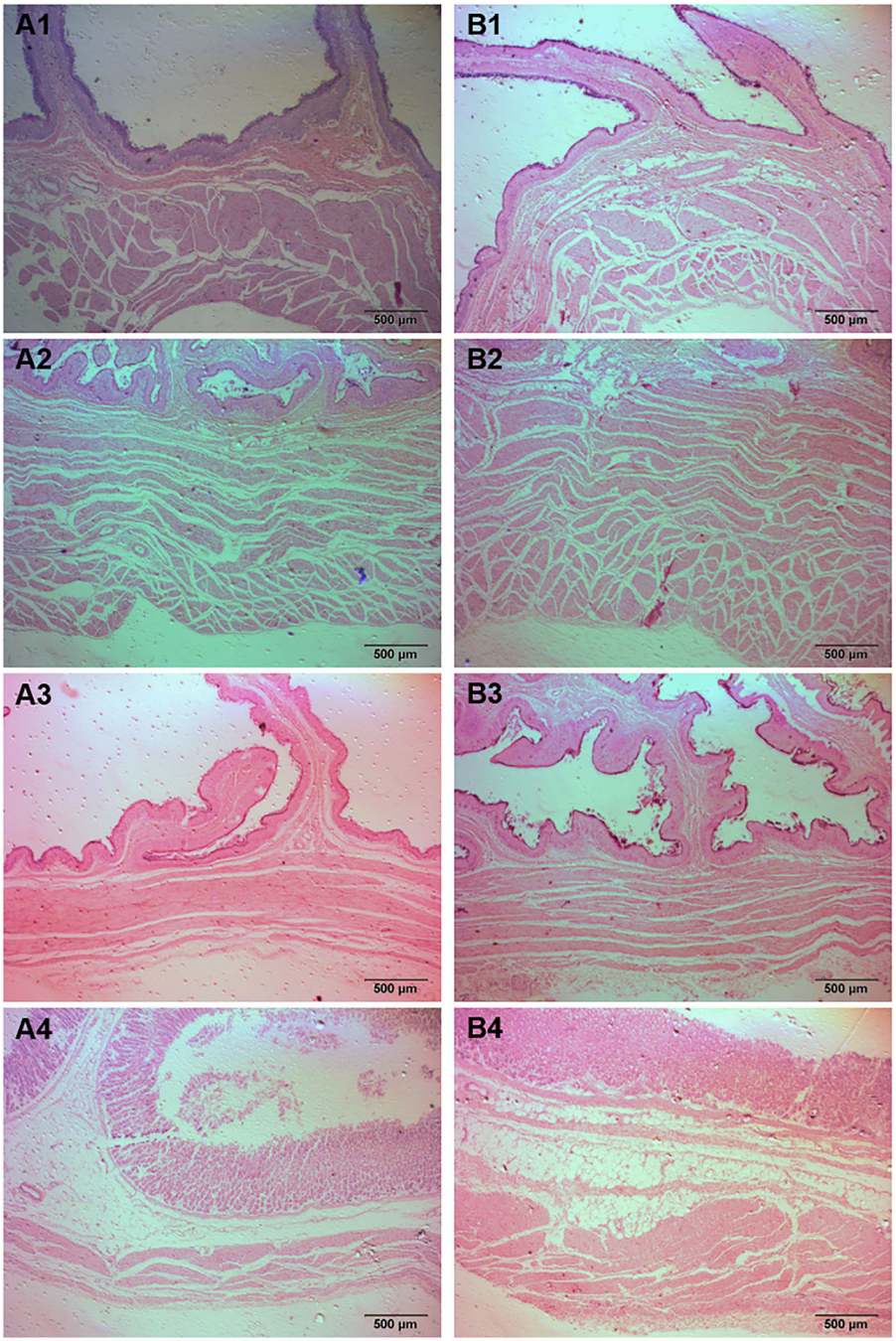

FIGURE 2
Histomorphology of stomachs in fattening sheep fed with ISL diet. **(A)** CON, **(B)** ISL, (1) Rumen, (2) Reticulum, (3) Omasum, (4) Abomasum.

the CON group ($P < 0.05$). The average daily feed intake (ADFI) of the ISL group was 1.03 ± 0.05 kg, while the CON group was 1.21 ± 0.04 kg. The average daily feed intake was significantly reduced ($P < 0.05$), and the feed conversion rate was both 0.17 ± 0.01, with no significant difference. During the fattening period when 8% ISL was added to the diet, the fattening sheep's body was healthy. Further research is needed to investigate the mechanism of the influence of ISL on the growth of fattening sheep.

3.2 Gastrointestinal morphology

The gastrointestinal tract of animals is the place where nutrients are digested and absorbed, and changes in its organizational structure are extremely important for animal feed intake and digestion and absorption capacity (24). At present, there are limited reports on the effects of ISL on the gastrointestinal development of fattening sheep. We selected the gastrointestinal tissue of the ISL group and the CON group fattening sheep

TABLE 2 Effect of ISL on the morphology of gastric tissue in fattening sheep.

Position	Item	CON (um)	ISL (um)
Rumen	Muscularis	1,010.62 ± 34.26	979.56 ± 27.46
Reticulum	Muscularis	1,335.28 ± 76.93[b]	1,926.77 ± 136.66[a]
Omasum	Muscularis	706.78 ± 10.17	728.23 ± 55.15
Abomasum	Muscularis	451.64 ± 21.53[b]	746.15 ± 42.11[a]

[a,b]Values within a row with different superscripts differ significantly at $P < 0.05$.

TABLE 3 Rumen fermentation characteristics of Isatis leaf diet treatment.

Item	Treatment		SEM	*P*-value
	CON	ISL		
Ammonia-nitrogen, mg/dL	6.21[b]	14.01[a]	1.865	0.001
Total volatile fatty acid, mM	66.10	68.94	2.709	0.311
Acetate, mM	45.23	47.57	2.555	0.374
Propionate, mM	12.21	12.54	0.844	0.704
Butyrate, mM	7.85	8.02	0.497	0.734
Valerate, mM	0.81	0.81	0.050	0.976
Acetate: propionate	3.71	3.94	0.386	0.565

[a,b]Values within a row with different superscripts differ significantly at $P < 0.05$.

to observe the morphological structure, and measured their muscle layer thickness (Figure 2, Supplementary Figure S1). The measurement results and statistical data are shown in the Table 2 and Supplementary Table S1. Compared with the CON group, the thickness of the reticulum and abomasum muscle layers in the ISL group was significantly increased ($P < 0.05$), and there was no significant change in the thickness of the rumen and omasum muscle layers. The difference in muscle layer thickness and villus length of each intestinal segment tissue was not significant.

3.3 Rumen fermentation and microbiota

Ammonia nitrogen is the main nitrogen source for rumen microbial fermentation and an important indicator affecting microbial activity. Suitable ammonia nitrogen is the primary condition to ensure the efficiency of microbial protein synthesis (25, 26). The concentration of ammonia nitrogen and volatile fatty acid (VFA) in the rumen are important indicators reflecting the fermentation status of feed in the rumen. After adding ISL feed, it was found that the levels of ammonia nitrogen in the ISL group were significantly higher than those in the CON group ($P < 0.01$). The total VFA, acetic acid, propionic acid, butyric acid, valeric acid, and acetic acid and propionic acid concentrations in the ISL group were increased compared to the CON group, but the difference was not significant (Table 3).

The rumen is the first stomach of ruminants, and rumen microorganisms are in dynamic equilibrium within the ruminant, maintaining normal growth of the body (27). Macrogenomic classification and sequencing of rumen microorganisms were conducted, and the rumen microbial population was analyzed based on genus level (Figure 3A). We detected Prevolella, Methanobrevibacter, Clostridium IV, Succiniclasticum, Selenomonas, and others between the ISL and CON groups. Among them, Prevolella, Methanobrevibacter, Succiniclasticum, Clostridium IV, and Selenomonas were the five dominant bacterial genera in the rumen of fattening sheep. Through further analysis of the rumen microbiota, significant changes were identified (accounting for 0.05% of the total sequence in at least one sample) (Table 4). A total of 6 genus level microbial changes were identified between the ISL group and the CON group, among which the relative abundance of Metanobrevibate and Centipeda in the ISL group was significantly upregulated compared to the CON group; the relative abundance of Butyrivibrio, Saccharoferments, Mogibacterium, and Pirellula was significantly downregulated ($P < 0.05$).

3.4 Fecal microbiota

The fecal microbiota is closely related to the intestinal microbiota, among which the dominant microbiota is closely related to the balance of the intestinal microbiota, playing an important role in the structural function of the intestine, the digestion, absorption, and metabolism of nutrients, as well as the immune regulatory function of the body (28). Its changes affect the growth and development of animals. Based on genus level analysis of fecal microbial populations in the ISL and CON groups (Figure 3B), Sporobacter, Bacteroides, Alistipes, Clostridium_XIVa, and Treponema were detected between the ISL and CON groups. Among them, Sporobacter, Bacteroides, and Alistipes were the three dominant genera of fecal bacteria detected in the ISL and CON groups. In the ISL and CON groups of fecal microbiota, a total of 12 significantly changing microbiota were screened at the genus level (Table 5). In the ISL group, Papilibacter, Pseudoflavonifractor, Butyricicoccus, Anaerovorax, and Metanocorpusculum were significantly upregulated in relative abundance compared to the CON group; Compared to the CON group, the relative abundance of Roseburia, Coprococcus, Clostridium XVIII, Butyrivibrio, Parasutterella, Macelliberoides, and Porphyromonas was significantly reduced ($P < 0.05$).

3.5 Rumen fluid metabolomics

Non-targeted LC-MS metabolomics analysis was conducted on 12 rumen fluid samples from fattening sheep in the ISL and CON groups, and differential metabolites were screened. A total of 164 differential metabolites were screened between the ISL and CON groups, including 62 anionic modes and 102 cationic modes (Supplementary Table S2).

Further hierarchical clustering analysis was conducted on the differential metabolites between groups (Figure 4). The red color represents an upregulation of metabolites relative to the CON group, while the blue color represents a downregulation. The differences in different metabolites between the ISL group and the CON group were significant in the rumen fluid of fattening sheep. There were 32 types of differential metabolites significantly downregulated in the ISL group relative to the CON group, such as 2E-Eicosenoic acid, Formaronetin, DL-2-Aminoadipic acid, Dihydrolipoate (dihydrolipoic acid), and Sulfadiazine.

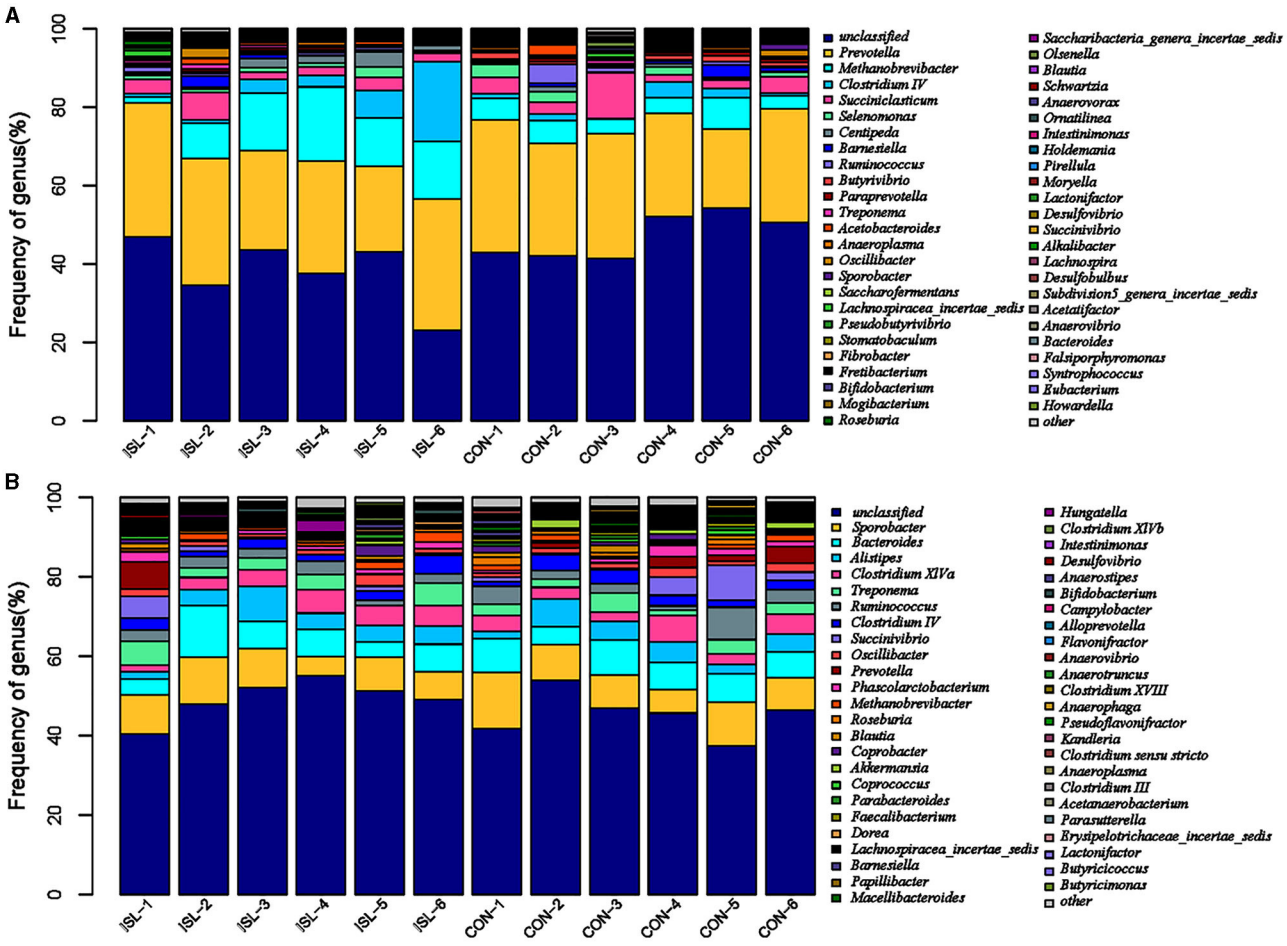

FIGURE 3
Distribution characteristics of horizontal microbiota in the ISL group group. **(A)** Distribution of rumen microbiota. **(B)** Distribution of fecal microbiota.

TABLE 4 **Significant changes in the main microbial communities in the rumen of the ISL diet group.**

Phylum	Genus	Treatment		SEM	*P*-value
		CON	ISL		
Euryarchaeota	Methanobrevibacter	5.082[b]	11.813[a]	2.105	0.006
Firmicutes	Centipeda	0.447[b]	1.698[a]	0.466	0.017
Bacillota	Butyrivibrio	1.095[a]	0.393[b]	0.164	0.001
	Saccharofermentans	0.325[a]	0.178[b]	0.051	0.011
	Mogibacterium	0.182[a]	0.118[b]	0.022	0.011
Planctomycetota	Pirellula	0.162[a]	0.013[b]	0.064	0.034

[a,b]Values within a row with different superscripts differ significantly at $P < 0.05$.

A total of 132 differential metabolites were significantly upregulated, such as D-galacturonic acid, (-)-Naringenin, Gly-Pro, N-Acetylglutamine, and L-NG-Monomethylarginine. A total of 24 pathways with the highest correlation between rumen fluid metabolites were screened between the ISL group and the CON group (Supplementary Table S3, Figure 5), mainly including Thiamine metabolism, Nicotinate and nicotinamide metabolism, Vitamin B6 metabolism, Tryptophan metabolism, Taurine and hydroxyrine metabolism, and Cysteine and methionine metabolism.

3.6 Serum metabolomics

Serum is an important component of the blood, containing about 1,000 endogenous small molecule metabolites, mainly

TABLE 5 Change characteristics of the main microbial communities in the feces of the ISL diet group.

Phylum	Genus	Treatment		SEM	*P*-value
		CON	ISL		
Bacillota	Roseburia	1.072[a]	0.655[b]	0.190	0.045
	Coprococcus	0.700[a]	0.338[b]	0.143	0.023
	Papillibacter	0.270[b]	0.472[a]	0.058	0.003
	Clostridium XVIII	0.227[a]	0.078[b]	0.065	0.039
	Pseudoflavonifractor	0.105[b]	0.182[a]	0.030	0.021
	Butyricicoccus	0.070[b]	0.122[a]	0.016	0.007
	Anaerovorax	0.028[b]	0.078[a]	0.017	0.011
	Butyrivibrio	0.068[a]	0.005[b]	0.025	0.021
Pseudomonadota	Parasutterella	0.148[a]	0.057[b]	0.026	0.003
Euryarchaeota	Methanocorpusculum	0.000[b]	0.147[a]	0.054	0.016
Bacteroidota	Macellibacteroides	0.525[a]	0.130[b]	0.161	0.027
	Porphyromonas	0.127[a]	0.012[b]	0.042	0.015

[a,b]Values within a row with different superscripts differ significantly at $P < 0.05$.

including plasma proteins, growth factors, hormones, inorganic ions, amino acids, glucose, nucleosides, lipids, and steroids (29). It is an important metabolomics research sample. Differential metabolite screening was conducted on serum samples. A total of 107 differential metabolites were screened between the ISL and CON groups, including 53 anionic and 54 cationic modes (Supplementary Table S4).

The hierarchical cluster analysis of serum differential metabolites (Figure 6) showed that compared to the CON group, a total of 67 differential metabolites were significantly upregulated, such as Formylanthranilic acid, Meclofenamate, Flurocortisone acetate, Imidazoleacetic acid, Glycylproline, and Ribothymidine; a total of 40 differential metabolites were significantly downregulated, such as Pyrocatechol, Bisindolylmaleimide I, Zafirlukast, beta-Octylglucoside, Enoxacin, and Visnadin. Metabolic pathway analysis was conducted on the selected serum differential metabolites, and a total of 25 key metabolic pathways were identified (Supplementary Table S5, Figure 7). The key metabolic pathways affecting serum metabolism were mainly: Valine, leucine and isoleucine biosynthesis, Pantothenate and CoA biosynthesis, beta-Alanine metabolism, Arginine and proline metabolism, Histidine metabolism, Alanine, Aspartate and glucose metabolism, etc.

3.7 Urine metabolomics

Urine is the final excreta of metabolic products in the body (30). Analyzing changes in urine metabolites, key metabolic pathways, and potential biomarkers is of great significance for studying the mechanism of influence on the growth performance of feeding ISL fattening sheep. A total of 187 differential metabolites were screened between the ISL group and the CON group urine samples, of which 77 were screened for anionic mode and 110 were screened for cationic mode (Supplementary Table S6).

Hierarchical cluster analysis was performed on the differential metabolites obtained from the analysis (Figure 8). Compared with the CON group, the ISL group significantly upregulated 46 differential metabolites in urine, such as Pyrocatechol, Picrotoxin, Pyridoxal (Vitamin B6), Guanosine, D-Galactalate, and Chlorogenic acid; A total of 141 differential metabolites were significantly downregulated, such as Acetyl-L-Cysteine, Thymidine, Barbituric acid, 2-Ethoxyethanol, Normanephrine, and Fluoxetine. A total of 42 key pathways with the highest correlation between urine samples metabolites in the ISL and CON groups were screened (Supplementary Table S7, Figure 9). The pathways with the highest correlation between feeding ISL on urine metabolites in fattening sheep were mainly Taurine and hydropotarine metabolism, Tryptophan metabolism, Pyrimidine metabolism, Phenylalanine metabolism, Riboflavin metabolism, and Vitamin B6 metabolism.

3.8 CircRNA bioinformatics

CircRNA, as a special type of endogenous non coding RNA, is widely present in the cytoplasm of eukaryotic cells and plays an important regulatory role in biological growth and development (31, 32). A total of 11 significantly differentially expressed circRNAs were screened between the ISL and CON groups, of which 10 were significantly upregulated and 1 was significantly downregulated (Supplementary Table S8, Figure 10A).

For the screened differentially expressed circRNAs, further research was conducted on the distribution of their target genes in annotation function (Figure 10B). The differentially expressed cricRNA target genes mainly participate in biological processes such as cellular processes, biological process regulation, biological regulation, cell composition, tissue and biogenesis, and are mainly located at positions such as cells, cellular parts, organelles, and organelles. The main molecular functions are binding activity, catalytic activity, and molecular functional regulation. To further examine the function of focusing on differentially expressed circRNA target genes between the ISL group and the CON group, enrichment analysis was conducted on the target genes, and

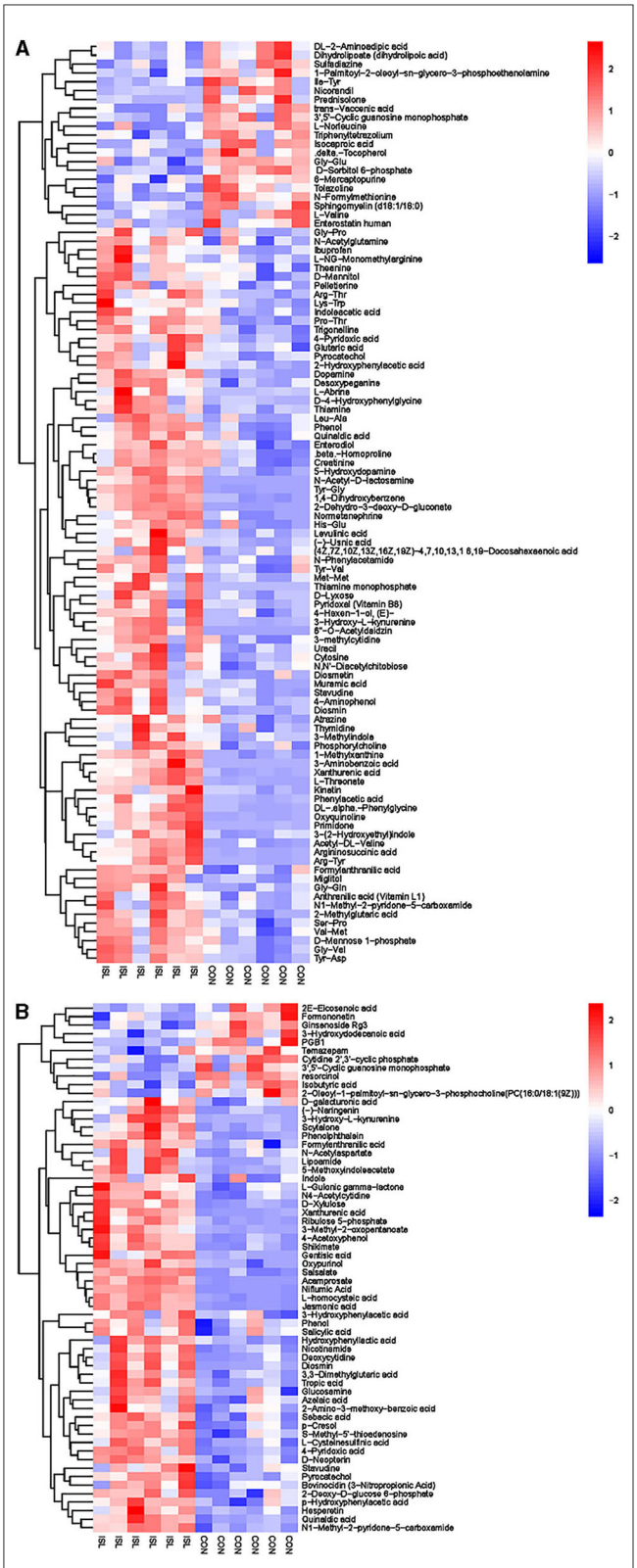

FIGURE 4
Hierarchical cluster analysis of rumen metabolites in fattening sheep. **(A)** Hierarchical clustering analysis of ISL groups in cationic mode. **(B)** Hierarchical cluster analysis of ISL group in anionic mode. The horizontal axis represents different experimental groups, the vertical axis represents the differential metabolites compared in the group, the blue color block represents the relative expression level of the corresponding position metabolites is down regulated, and the red color block represents the up regulation.

the top 30 functions with the highest enrichment degree were selected (Supplementary Table S9, Figure 10C). The circRNA target genes of fattening sheep fed with ISL were mainly enriched in the phosphorylation modification of regulating histones and the regulation of the nervous system. Discovery of alkaloids from the Isatis leaf with neuroprotective activity, which is consistent with the results of circRNA sequencing bioinformatics analysis (33, 34).

3.9 LncRNA bioinformatics

LncRNAs are a type of RNAs with a length >200 nt and no protein encoding potential (35). LncRNA plays an important role in regulating growth and development, especially in regulating skeletal muscle growth and development (36). By analyzing the differential changes in lncRNA and its functional impact on target genes, it is of great significance to study the effects of ISL on the growth and development of fattening sheep. A total of 160 transcripts (lncRNA, mRNA) with significant differences were screened (Supplementary Table S10, Figure 11A), and 94 transcripts were significantly upregulated, including 64 lncRNAs and 30 mRNA, significantly downregulated 66 transcripts, 13 lncRNAs, and 53 mRNA.

The distribution analysis of the target genes of differential transcripts was conducted in the annotation function (Figure 11B). The target genes of transcripts mainly participate in biological processes such as cellular processes, metabolic processes, biological regulation, and biological regulation, mainly located in cells, organelles, and cellular parts. The main molecular functions focus on binding activity and catalytic activity. Enrichment analysis was conducted on the target genes of differential transcripts, and the top 30 functions with the highest enrichment degree were selected (Supplementary Table S11, Figure 11C). The target genes of differential transcripts are mainly enriched in immune regulatory functions, such as defense against viruses and immune responses. ISL has anti-inflammatory and antibacterial functions, and contains various antiviral substances, which can enhance the body's immunity and play an important role in regulating the health of fattening sheep (18, 19).

3.10 MiRNA bioinformatics

MiRNAs are quite conserved in species evolution, and the expression of miRNAs found in animals has strict tissue specificity and timing (37). MiRNA plays various roles in cell growth and development, including regulating development, differentiation, apoptosis, and proliferation, and also plays a significant role in biological development (38). Differential expression analysis was conducted on miRNAs in the ISL and CON groups, and a total of 312 significantly different miRNAs were screened, of which 173 were significantly upregulated and 139 were significantly downregulated (Supplementary Table S12, Figure 12A).

Functional enrichment analysis was conducted on differentially targeted miRNAs obtained through screening, and gene functions of differentially targeted miRNAs were annotated and classified (Figure 12B). The differentially targeted miRNAs mainly participate in biological processes such as cellular

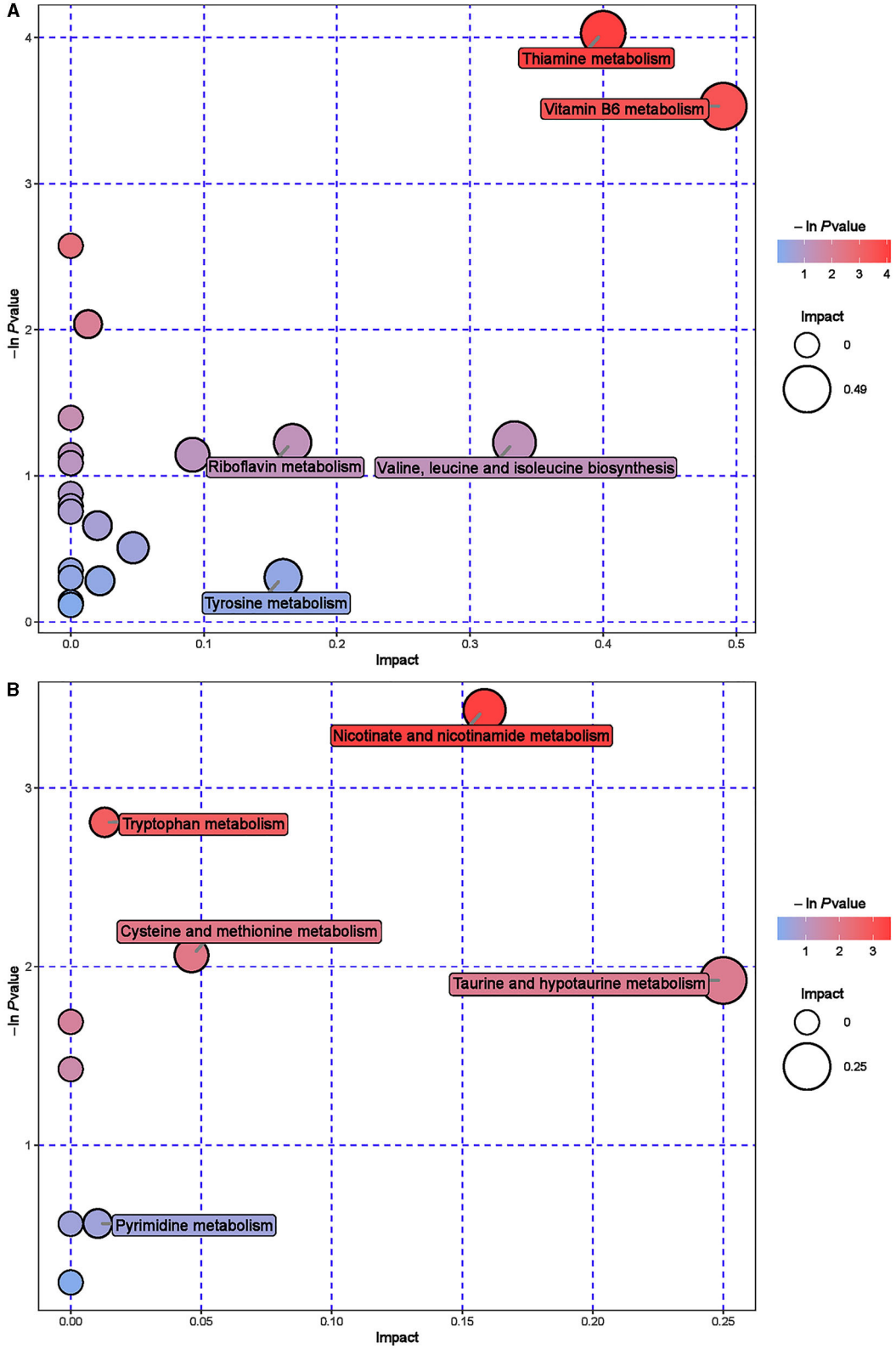

FIGURE 5
Pathway analysis of rumen metabolomics in fattening sheep. **(A)** Pathway analysis of ISL group in cationic mode. **(B)** Pathway analysis of ISL group in anionic mode. The color and size of bubbles indicate the impact of mint treatment on sample metabolism, while larger red bubbles indicate a greater impact on the pathway.

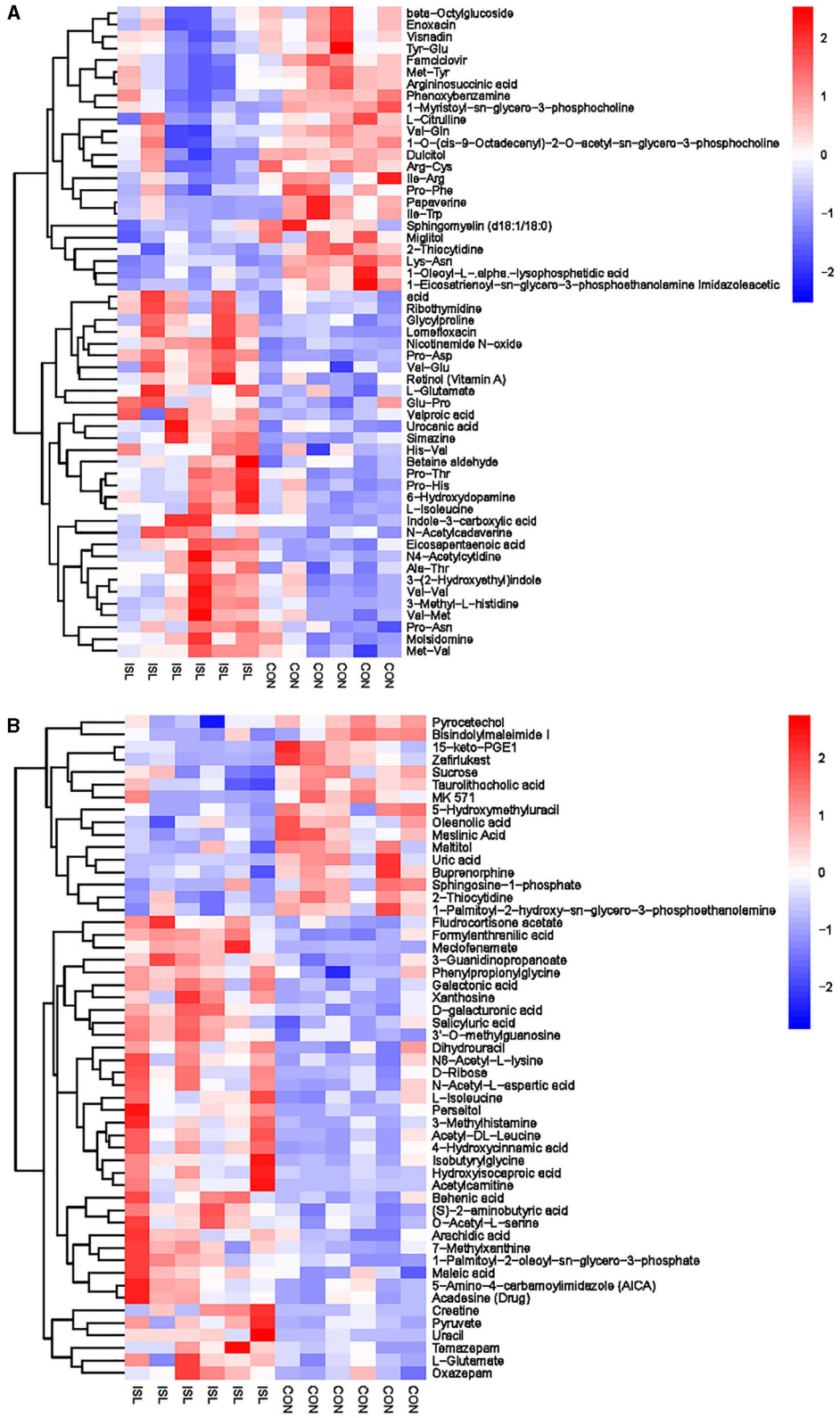

FIGURE 6
Hierarchical cluster analysis of serum metabolites in fattening sheep. **(A)** Hierarchical cluster analysis of ISL group serum in cationic mode. **(B)** Hierarchical cluster analysis of ISL group serum in anionic mode.

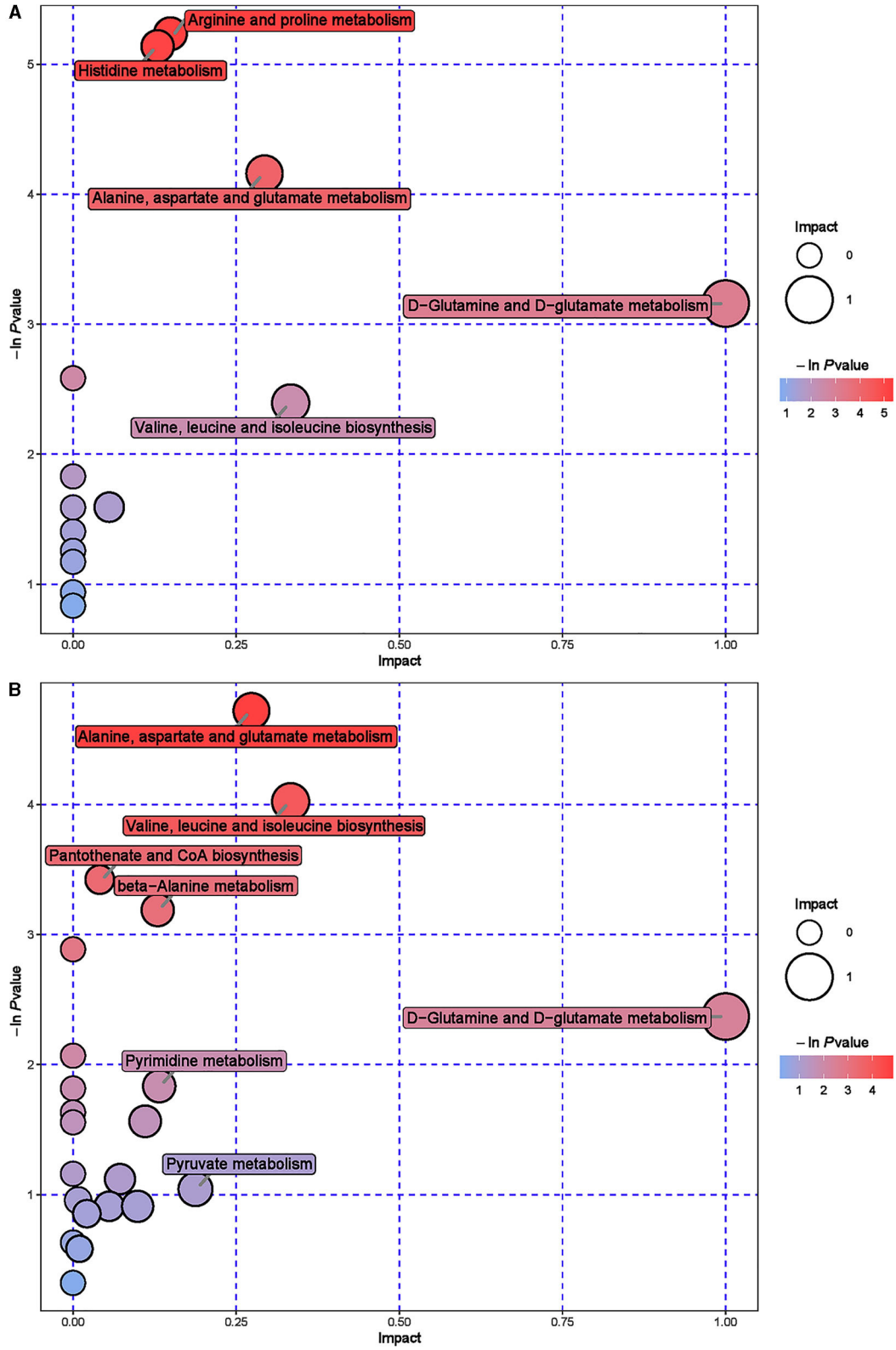

FIGURE 7
Pathway analysis of serum metabolomics. **(A)** Pathway analysis of ISL group serum in cationic mode. **(B)** Pathway analysis of ISL group serum under anionic mode.

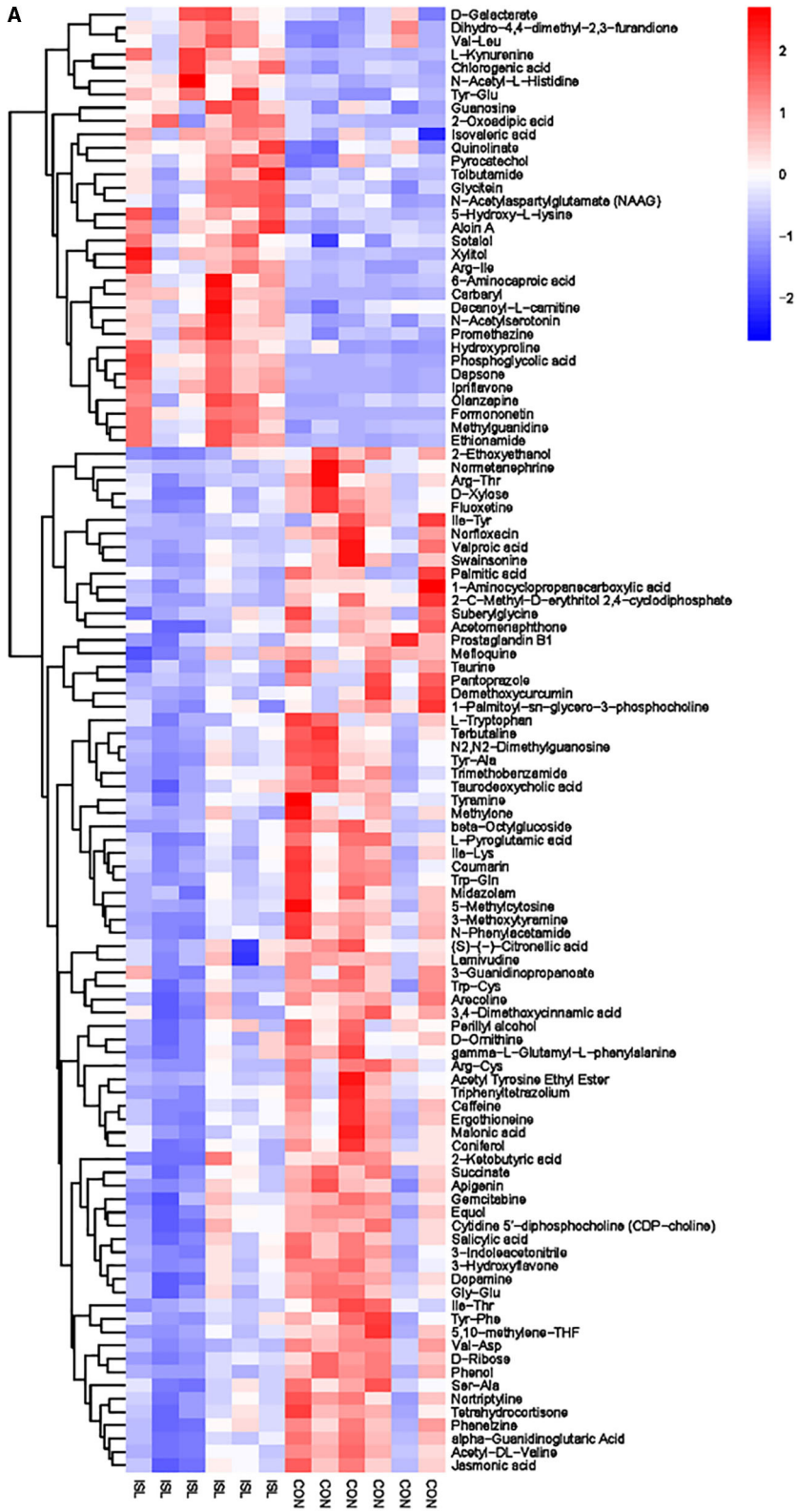

FIGURE 8 (Continued)

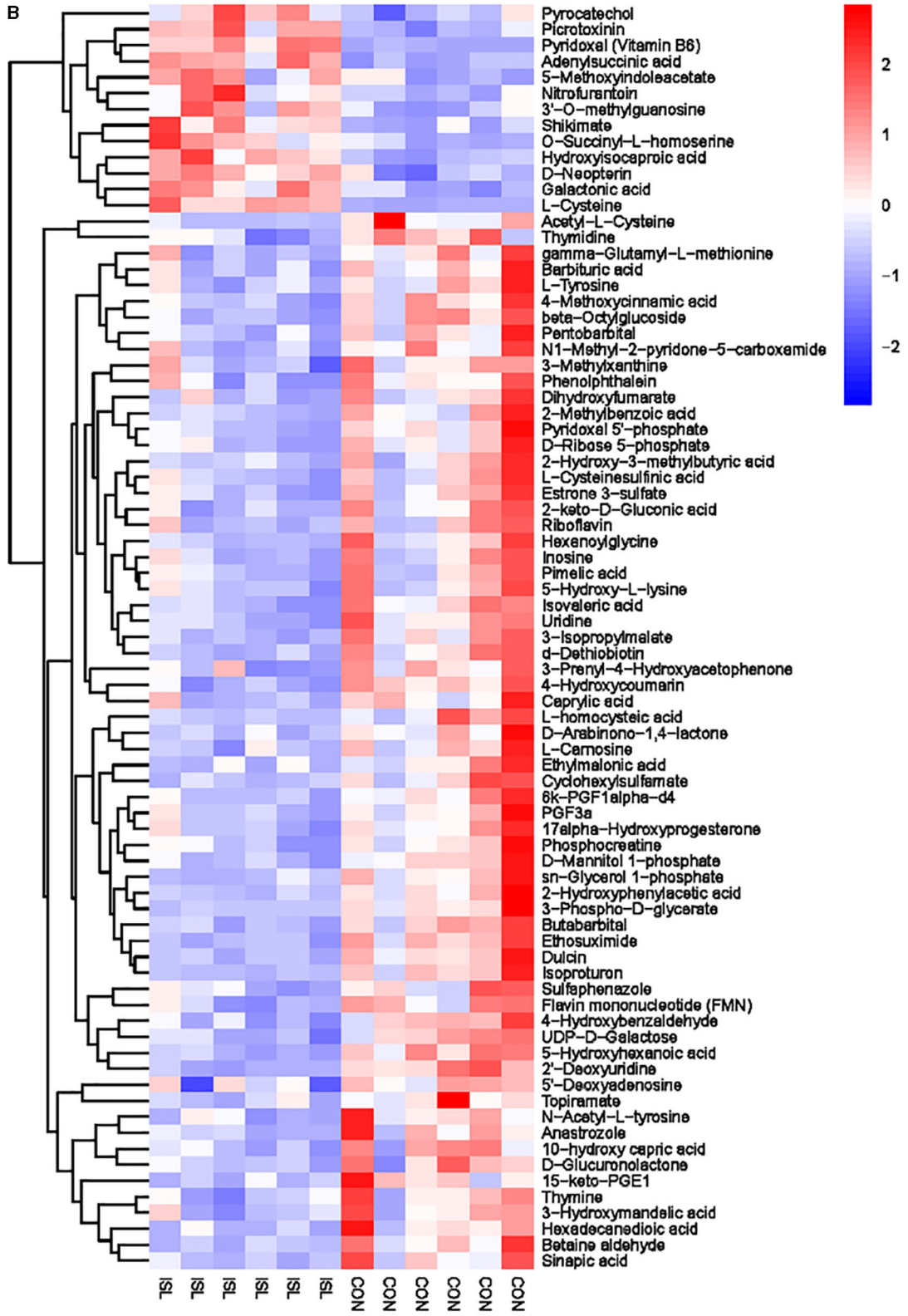

FIGURE 8 (Continued)
Hierarchical cluster analysis of urine metabolites in fattening sheep. **(A)** Hierarchical cluster analysis of ISL group urine in cationic mode. **(B)** Hierarchical cluster analysis of ISL group urine in anionic mode.

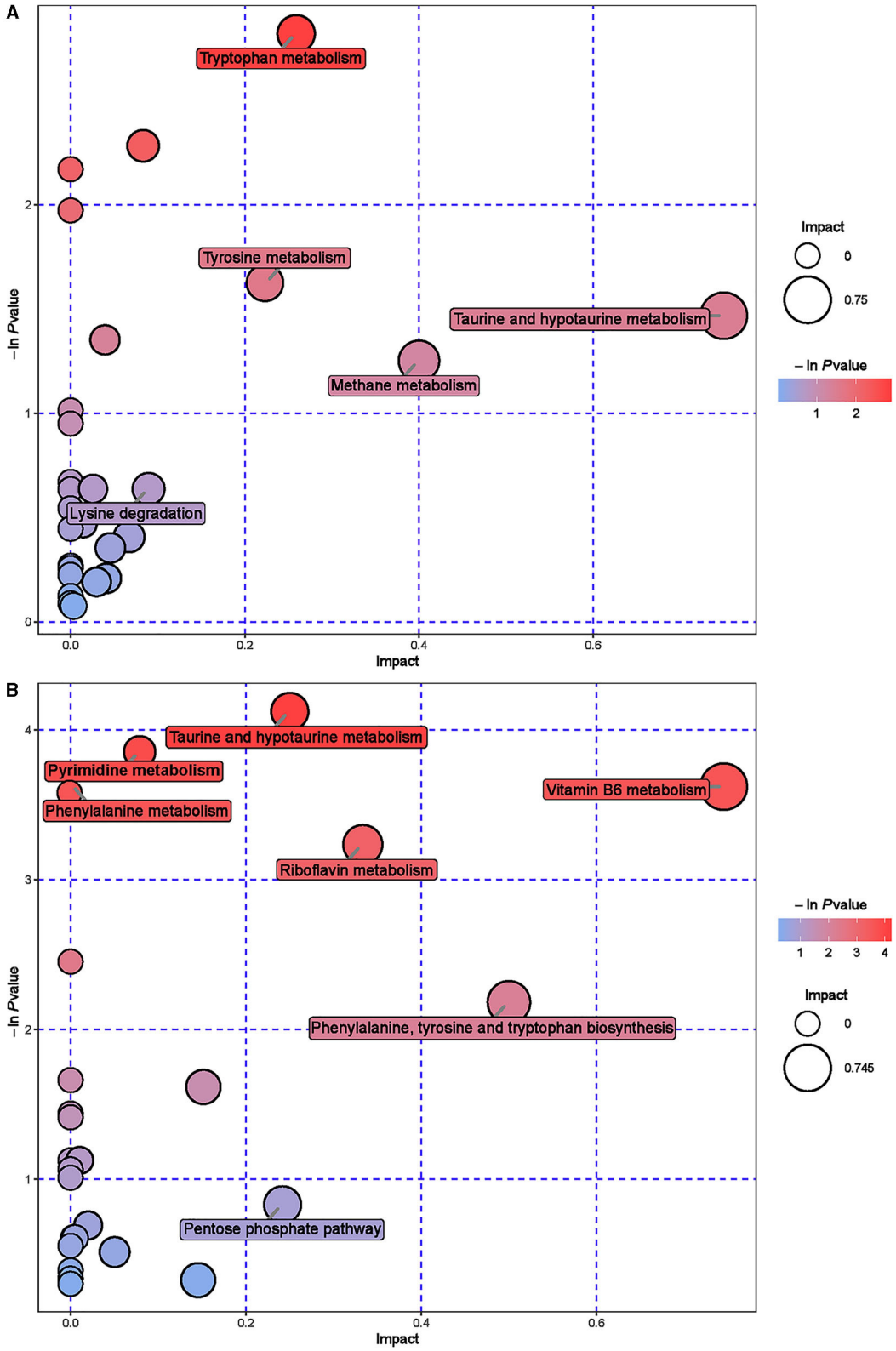

FIGURE 9
Pathway analysis of urine metabolomics. **(A)** Pathway analysis of ISL group urine in cationic mode. **(B)** Pathway analysis of ISL group urine under anionic mode.

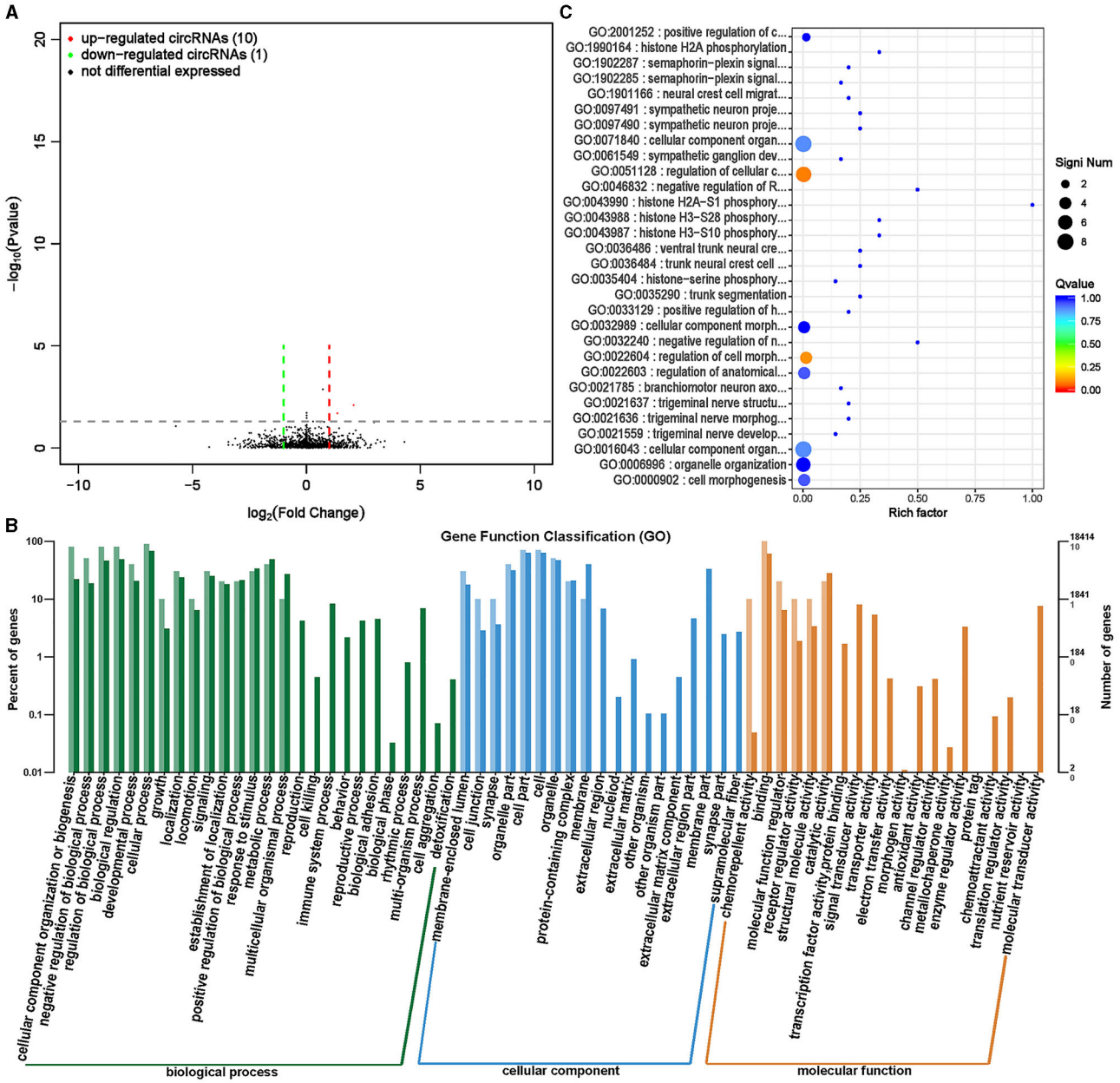

FIGURE 10
Differential expression analysis of circRNA between ISL group and CON group. **(A)** Volcano map of circRNA expression differences between ISL group and CON group. The horizontal axis represents the fold change [log (B/A)] value of the transcript expression difference between different groups, while the vertical axis represents the *P*-value of the transcript expression change. The smaller the *P*-value, the greater the log (*P*-value), and the more significant the difference. Red represents upregulated transcripts, green represents downregulated transcripts, and black represents non differential transcripts. **(B)** Histogram of host gene functional annotation classification for differentially expressed circRNA between ISL and CON groups. The horizontal axis represents the functional classification, while the vertical axis represents the number of genes within the classification (right) and their percentage in the total number of annotated genes (left). Light colors represent host genes, while dark colors represent all genes. **(C)** The top 30 functional scatter plots show significant enrichment of circRNA between the ISL group and the CON group. The vertical axis represents functional annotation information, while the horizontal axis represents the Rich factor corresponding to the function. The size of the Q-value is represented by the color of the dot. The smaller the Q-value, the closer the color is to red. The number of differentially expressed circRNA host genes is represented by the size of the dot.

processes, single biological processes, biological regulation, and metabolic processes, mainly located in cells, organelles, and membranes. The main molecular functions are focused on binding and catalytic activity. The top 10 functions with the highest enrichment in the three ontologies were selected (Supplementary Table S13, Figure 12C). The mRNA targeted by miRNAs is mainly enriched in transcription regulation and signal transduction functions, and the molecular function is mainly binding activity, including protein, calcium ions, DNA, and ATP. MiRNA bioinformatics analysis found that

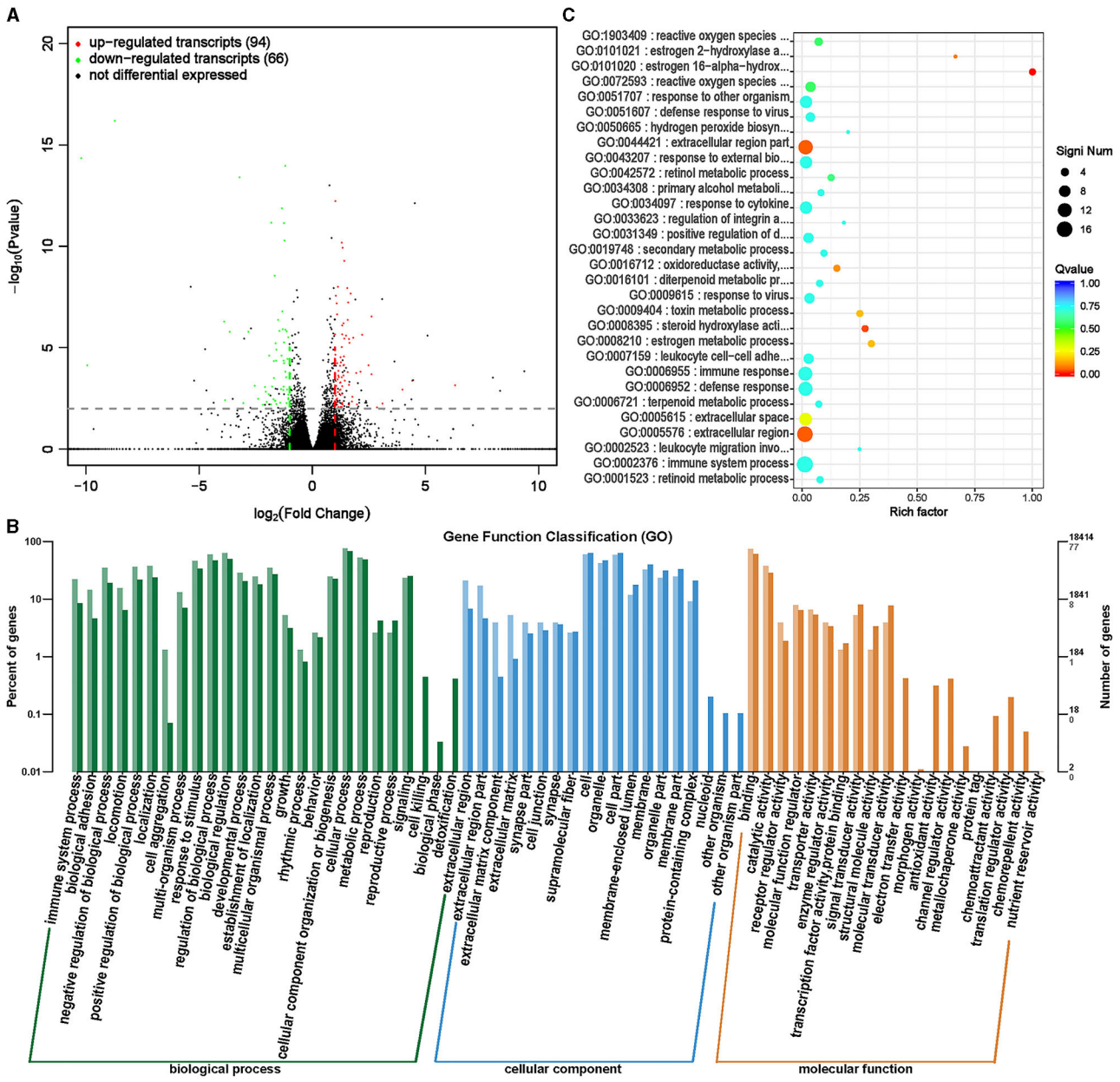

FIGURE 11
Differential expression analysis of transcriptome between ISL group and CON group. **(A)** Volcano map of transcript expression differences between ISL group and CON group. **(B)** Histogram of functional annotation classification of genes corresponding to ISL and CON differential transcripts. **(C)** The top 30 functional scatter plots show significant enrichment of differentially expressed transcripts in the ISL and CON groups.

differential miRNAs have a regulatory effect on energy metabolism, thereby affecting the growth performance of fattening sheep fed with ISL.

4 Discusses

Most reports have shown that using Chinese herbal medicine as a feed additive in animal production can improve the growth performance of livestock and poultry (39, 40). As a plant rich in protein, minerals, and multiple vitamins, ISL is rich in amino acids such as phenylalanine and valine, which are essential amino acids in livestock (41). Therefore, adding an appropriate amount of ISL to livestock feed can enhance disease resistance and muscle performance.

In this study, after 2 months of feeding, it was found that the average daily gain of fattening sheep in the ISL group was significantly lower than that in the CON group ($P < 0.05$); the average daily feed intake of the ISL group decreased by 25% compared to the CON group. This may be due to the rich dietary fiber in the leaves, which are consumed by fattening sheep, leading to a stronger sense of fullness, reduced appetite, and reduced feed intake, resulting in a decrease in the average daily feed intake of the ISL group. A decrease in appetite and calorie intake in the

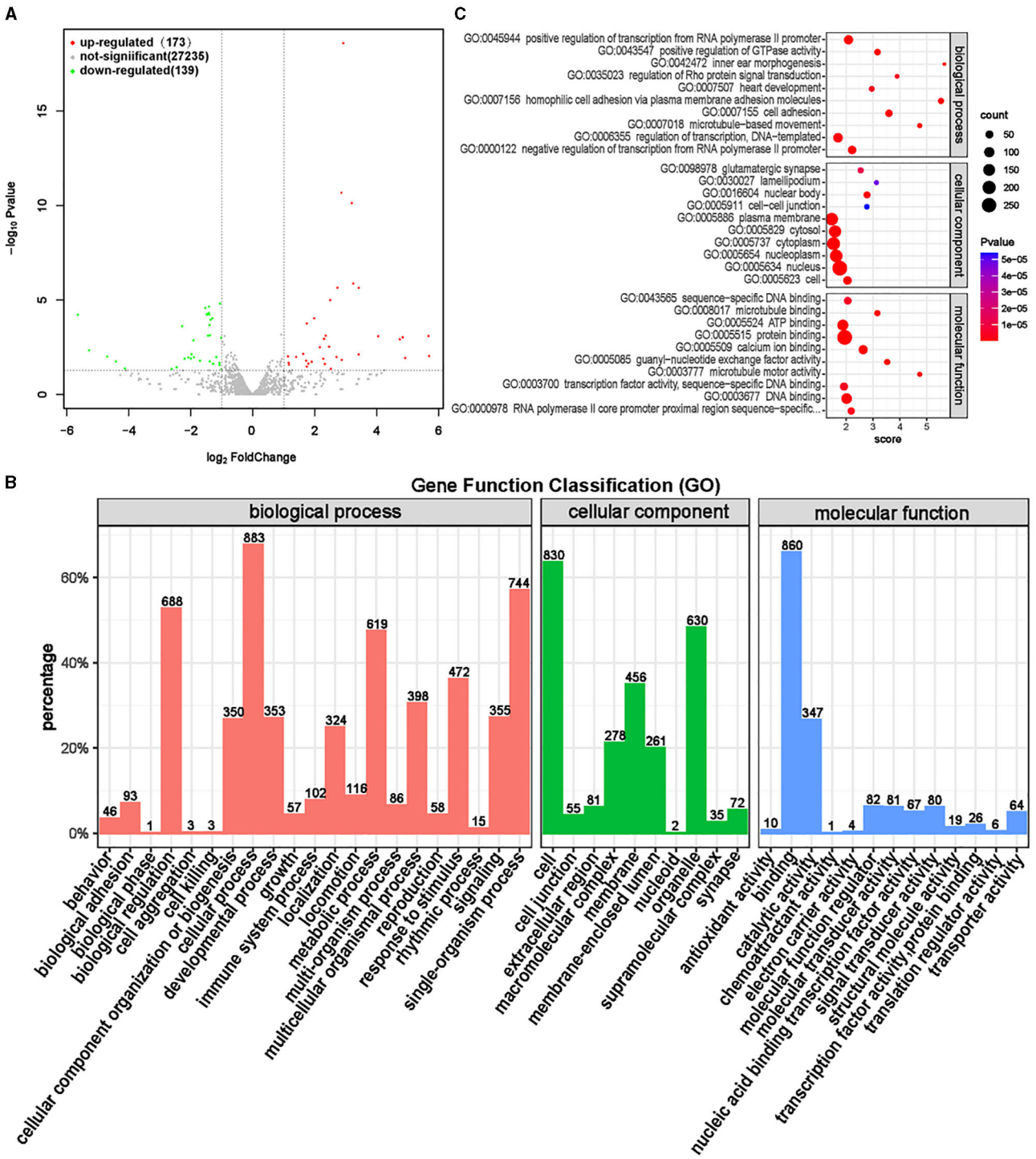

FIGURE 12
Differential expression analysis of miRNA between ISL group and CON group. **(A)** Volcano map of miRNA expression differences between ISL group and CON group. **(B)** ISL group and CON group differentially expressed miRNA GO annotation classification histogram. **(C)** The functional scatter plot shows a significant difference in miRNA enrichment between the ISL group and the CON group, with a top 10 * 3 degree (biological process, cellular component, and molecular function each with a top 10 degree).

diet can help control weight and reduce body fat rate, which may be the reason for the decrease in average daily weight gain in the ISL group. The rumen of ruminant animals utilizes microbial fermentation to degrade crude fibers in feed, and the mucosal muscle layers of the reticulum, omasum, and abomasum contract and relax, providing the power for digestion of crude feed. The more developed the muscle layer, the stronger the gastric digestion ability (42, 43). ISL contains relatively rich cellulose, which can promote gastrointestinal contraction and peristalsis, which may be the reason why the thickness of the reticulum and abomasum

muscle layer in the ISL group is significantly higher than that in the CON group.

The appropriate concentration of ammonia nitrogen is the primary condition to ensure the efficiency of microbial protein synthesis (44). The protein levels in the diet of ISL were higher than those in the basal diet of the CON group, which may be the reason why the ammonia nitrogen levels in the ISL group were significantly higher than those in the CON group. Gastrointestinal microbiota can convert nitrogen sources in feed into bacterial proteins that can be absorbed and utilized by ruminants, playing an important role in animal metabolism and health. Analysis of rumen microbiota showed that compared to the CON group, the relative abundance of Metanobrevibate and Centipeda in the ISL group was significantly upregulated; the relative abundance of Butyrivibrio, Saccharofermentans, Mogibacterium, and Pirelula was significantly reduced. Methanobrevibater is the main hydrogen trophic methanogen in the rumen, which plays an important role in energy metabolism and adipose tissue deposition in animals. The abundance of methanogens is associated with lower body fat formation (45). Compared to forage, high starch diets can reduce rumen pH and inhibit the growth of methanogens, while the addition of ISL reduces the starch content of the diet, which may be the reason for the upregulation of relative abundance of Metanobrevibate (46). Butyrivibrio can induce the production of endotoxin, which can cause inflammatory reaction, metabolic and immune disorders, obesity, insulin resistance, diabetes, and other metabolic diseases, which is consistent with the fact that feeding Isatis leaf reduces the average daily weight gain and fat accumulation of fattening sheep (47, 48). Saccharoferments in the rumen microbiota can promote the production of low-density lipoprotein cholesterol (LDL-C), reduce the relative abundance of Saccharoferments, and lower blood lipids (49). Mogibacterium is usually abundant in the colon mucosa of human colorectal cancer patients, so an increase in its relative abundance may damage the health of goat rumen epithelium (50, 51). Analysis of fecal microbiota showed that compared to the CON group, the relative abundance of Papilibacter, Pseudoflavonifractor, Butyricicoccus, Anaerovorax, and Metanocorpusculum in the ISL group was significantly upregulated; Roseburia, Coprococcus, Clostridium XVIII, Butyrivibrio, Parasutterella, Macellibacteroides, and Porphyromonas were significantly downregulated. The relative abundance of Anaerotruncatus, Butyricoccus, and Papilibacter is positively correlated with the production of lipopolysaccharides (LPS). Butyricoccus can protect the intestinal barrier by producing short chain fatty acids (52); studies have found that high abundance of Parasutterella is associated with the activation of the human fatty acid synthesis pathway. In weight loss intervention trials, the abundance of Parasutterella was significantly reduced, and Parasutterella increased or was a mechanism for weight gain (53). In summary, it was found that the effect of feeding ISL on gastrointestinal microbiota mainly focuses on upregulating the beneficial genera for the body's immune system and downregulating the genera related to obesity. Therefore, ISL may affect growth performance by promoting the health of fattening sheep and reducing fat deposition.

To further explore the mechanism of the effect of adding ISL to the diet on the growth performance of fattening sheep, we conducted metabolomics analysis on rumen fluid, serum, and urine. Among them, 164 differential metabolites in rumen fluid were screened between the ISL and CON groups, with 24 key metabolic pathways; a total of 107 serum differential metabolites were screened, with 25 key metabolic pathways; there were 187 differential metabolites in urine and 42 key metabolic pathways. In the metabolism of rumen fluid, thiamine (vitamin B1) is an important component of co-carboxylase in glucose metabolism, mainly maintaining the normal metabolism of carbohydrates (54); niacin and niacinamide are two important members of the vitamin B family, playing important roles in metabolic processes, especially in fat and sugar metabolism; vitamin B6 is involved in the normal metabolism of sugars, proteins, and fats, and the regulation of these B-group vitamin metabolic pathways can promote the body's fat catabolism (55). In serum metabolism, beta-Alanine is produced through the metabolism of fat and glycogen, and plays an important role in cellular metabolism. In urine metabolism, taurine and taurine are involved in fat metabolism, helping the body utilize fat for energy supply while also regulating the immune system (56–58); Riboflavin (vitamin B2) is involved in energy metabolism in the body and is related to the metabolism of carbohydrates, proteins, nucleic acids, and fats (59). Based on the metabolomics analysis of rumen fluid, serum, and urine, the addition of ISL to the diet mainly regulates energy metabolism related pathways, especially fat metabolism. ISL can increase the consumption of body fat, reduce fat deposition, and thus lead to a decrease in the average daily weight gain of fattening sheep.

In addition, we also conducted transcriptomic analysis of rumen epithelial tissue to understand the changes in genes caused by feeding ISL at the transcriptome level, which helps us to gain a deeper understanding of the impact of ISL on the growth performance of fattening sheep. GO enrichment analysis of circRNA, lncRNA, and miRNA showed that these differential genes play important regulatory roles in normal cellular physiological processes, development, and immune regulation. It is of great significance for improving the physical health of fattening sheep.

The feeding experiment found that although feeding a diet containing ISL reduced the average daily gain of fattening sheep, it also reduced the average daily feed intake of fattening sheep. Based on the above analysis, the reason for the decrease in average daily feed intake of fattening sheep may be due to the rich cellulose content in the leaves (60), which creates a sense of satiety in fattening sheep. At the same time, the bitter taste of the ISL reduces the consumption of fattening sheep, leading to a decrease in feed intake.

Research has found that increasing dietary fiber can reduce fat deposition and improve lean meat percentage in the carcass (61–63); Feeding ISL can promote gastrointestinal health, increase the body's immunity and disease resistance. This study found that fattening sheep fed with a diet supplemented with ISL have a healthier body. The reason for the decrease in average daily weight gain may not only be due to ISL's ability to increase satiety and reduce food intake, but also due to ISL's ability to promote fat metabolism and reduce fat deposition. Although ISL reduces the average daily weight gain of fattening sheep, it can reduce the fat content of fattening sheep meat, which also has important application value for fattening sheep breeding.

5 Conclusion

In summary, the addition of ISL to the diet significantly increased the thickness of the reticulum and abomasum muscle layer in fattening sheep, slowed down the flow rate of feed at the end of the digestive tract, promoted further degradation of coarse feed, significantly increased ammonia nitrogen levels, and improved the composition of rumen microorganisms and intestinal microbiota. Compared to the CON group, the ISL group significantly increased the relative abundance of Methanobrevibacter and Centipeda in the rumen microbiota, while significantly reduced the relative abundance of Butyrivibrio, Saccharofermentans, Mogibacterium, and Pirellula; the relative abundance of intestinal microbiota Papilibacter, Pseudoflavonifractor, Butyricicoccus, Anaerovorax, and Methanocorpusculum was significantly upregulated; the relative abundance of Roseburia, Coprococcus, Clostridium XVIII, Butyrivibrio, Parasutterella, Macellibacteroides, and Porphyromonas significantly decreased. Metabolomics analysis of rumen fluid, serum, and urine showed that differential metabolites and differential metabolic pathways are mainly enriched in regulating energy metabolism, especially fat metabolism, to affect fat deposition and reduce fat rate in fattening sheep. Transcriptome analysis of rumen epithelial tissue revealed that whether circRNA, lncRNA, or miRNA, these differential genes play important regulatory roles in normal cellular physiological processes, development, and immune regulation. These results fully demonstrate that feeding fattening sheep with the addition of ISL diet has a positive effect on body health, improves the gastrointestinal microbiota of fattening sheep, enhances their immunity and resistance, promotes fat metabolism, and reduces fat accumulation in the body. ISL can improve the health level of fattening sheep and reduce fat, which is of great significance for healthy breeding of fattening sheep.

Data availability statement

The original contributions presented in the study are publicly available. This data can be found here: https://www.ncbi.nlm.nih.gov/bioproject; PRJNA1051176, PRJNA1051169, and PRJNA1050748.

Ethics statement

The animal studies were approved by the Animal Protection Committee of Anhui Agricultural University. The studies were conducted in accordance with the local legislation and institutional requirements. Written informed consent was obtained from the owners for the participation of their animals in this study.

Author contributions

ZC: Conceptualization, Data curation, Writing—original draft. MY: Conceptualization, Formal analysis, Investigation, Writing—original draft. JZ: Conceptualization, Investigation, Writing—original draft. ZhZ: Investigation, Writing—original draft. ZL: Investigation, Writing—original draft. CY: Investigation, Writing—original draft. SS: Investigation, Writing—original draft. LW: Investigation, Writing—original draft. YL: Writing—review & editing. ZiZ: Funding acquisition, Writing—review & editing. HC: Funding acquisition, Writing—review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as 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.2024.1332457/full#supplementary-material

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Benzoic acid supplementation improves the growth performance, nutrient digestibility and nitrogen metabolism of weaned lambs

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Nitrogen is one of the essential components of proteins and nucleic acids and plays a crucial role in the growth and development of ruminants. However, the nitrogen utilization rate of ruminants is lower than that of monogastric animals, which not only reduces protein conversion and utilization, but also increases manure nitrogen discharge as well as causing environmental pollution. The lamb stage is an important period in the life of sheep, which can affect the production performance and meat quality of fattening sheep. The purpose of this experiment was to explore effects of benzoic acid supplementation on growth performance, nutrient digestibility, nitrogen metabolism and plasma parameters of weaned lambs. A total of 40 weaned male Hu sheep lambs with similar body weight were randomly divided into 4 groups: control with no benzoic acid (0 BA) and the lambs in other 3 groups were fed 0.5, 1, and 1.5% benzoic acid on the basis of experimental diet (0.5, 1, and 1.5 BA, respectively). The experiment lasted for 60 days. Results showed that the average daily gain of 1 BA group was significantly increased ($p < 0.05$) when compared to 0 and 1.5 BA groups, while an opposite tendency of dry matter intake to average daily gain ratio was observed. The dry matter, organic matter, neutral detergent fiber and acid detergent fiber digestibility of 1 BA group was significantly increased ($p < 0.05$) as compared with 0 and 1.5 BA groups as well as plasma albumin content. Also, the urinary hippuric acid and hippurate nitrogen concentrations in 1 and 1.5 BA groups were higher ($p < 0.05$) than those in 0 and 0.5 BA groups. Additionally, the nitrogen intake in 0.5 and 1 BA groups was significantly increased ($p < 0.05$) when compared to other groups. At 1 h after morning feeding, the plasma benzoic acid concentration of 1 BA group reached up to maximum value and was higher ($p < 0.05$) than other groups, and then began to decrease. Similarly, the hippuric acid concentration in plasma of 1 and 1.5 BA groups was higher ($p < 0.05$) than that of 0 BA group from 1 to 4 h post morning feeding. At 3 h after feeding, the urea nitrogen concentration in plasma of 0 BA group was higher ($p < 0.05$) than that of 1.5 BA group. Overall, the appropriate supplementation of benzoic acid (1%) in the diet can improve growth performance and nitrogen metabolism of weaned lambs.

KEYWORDS

benzoic acid, weaned lambs, hippuric acid, growth performance, nitrogen metabolism

1 Introduction

With the shortage of feed resources and further aggravation of environmental pollution, it is difficult to increase animals' food production by simply expanding the number of breeding heads and ignoring environmental pollution. Technological innovation is one of the main ways to transform animals' production mode and improve animals' output rate and resource utilization rate, which are beneficial for establishment of resource-saving and environment-friendly society in the future (1). A large amount of nitrogen substances excreted by livestock is an important cause of environmental pollution in animal husbandry (2, 3). These nitrogen substances mainly derive from the undigested crude protein and degradation of amino acids in feed (4, 5). Nitrogen is one of the essential components of proteins and nucleic acids and plays a crucial role in the growth and development of ruminants. However, under the current dietary feeding system, the nitrogen utilization rate of ruminants is lower than that of monogastric animals (6). The nitrogen utilization rate of ruminants is approximately 20 to 36%, and the remaining 64 to 80% of nitrogen is excreted in the form of feces and urine (7), which not only reduces protein conversion and utilization, but also increases manure nitrogen discharge (8–10). Lower nitrogen utilization of ruminants severely restricts the economic benefits of livestock farms. Therefore, increasing the efficiency of nitrogen conversion and utilization in ruminants by nutritional strategy is of great significance for ruminants industry as well as reducing the environmental pollution (11).

Hippuric acid (HA), also known as benzoylglycine, is the glycine conjugate of benzoic acid (BA), and it is found in high concentrations in the urine of herbivores (12). For herbivores, including ruminants, polyphenols in plant-based diets can form BA under the joint action of gut microorganisms, which are subsequently absorbed through the gut and transported to the liver for metabolism (13). In animals' liver mitochondria, BA and glycine are catalyzed by enzymes to form HA, which is then excreted in the urine (14). In early research, Doak (15) found that urea nitrogen accounted for 76.4% of urinary nitrogen in wether, and the proportions of allantoin nitrogen and HA nitrogen were 4.1 and 2.6%, respectively. Bristow et al. study analyzed the nitrogen content in the urine of cattle and sheep, and found that 69% of the total nitrogen in bovine urine was in the form of urea, 7.3% in the form of allantoin, and 5.8% in the form of HA. In sheep urine, the nitrogen in the form of urea accounted for 83% of the total nitrogen content, and both HA nitrogen and allantoin nitrogen accounted for 4.3% of total nitrogen (16). Thus, nitrogen excreted by HA can be used as another way of nitrogen excretion in ruminants.

BA is an important precursor in the process of HA formation, and its content directly affects the excretion of HA. As a weakly acidic aromatic acid organic compound, BA is widely used as organic acidifier and preservative in food, medicine and feed industry because of its broad antibacterial effect and strong antibacterial ability (17, 18). BA can improve the growth performance and feed conversion of monogastric animals by promoting the production and activation of gastrointestinal digestive enzymes (19), enhancing gut absorption capacity (20), improving intestinal barrier (21) and regulating intestinal microbiota (22). Nevertheless, little attention has been paid to the effects of BA on urea metabolism, nitrogen metabolism and growth performance of ruminants. Noticeably, both HA synthesis and urea cycle occur in the liver mitochondria of ruminants. Among them, the urea cycle mainly consists of 1 molecule NH_3 and 1 molecule CO_2 catalyzed by carbamyl phosphate synthetase I to produce carbamyl phosphate (23). The NH_4^+ produced by glutamine in mitochondria can be used to synthesize carbamyl phosphate as well as glycine. When the entry of BA into liver cells increases, HA synthesis is elevated, and the consumption of glycine correspondingly increases, thus up-regulating the synthesis of glycine in liver mitochondria. If the amount of NH_4^+ in the mitochondria used to synthesize glycine increases, does the amount used to synthesize carbamyl phosphate decrease, then reducing urea production? However, there is a lack of relevant research at present. Therefore, this study was carried out to evaluate the effects of different levels of BA supplementation on nutrient digestibility, urea metabolism, nitrogen metabolism and growth performance of weaned lambs.

2 Materials and methods

2.1 Ethic statement

All animal care and handing procedures in this study were conducted under the guidance of the Care and Use of Laboratory Animals in China and were approved by (protocol number: 2020022) the Animal Care Committee of Xinjiang Agricultural University (Urumqi, Xinjiang, China).

2.2 Experimental animals and feeding management

The animal experiment was conducted at a commercial sheep farm located at Agricultural Science and Technology Park, Changji, China. A total of 40 healthy male Hu sheep lambs with similar age and body weight (BW, 17.27 ± 1.52 kg) after weaning were used. After marking with ear tags, the lambs were randomly allocated to 4 groups, each with 10 animals. All lambs were fed a same basal diet that was formulated according to the NRC (24). Feed compositions and nutrient levels of experimental diet are presented in Table 1. Lambs in each group were supplemented with 0, 0.5, 1 and 1.5% BA (Purchased from Henan Xizheng Industry Co., China; Purity ≥ 99.5%) in the basal diet, and the treatments were labeled as 0, 0.5, 1, and 1.5 BA groups, respectively. The additive amount of BA was based on the previous studies in beef cattle (25) and grow-finisher pigs (26).

The current study was performed from February to April of 2023. All animals of 4 treatments were reared in 40 pens with 1 lamb in each pen (1 × 1.2 m). The 40 pens were located inside a barn open on two sides and arranged in two rows of 20, separated by the central feeding lane. The pens are enclosed by horizontal metal rail bars, which also delimit the pens at the feeding lane. The floor had a concrete base covered with barley straw bedding, of which one fresh flake (around 1.5 kg) per pen was added over the permanent

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TABLE 1 Feed ingredients and nutrient levels of the diet (DM basis).

Ingredients, %	Content	Nutrient levels[b], %	Content
Corn	11.88	DM	93.04
Wheat bran	4.15	OM	95.61
Soybean meal	6.47	CP	15.03
Stone powder	0.23	EE	4.14
NaCl	0.12	NDF	26.40
Premix[a]	0.23	ADF	12.44
Alfalfa hay	38.46	Ca	1.02
Whole corn silage	38.46	P	0.36
Total	100.00		

DM, dry matter; OM, organic matter; CP, crude protein; EE, ether extract; NDF, neutral detergent fiber; ADF, acid detergent fiber; [a]The Premix provided the following per kg of diets: S (sulphur) 200 mg, Fe (ferrous) 25 mg, Zn (zinc) 40 mg, Cu (cuprum) 8 mg, Mn (manganese) 40 mg, I (iodine) 0.3 mg, Se (selenium) 0.2 mg, Co (cobalt) 0.1 mg, VA 940 IU, VD 111 IU, VE 20 IU; [b]Nutrient levels were measured values.

bedding once a day. The lambs were untethered and did not have any access to a paddock area. BA was fully mixed with the basal diet. Lambs were fed twice daily at 10:00 and 17:00, respectively, allowing 5 to 10% orts, and given free access to drinking clean water. Before feeding trail, the experimental shed was cleaned and sterilized, and parasites were eliminated. A 5-d adaptive phase was followed by 60 days of experimental period.

2.3 Growth performance measurement

Before morning feeding, the BW of all lambs was measured on d 0, 30, and 60, and the average daily gain (ADG) was calculated by initial and final BW. The feed intake was recorded according to the difference of feed offered and refused and converted into dry matter intake (DMI). Feed conversion ratio (F:G) was determined through dividing DMI by ADG.

2.4 Urine, feces and blood samples collection

From d 50 to 55 of the experiment, 6 lambs in each group were randomly selected to collect urine and fecal samples. A self-made urine collection device was used to collect lamb urine samples, and urine was collected every 4 h throughout the day to record the daily urine output of lambs in detail. All the urine of per lamb during the digestion and metabolism experiment were fully shook. The total urine weight was determined by an electronic balance (Deante Sensor Technology Co., Ltd., Tianjin, China), and 10% of the total urine was subsampled and stored in urine sample bottles. Immediately, the pH of urine was determined by a portable pH meter (Ruibin Technology Co., Ltd., Guangzhou, China). Next, the per 100 mL of urine samples were mixed with 10 mL of 10% sulphuric acid for acidification (27) and preserved at−20°C for analysis of BA content and nitrogen metabolism.

In addition, fecal samples were collected in nylon sieve plates placed under the floor of the individual lamb stall. The feces were collected every 4 h throughout the day. After the lamb fecal samples were thoroughly mixed for 6 consecutive days, 10% of the total amount was randomly weighed. Meanwhile, the fresh feed and orts were sampled daily. The fecal samples were mixed per lamb and subsampled. All feed, orts and fecal samples (the 100 g feces were mixed with 10 mL of 10% sulphuric acid) were dried at 65°C in a forced-air oven (Hengmai drying equipment Co., Ltd., Changzhou, China) for 48 h to a constant weight. Then, air-dried samples were ground to pass through a 1-mm sieve (Xulang machinery Equipment Co., Ltd., Guangzhou, China) for measurement of nitrogen metabolism and apparent digestibility.

Before morning feeding (0 h) and 1, 2, 3, and 4 h after morning feeding on d 40, 6 lambs from each treatment were randomly selected to collect blood samples. During each sampling time point, a total of 5 mL blood was sampled from the jugular vein of each lamb using evacuated tubes containing no anticoagulant. Then, blood samples were centrifuged at 3,500 $\times g$ and 4°C for 15 min to collect plasma. The plasma was stored at−20°C for further analysis.

2.5 Urine, feces and blood samples analysis

The feed and fecal samples were analyzed for DM (method 934.01), organic matter (OM, method 942.05), CP (method 990.93), ether extract (EE, method 920.39), Calcium (Ca) and Phosphorus (P) reference to the AOAC procedures (28). In addition, the neutral detergent fiber (NDF) and acid detergent fiber (ADF) contents were determined using an ANKOM fiber analyzer (A2000i, Ankom Technology Corp., Macedon, New York, USA). The chemical composition contents in feed and feces, and DMI and fecal weight were used to calculate the apparent digestibility (29).

Plasma samples collected on d 40 before morning were used to measure contents of biochemical parameters, including glucose (GLU), total bilirubin (T-Bil), direct bilirubin (D-Bil), alanine transaminase (ALT), aspartate transaminase (AST), alkaline phosphatase (ALP), glutamyl transferase (GT), total protein (TP), triglyceride (TG), total cholesterol (TC) and albumin (ALB), with an automatic biochemical analyzer (ZY KHB-1280, Huaren Biotechnology Co., Ltd., Nanjing, China). Plasma samples collected at dynamic points were used for the determination of BA, HA and urea nitrogen. The BA and HA concentrations in plasma and urine were analyzed by high-performance liquid chromatography (HPLC) following the procedures of Kubota et al. (30). Briefly, 100 μL of plasma (200 μL of urine after 10-fold dilution) was transferred to another centrifuge tube, and 200 μL (400 μL of urine) acetonitrile precipitated protein containing o-chlorobenzoic acid as the internal target was added. The samples were swirled and mixed for 20 s, and centrifuged at 9,500 $\times g$ for 1 min. Subsequently, 10 μL of mixture was collected and measured using liquid chromatograph. Determination conditions: IC YS-50 weakly acidic cation exchange column 4.6 × 125 mm was used; flow rate 1.0 mL/min; column temperature 30°C; detection wavelength 235 nm; a linear elution; sample size was 10 μL. In addition, urea nitrogen was determined using commercial kit (NO.RATA-A 7170 Huaying, Beijing, China) reference to the instructions.

2.6 Statistical analysis

All data were analyzed with one-way ANOVA procedure of the SPSS statistical software (version 22.0 for Windows; SPSS, Chicago, USA), with each lamb as an experimental unit. Orthogonal polynomial contrasts were completed to detect the linear and quadratic effects of benzoic acid levels. Duncan test was conducted to determine the differences among four treatments. Data were presented as mean and standard error of mean. The significance level was indicated at $p \leq 0.05$, and a trend was declared at $0.05 < p \leq 0.10$. Besides, the dynamic changes of BA, HA and urea nitrogen were used to draw broken line graphs using GraphPad Prism software (version 8.0 for Windows; GraphPad Prism, San Diego, USA).

3 Results

3.1 Growth performance of weaned lambs

As shown in Table 2, the initial BW and BW on d 30 did not show significant difference ($p > 0.05$) among all groups. However, the final BW showed quadratic variation tendency ($p = 0.006$), and the 1 BA group had the highest value. The DMI and ADG were similar ($p > 0.05$) among all groups from d 1 to 30, and F:G was significantly lower in the 1BA group as compared to the 0 BA group ($p < 0.05$). From d 31 to 60, the DMI and ADG of 1 BA group were higher ($p < 0.05$) than those of 0 BA and 1.5 BA groups, whereas F:G displayed an opposite trend. No significant difference ($p > 0.05$) of DMI was observed among all group during the whole experimental period. Compared with 0 BA and 1.5 BA groups, the ADG in 1 BA group was significantly elevated ($p < 0.05$) from d 1 to 60. The F:G of 1 BA group had minimum value and lower ($p < 0.05$) than 0 and 1.5 BA groups.

3.2 Nutrients digestibility of weaned lambs

Obviously, the apparent digestibility of CP, EE, Ca and P was similar ($p > 0.05$) among four groups. The DM, OM and ADF digestibility of 1 BA group were higher ($p < 0.05$) than those of 0 BA group (Table 3). Compared with other groups, the NDF digestibility in 1 BA group was significantly increased ($p < 0.05$).

3.3 Urinary pH and nitrogen excretion of weaned lambs

No obvious difference ($p > 0.05$) of urinary pH, output, total nitrogen excretion and other ingredients nitrogen excretion was found among four groups (Table 4). The HA and hippurate nitrogen in 1 and 1.5 BA groups were higher ($p < 0.05$) than those in 0 and 0.5 BA groups. Likewise, compared with 0 BA group, the HA excretion and hippurate nitrogen excretion in BA treatments were significantly increased ($p < 0.05$). An opposite trend of urea nitrogen was observed between 0 BA group and BA treatments. However, the urea nitrogen excretion was similar ($p > 0.05$) among all groups. In addition, total nitrogen and other ingredients nitrogen in urine of 1.5 BA group were higher ($p < 0.05$) than that of 0 and 0.5 BA groups.

3.4 Correlation analysis of hippurate nitrogen and urea nitrogen in weaned lambs

As can be seen in Figure 1, the HA nitrogen content in the urine of lambs was significantly negatively correlated with the urea nitrogen content ($p = 0.0052$), and the amount of HA nitrogen excreted was significantly positively correlated with the amount of urea nitrogen excreted ($p = 0.0242$).

3.5 Nitrogen metabolism of weaned lambs

There was no significant difference ($p > 0.05$) of fecal nitrogen, urinary nitrogen and nitrogen retention among all groups (Table 5). Nevertheless, the nitrogen intake in 0.5 and 1 BA groups was significantly enhanced ($p < 0.05$) when compared to 0 and 1.5 BA groups.

3.6 Blood biochemical parameters of weaned lambs

The concentrations of GLU, T-Bil, D-Bil, ALT, AST, ALP, GT, TP, TG and TC in plasma were similar ($p > 0.05$) among four groups (Table 6). Compared with 0 BA and 1.5 BA groups, the plasma ALB content of 1 BA group was significantly increased ($p < 0.05$).

3.7 Dynamic changes of benzoic acid, hippuric acid and urea nitrogen in plasma of weaned lambs

As shown in Figure 2A, at 1 h after morning feeding, the plasma BA concentration of 1 BA group reached up to maximum value and was higher ($p < 0.05$) than other groups, and then began to decrease. At 1 post feeding, the concentration of BA in 1.5 BA group was significantly increased ($p < 0.05$) as compared with 0 and 0.5 BA groups. During the whole sampling process, the BA content in 0 and 0.5 BA groups was always at a lower level. Similarly, the HA concentration in plasma of 1 and 1.5 BA groups was higher ($p < 0.05$) than that of 0 BA group from 1 to 4 h post morning feeding (Figure 2B). After lambs supplemented with BA, the plasma HA concentration gradually increased and reached the highest value at 1 h post feeding and then began to display a fluctuation change. The HA concentration of 0, 0.5, and 1.5 BA groups at 4 h was close to corresponding 0 h. On the contrary, at 3 h after feeding, the urea nitrogen concentration in plasma of 0 BA group was higher ($p < 0.05$) than that of 1.5 BA group (Figure 2C). No obvious difference ($p > 0.05$) of urea nitrogen concentration was observed at other time points among all groups.

TABLE 2 Effects of benzoic acid supplementation on dry matter intake and average daily gain of weaned lambs.

Items	Groups				SEM	*P*-value		
	0 BA	0.5 BA	1 BA	1.5 BA		Treatment	Linear	Quadratic
Body weight, kg								
Initial BW	17.24	17.26	17.33	17.27	0.24	0.999	0.947	0.934
d 30 BW	21.05	21.85	22.70	21.01	0.41	0.428	0.840	0.138
Final BW	26.84^{b}	29.32^{ab}	30.93^{a}	27.02^{b}	0.59	0.034	0.662	0.006
Day 1 to 30								
DMI, g/d	601.80	599.29	598.35	579.71	10.99	0.890	0.500	0.717
ADG, g/d	127.10	153.00	179.17	125.00	9.87	0.172	0.818	0.044
F:G	4.73^{a}	3.92^{ab}	3.34^{b}	4.64^{a}	0.13	0.083	0.672	0.004
Day 31 to 60								
DMI, g/d	834.35^{b}	861.27^{ab}	873.85^{a}	828.12^{b}	6.64	0.043	0.917	0.006
ADG, g/d	192.83^{b}	248.83^{a}	274.33^{a}	200.00^{b}	9.55	0.003	0.525	<0.001
F:G	4.33^{a}	3.46^{ab}	3.19^{b}	4.14^{a}	0.15	0.031	0.342	0.002
Day 1 to 60								
DMI, g/d	718.07	730.28	736.10	703.02	10.42	0.710	0.656	0.320
ADG, g/d	159.97^{b}	200.92^{ab}	226.75^{a}	162.50^{b}	8.52	0.009	0.624	0.001
F:G	4.49^{a}	3.63^{ab}	3.25^{b}	4.33^{a}	0.12	0.055	0.024	0.008

BA, benzoic acid; BW, body weight; DMI, dry matter intake; ADG, average daily gain; SEM, standard error of mean. 0 BA, control with no BA (Henan, China); 0.5 BA, supplementation of 0.5% BA on the basis of experimental diet; 1 BA, supplementation of 1% BA on the basis of experimental diet; 1.5 BA, supplementation of 1.5% BA on the basis of experimental diet. In the same row, values with different superscripts differ significantly ($p < 0.05$). F:G, DMI/ADG.

TABLE 3 Effects of benzoic acid supplementation on nutrients digestibility of weaned lambs.

Items	Groups				SEM	*P*-value		
	0 BA	0.5 BA	1 BA	1.5 BA		Treatment	Linear	Quadratic
DM	64.19^{b}	67.41^{a}	70.12^{a}	65.83^{ab}	0.71	0.011	0.143	0.004
OM	68.55^{c}	72.12^{ab}	73.84^{a}	70.37^{bc}	0.64	0.009	0.120	0.002
CP	59.09	61.28	65.42	64.03	1.10	0.174	0.057	0.398
NDF	64.77^{b}	65.56^{b}	69.55^{a}	64.49^{b}	0.73	0.033	0.568	0.029
ADF	60.15^{c}	64.69^{ab}	66.49^{a}	62.79^{bc}	0.68	0.001	0.029	<0.001
EE	73.99	79.49	81.03	75.03	1.11	0.052	0.592	0.008
Ca	44.80	46.59	51.99	47.36	1.25	0.215	0.236	0.196
P	39.16	43.86	47.83	41.29	1.57	0.249	0.455	0.082

BA, benzoic acid; DM, dry matter; OM, organic matter; CP, crude protein; NDF, neutral detergent fiber; ADF, acid detergent fiber; EE, ether extract; SEM, standard error of mean. 0 BA, control with no BA (Henan, China); 0.5 BA, supplementation of 0.5% BA on the basis of experimental diet; 1 BA, supplementation of 1% BA on the basis of experimental diet; 1.5 BA, supplementation of 1.5% BA on the basis of experimental diet. In the same row, values with different superscripts differ significantly ($p < 0.05$).

4 Discussion

Lamb nutrition is an increasingly important issue in today's sheep production. The feeding management of lambs has long-term influence on future production performance of meat quality of fattening sheep (31). Due to the immature gastrointestinal tracts, the lambs are easily affected by harmful microorganisms. After weaning, lambs undergo the changes of feed type and rearing pattern, resulting in decreased nutrient digestibility and growth rate (32). Relieving the weaning stress of lambs is of great significance for improving the healthy growth of lambs. As an aromatic carboxylic acid, BA has multiple health benefits, including antibacterial and anti-inflammatory activities (18). Previously, a research has found that dietary supplementation of BA could increase ADG of weanling pig, but had no obvious difference of feed efficiency (33). In the current study, 1% BA supplementation significantly increased ADG and feed efficiency when compared to 0 BA and 1.5 BA groups. The different reason may be that the animals used in experiment was different. BA can produce esterification products with bacteria, which then affect the metabolism of pathogenic bacteria. Besides, BA can interfere with the DNA activity of pathogenic bacteria, thereby decreasing their

TABLE 4 Effects of supplementing different levels of benzoic acid on urinary pH and nitrogen excretion in weaned lambs.

Items	Groups				SEM	*P*-value		
	0 BA	0.5 BA	1 BA	1.5 BA		Treatment	Linear	Quadratic
Urinary pH	6.65	6.58	6.54	6.52	0.02	0.174	0.003	0.346
Urinary output, mL	460.83	487.27	480.40	451.77	39.71	0.138	0.008	0.105
Hippuric acid, mg/mL	6.59^{c}	20.95^{b}	27.61^{a}	28.20^{a}	1.86	<0.001	<0.001	<0.001
Hippuric acid excretion, g/d	2.84^{b}	9.89^{a}	13.29^{a}	12.60^{a}	1.26	0.005	0.001	0.064
Hippurate nitrogen, g/L	0.51^{c}	1.64^{b}	2.16^{a}	2.20^{a}	0.15	<0.001	<0.001	<0.001
Hippurate nitrogen excretion, g/d	0.22^{b}	0.77^{a}	1.04^{a}	0.98^{a}	0.10	0.005	0.001	0.064
Urea nitrogen, g/L	5.73^{a}	4.87^{b}	4.75^{b}	4.68^{b}	0.23	0.041	0.013	0.155
Urea nitrogen excretion, g/d	2.68	2.44	2.28	1.99	0.15	0.741	0.278	0.959
Other ingredients nitrogen, g/L	1.63^{b}	1.56^{b}	3.12^{ab}	4.55^{a}	0.46	0.057	0.011	0.374
Other ingredients nitrogen excretion, g/d	0.66	0.72	1.42	1.59	0.17	0.108	0.022	0.856
Total nitrogen in urine, g/L	7.88^{b}	8.07^{b}	10.00^{ab}	11.44^{a}	0.54	0.048	0.008	0.521
Urinary total nitrogen excretion, g/d	3.56	3.93	4.74	4.57	0.37	0.671	0.271	0.728

BA, benzoic acid; SEM, standard error of mean. 0 BA, control with no BA (Henan, China); 0.5 BA, supplementation of 0.5% BA on the basis of experimental diet; 1 BA, supplementation of 1% BA on the basis of experimental diet; 1.5 BA, supplementation of 1.5% BA on the basis of experimental diet. In the same row, values with different superscripts differ significantly ($p < 0.05$).

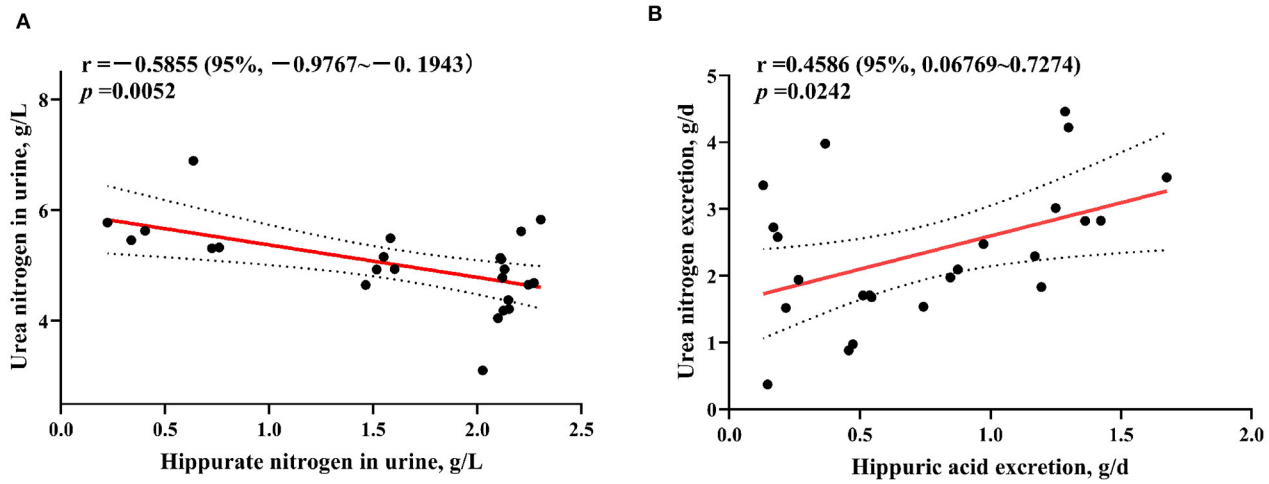

FIGURE 1
Analysis of the content of hippurate nitrogen and urea nitrogen in the urine of weaned lambs **(A)** and the correlation between hippurate nitrogen and urea nitrogen excretion **(B)**.

growth (34). These effects are conducive to reducing the incidence rate of diarrhea and relieving the negative impact on growth rate of lambs caused by weaning stress. Our results showed that appropriate addition (1%) of BA improved growth performance of weaned lambs. The improved growth performance of animals is usually associated with higher apparent digestibility and nitrogen metabolism. Hence, we conducted the following experiment to study the influence of BA on apparent digestibility and nitrogen metabolism of weaned lambs.

The gastrointestinal tracts of weaned lambs have insufficient digestive enzyme secretion, thus the nutrients digestibility of lambs are low (35). In our study, 0.5 and 1% dietary supplementation of BA significantly increased the nutrients intake of lambs. In general, the typical feeds fed with young ruminants have relatively high values of acid binding capacity, which affect the nutrients intake of animals (36). A previous study found that dietary supplementation with BA could decrease acid binding capacity of feeds (37), which might explain the positive effects of BA on nutrients intake. The digestibility of DM and OM are key parameters to reflect the utilization ability of feed by animals (38). In the current study, the DM and OM digestibility in 1 BA group showed highest values and were significantly elevated as compared with 0 BA group, indicating that the lambs given to 1% BA could obtain more nutrients and accelerate growth, which were matched to ADG results. Organic

TABLE 5 Effects of supplementing different levels of benzoic acid on nitrogen metabolism in weaned lambs (g/d).

Items	Groups				SEM	*P*-value		
	0 BA	0.5 BA	1 BA	1.5 BA		Treatment	Linear	Quadratic
Nitrogen intake	18.75^{b}	19.80^{a}	20.35^{a}	18.69^{b}	0.20	0.001	0.746	<0.001
Fecal nitrogen	7.63	7.65	7.04	6.72	0.18	0.187	0.054	0.626
Urinary nitrogen	3.56	3.93	4.74	4.57	0.37	0.671	0.271	0.728
Nitrogen retention	7.56	8.21	8.57	7.41	0.40	0.257	0.626	0.118

BA, benzoic acid; SEM, standard error of mean. 0 BA, control with no BA (Henan, China); 0.5 BA, supplementation of 0.5% BA on the basis of experimental diet; 1 BA, supplementation of 1% BA on the basis of experimental diet; 1.5 BA, supplementation of 1.5% BA on the basis of experimental diet. In the same row, values with different superscripts differ significantly ($p < 0.05$).

TABLE 6 Effects of supplementing different levels of benzoic acid on blood biochemical parameters in weaned lambs.

Items	Groups				SEM	*P*-value		
	0 BA	0.5 BA	1 BA	1.5 BA		Treatment	Linear	Quadratic
GLU, mmol/L	5.17	4.90	5.40	5.29	0.12	0.536	0.451	0.768
T-Bil, μmol/L	3.46	3.26	3.30	3.18	0.09	0.725	0.329	0.808
D-Bil, μmol/L	1.15	1.18	1.16	1.16	0.03	0.990	0.964	0.850
ALT, U/L	15.53	13.77	15.87	13.97	0.91	0.817	0.765	0.973
AST, U/L	87.50	105.97	98.22	104.22	3.87	0.339	0.229	0.424
ALP, U/L	326.45	236.85	293.27	214.87	21.90	0.260	0.159	0.897
GT, U/L	71.83	69.13	75.22	66.75	2.44	0.673	0.689	0.574
TP, g/L	49.12	52.18	49.95	54.58	0.94	0.159	0.089	0.663
TG, mmol/L	0.36	0.34	0.37	0.37	0.02	0.956	0.845	0.750
TC, mmol/L	1.21	1.22	1.36	1.28	0.03	0.295	0.199	0.472
ALB, g/L	23.23^{b}	24.22^{ab}	25.68^{a}	22.87^{b}	0.40	0.044	0.614	0.204

BA, benzoic acid; GLU, glucose; T-Bil, total bilirubin; D-Bil, direct bilirubin; ALT, alanine transaminase; AST, aspartate transaminase; ALP, alkaline phosphatase; GT, glutamyl transferase; TP, total protein; TG, triglyceride; TC, total cholesterol; ALB, albumin; SEM, standard error of mean. 0 BA, control with no BA (Henan, China); 0.5 BA, supplementation of 0.5% BA on the basis of experimental diet; 1 BA, supplementation of 1% BA on the basis of experimental diet; 1.5 BA, supplementation of 1.5% BA on the basis of experimental diet. In the same row, values with different superscripts differ significantly ($p < 0.05$).

acid is an effective alternative to enhance nutrient digestibility in animals industry production. The organic acid has multifunctional effects, including reduction of gastrointestinal pH, enhancement of gastrointestinal retention time, stimulation of pancreatic secretions and promotion of gastrointestinal morphology, thus improving nutrient digestibility (39, 40). Our results might relate to the ability of BA to regulate the gastrointestinal pH and digestive enzyme levels and improve gut morphology. In the future, the effects of BA on gastrointestinal development of lambs need in-depth investigation. In addition, we also found that the NDF and ADF digestibility of 1 BA group were higher than 0 and 1.5 BA groups. In ruminants, the microbial community in the rumen is responsible for crude fiber utilization. Unfortunately, the effect of BA on ruminal microbial community has not been reported. A recent study verified that dietary supplementation of BA could stabilize the microbiota fluctuation caused by weaning stress and speed up rapid colonization of dominant bacteria in the gut of piglets (41). Even though the microbial community between monogastric animals and ruminants existed difference, these results indicated that BA could affect the microbial community. We speculated that the positive effects of BA on NDF and ADF digestibility might be associated with the regulation of microbiota in the gastrointestinal tracts. Lastly, the activity of digestive enzyme plays an important role in the nutrients digestion (42). Therefore, more experiments are needed to explore the effects of BA on microbial community and digestive enzyme activity of weaned lambs.

Previously, studies have reported that with the elevation of dietary BA supplementation, the urinary pH was linearly reduced (43), and besides, the HA concentration in urine was linearly increased (44, 45), which also displayed similar findings in our research. The reason for reduced urinary pH may be due to the elevation of urinary HA content. After absorption, the BA is conjugated with glycine through glycine-N-acylase, and then transformed into HA in the liver (46). Moreover, the urea nitrogen concentration in 1% BA supplementation treatments was significantly decreased (3 h), suggesting that the nitrogen conversion of 0 BA lambs was low. As mentioned earlier, the HA synthesis and urea cycle occur in the hepatic mitochondria of ruminants, and together consume NH_4^+ produced from dietary amino acid metabolism (47). Thus, when BA enters the animal's body, does the increase of HA synthesis have the effect of reducing urea production? In the current study, results showed that with the increase of BA intake, the urinary HA content and HA excretion increased linearly, while urinary urea nitrogen content decreased linearly. At the same time, it was found that there was a significant correlation between urinary urea nitrogen excretion and HA

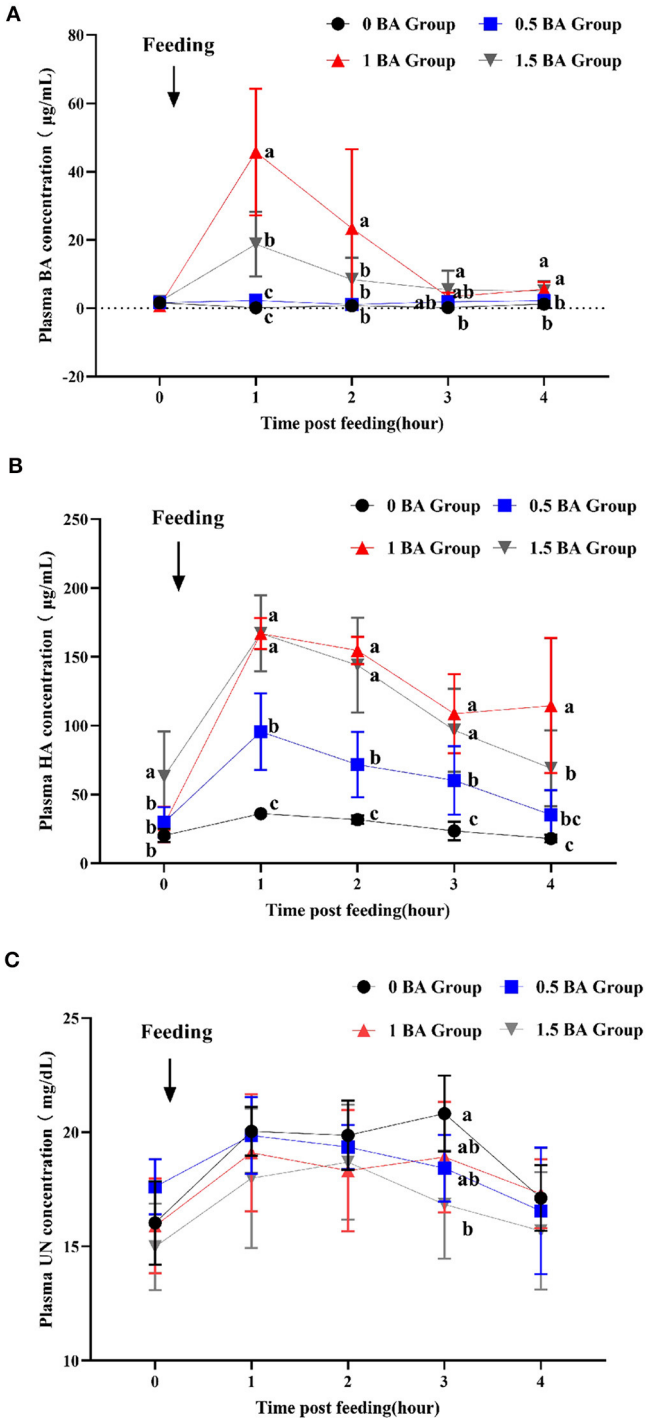

FIGURE 2
Effects of supplementing different levels of benzoic acid on dynamic changes of benzoic acid **(A)**, hippuric acid **(B)**, and urea nitrogen **(C)** in plasma in weaned lambs. BA, benzoic acid; HA, hippuric acid; UN, urea nitrogen. 0 BA, control with no BA (Henan, China); 0.5 BA, supplementation of 0.5% BA on the basis of experimental diet; 1 BA, supplementation of 1% BA on the basis of experimental diet; 1.5 BA, supplementation of 1.5% BA on the basis of experimental diet. Different letters (a, b, and c) represent statistically significant differences ($P < 0.05$).

nitrogen excretion. This result confirmed our previous hypothesis that the elevation HA excretion by adding BA to the diet reduced urea nitrogen excretion. However, there were unsatisfactory results in terms of total urinary nitrogen output. In addition to hippuric acid nitrogen and urea nitrogen, there are also nitrogen produced by purine derivatives in urinary total nitrogen. In previous studies of crossbred Bulls, it was found that nitrogen produced by purine derivatives (such as allantoin nitrogen) increased linearly

as feed intake increased (48). In our experiment, other component nitrogen excretion of weaned lambs increased significantly, and the unsatisfactory total urinary nitrogen excretion might be due to the higher feed intake of 0.5 and 1% BA lambs. In addition, according to our unpublished data, after feeding BA to lactating lambs, metabolites of jejunum contents were mainly enriched in pyrimidine and purine metabolism pathways, indicating that BA intake also affected the metabolism of purine derivatives in lambs. A previous study in rabbits found that intake of sodium benzoate led to an increase in urinary HA excretion and a decrease in urinary urea nitrogen excretion (49), which were consistent with the results of our experiment. Nousiainen et al. (50) reported that increased content of urinary and blood urea nitrogen, indicated a reduced nitrogen efficiency of cows. The 0.5 and 1 BA groups showed higher nitrogen intake when compared to 0 and 1.5 BA groups. According to our results, appropriate supplementation of BA in the diet could improve nitrogen utilization of weaned lambs, which was helpful for promoting growth.

Recently, a study reported that the improvement of ADG was attributed to the elevated nitrogen retention in pigs fed diets with BA (51). In the current study, we did not find obvious difference of nitrogen retention among all groups. However, the 1 BA group had highest value of nitrogen retention. A previous study found that dietary supplementation of BA could improve the intestinal morphology and up-regulate the expression of glucagon-like peptide 2 gene in the intestinal mucous (37), which might enhance nitrogen retention in animals fed ration with BA. We also found that the addition of 1.5% BA to the basal ration decreased nitrogen intake and reduced nitrogen utilization in lambs, thus negatively affecting growth performance. The possible reason might be related to elevation of glycine synthesis required to transform BA into HA in the liver, which might affect the amino acid metabolism and reduce amino acid concentration for synthesis of protein (46). Excessive utilization of protein and amino acid for transformation of HA reduces nitrogen retention. Thus, according to our results, the appropriate supplementation level of BA in the diet of lambs was 1%.

As important indexes related to health condition of animals, the changes of serum biochemistry can be utilized to estimate the body's physiological metabolism and organ functions (52). The T-Bil, D-Bil, ALT, AST, ALP and GT contents in plasma can be used to evaluate the liver function, and the changes of GLU, TG and TC concentrations are associated with fat metabolism. Furthermore, the plasma concentrations of TP and ALB are key parameters of protein metabolism (52). In the present experiment, the plasma contents of GLU, T-Bil, D-Bil, ALT, AST, ALP, GT, TP, TG and TC were similar among four treatments, suggesting that BA supplementation did not have adverse effects on lipid metabolism and hepatic function of weaned lambs. A previous study found that BA supplementation significantly increased the contents of ALB in blood, and reduced the urea nitrogen content (53). Consistent with previous study, our study found that 1% BA supplementation increased plasma ALB content and reduced urea nitrogen concentration, indicating that BA could improve the anabolism of protein to a certain degree, which was conducive to promoting growth of lambs.

Previous studies have verified that the HA is formed by BA biosynthesis reaction and can be excreted from the body's urine within 4 h (54, 55). We studied the dynamic changes of BA, HA and urea nitrogen in plasma of weaned lambs fed BA for the first time. Results showed that after feeding BA 1 h, the plasma concentrations of BA and HA reached up to highest value and then decreased gradually, and the 1 and 1.5 BA groups were higher than other groups. BA is absorbed through the animal's intestine and enters the bloodstream, where it is then transported to the liver for metabolism (13). Our findings indicated that after BA transported to the liver, the process of BA and glycine producing HA under the catalysis of enzyme was very rapid. In animals, BA and glycine are catalyzed by enzymes to form HA, which is then excreted in the urine. In addition, BA can also be metabolized by gut microbes (56, 57). The microbial community of lambs were still in developmental stage, and higher levels of BA and HA may have positive effects on microbial community. However, the potential mechanism of action still needs exploration. As the main products of protein metabolism of animals' body, urea nitrogen are important indexes to reflect protein utilization. The decrease of these contents indicates that the protein utilization is improved, which contributes to nitrogen deposition, and besides, high urea nitrogen content can also have adverse effects on animal health (58). In this study, dietary supplementation of BA effectively decreased plasma urea nitrogen content after morning feed from 2 to 3 h, which was in line with urinary urea nitrogen content mentioned earlier. This result was conducive to improving nitrogen metabolism of lambs. Future research should be paid more attention to the potential mechanisms of BA on the nitrogen metabolism of lambs.

Taken together, BA supplementation had the ability to improve nitrogen metabolism of waned lambs, which was conducive to promoting growth of lambs. However, some limitations need to be acknowledged in the current study. First of all, the absorption pathway of BA in the gastrointestinal tracts requires further elucidation. In the subsequent experiment, we will explore the potential mechanism of action using *in vitro* and *in vivo* studies. In addition, future studies to understand the functions of microbial community in the gastrointestinal tracts using metagenomics or culturomics as well as host function using transcriptomics are needed to provide more information on the role of microorganism in the digestive organ of weaned lambs and their response to dietary BA supplementation.

5 Conclusions

Supplementation of BA (1%) improved the ADG, feed efficiency and DM, OM, NDF and ADF digestibility of weaned lambs. In addition, dietary supplementation with 1% BA significantly increased urinary HA contents and excretion as well as nitrogen intake. After supplementation with BA, the plasma and urinary urea nitrogen contents were reduced. Thus, based on our findings, the appropriate supplementation of BA (1%) in the diet improves the growth performance and nitrogen metabolism of weaned lambs.

Data availability statement

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

Ethics statement

All procedures involving animal care and management used in this experiment were authorized (protocol number: 2020022) by the Institutional Animal Care and Use Committee of Xinjiang Agricultural University (Urumqi, Xinjiang, China). The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

WZ: Conceptualization, Investigation, Visualization, Writing – original draft, Writing – review & editing. SS: Investigation, Visualization, Writing – review & editing. YaqZ: Investigation, Writing – review & editing. YanZ: Writing – original draft. JW: Investigation, Writing – review & editing. ZL: Investigation, Writing – review & editing. KY: Conceptualization, Data curation, Funding acquisition, Methodology, Project administration, Supervision, Writing – review & editing.

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Conflict of interest

JW and ZL were employed by Xinjiang Shangpin Meiyang 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

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Effect of tea polyphenols on intestinal barrier and immune function in weaned lambs

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Introduction: The purpose of this study was to explore the effects of tea polyphenols on growth performance, cytokine content, intestinal antioxidant status and intestinal barrier function of lambs, in order to provide reference for intestinal health of ruminants.

Methods: Thirty weaned lambs (average initial weight 9.32 ± 1.72 kg) were randomly divided into five groups with six lambs in each group. The control group did not add anything but the basic diet mainly composed of Pennisetum and Corn, and the other four groups added 2, 4, 6g/kg tea polyphenols and 50mg/kg chlortetracycline to the basic diet, respectively. The experiment lasted for 42 days.

Results: Dietary tea polyphenols improved the growth and stress response and reduced intestinal permeability of lambs ($p > 0.05$), while CTC did not affect the final lamb weight ($p > 0.05$). Both tea phenols and CTC significantly reduced inflammatory factors and enhanced the immune system ($p > 0.05$). Dietary tea polyphenols increased villus height, villus height/crypt depth, secretory immunoglobulin A ($p > 0.05$), and antioxidant enzymes, while decreasing MDA and apoptosis in the intestine ($p > 0.05$). However, compared with other groups, the content of T-AOC in jejunum did not change significantly ($p > 0.05$). Tea polyphenols also increased claudin-1 levels in the duodenum, jejunum, and ileum more than CTC ($p > 0.05$). CTC had a limited effect on the mRNA expression of *Occludin* and *ZO-1*, while tea polyphenols increased these in both the duodenum and ileum ($p > 0.05$).

Conclusion: This study demonstrated that tea polyphenols can effectively improve the intestinal barrier of weaned lambs, and that they have anti-inflammatory and antioxidant effects similar to those of antibiotics. Thus, tea polyphenols could be used to replace antibiotics in ensuring safety of livestock products and in achieving the sustainable development of modern animal husbandry.

KEYWORDS

tea polyphenol, weaned lamb, intestinal barrier, antioxidant capacity, immune function

1 Introduction

The intestine, a critical organ for nutrient digestion and absorption, is also the largest immune organ in the body. Therefore, effective gastrointestinal function is very important for animal health, growth and production performance (1). However, when lambs are weaned, incomplete intestinal development and changes in the diet structure produce weaning stress.

It leads to atrophy of the intestinal villi or deepening of crypts, resulting in a sudden drop in the capacity of young animals to digest and absorb nutrients (2). In addition, some studies have shown that weaning may promote oxidative stress, which damages the intestinal barrier (3), and can lead to intestinal cell apoptosis, intestinal immune function imbalance (4, 5), diarrhea, and weakened disease resistance (6). Therefore, alleviating the negative effects of weaning stressors has become an urgent challenge for the development of healthy animals. Antibiotics have been widely used to alleviate weaning stress (7); however, their drug residues and the development of resistance have led to a global ban on feeding large quantities of antibiotics. Therefore, researchers are exploring the use of natural plant extracts with anti-inflammatory and antioxidant properties as alternatives to antibiotics for science-based farming.

Tea, the world's second most popular beverage after water, has seen a steady annual growth rate of approximately 4.4% over 10 years. China, as the largest producer and consumer of tea, is a rich source of tea polyphenols (8). According to its chemical structure, tea polyphenols can be divided into catechins, flavonoids, flavonols, phenolic acids and peptides, and anthocyanins, which are the general names of the polyphenols in tea. In addition, there are some other small amounts of polyphenols, such as epigallocatechin gallate, flavonoid glycosides and tannins (9). It is reported that tea polyphenols can inhibit inflammatory mediators such as cytokines, oxygen free radicals and histamine, thus contributing to the healthy growth of animals. In addition, it also has the function of stimulating signal pathways such as Nrf2, Akt and NF-κB to reduce inflammatory damage. As direct antioxidants and effective free radical scavengers, tea polyphenols can enhance the intestinal barrier function and improve the antioxidant capacity of animals, which is attributed to their phenolic hydroxyl groups (10). As a natural plant, tea polyphenols have also been proved to have the ability to regulate intestinal microbial diversity, promote intestinal health and prevent chronic metabolic diseases. In addition, many studies show that tea polyphenols have preventive effects on cardiovascular diseases, cancer, obesity, diabetes and allergic diseases (11).

Wei et al. (12) found that mice administered cyclophosphamide can recover their original level of intestinal tight junction protein and improve their antioxidant enzyme activity after being fed catechins. Studies have found that the benefits of adding green tea extract to broiler diet include increasing weight gain, improving feed conversion rate, reducing low-density lipoprotein and low-density lipoprotein cholesterol and reducing pathogenic bacteria in cecum, which is similar to the results of adding green tea by-products to pigs to improve growth performance (13, 14). In addition, tea polyphenols in monogastric animals can significantly reduce intestinal inflammation and oxidative stress, improve intestinal structure, protect intestinal mucosal health, and prevent intestinal diseases to some extent (15). However, there are few applications of tea polyphenols in ruminants; therefore, this study aimed to evaluate the effects of tea polyphenols on the intestinal morphology, intestinal barrier function, antioxidant index, immune index, and intestinal cell apoptosis of weaned lambs. Lambs were chosen as the object of study because intensive goat production is an important and expanding enterprise in China, and lambs are very susceptible to diseases. These data are necessary before considering the use of tea polyphenols as immunomodulatory interventions in clinical practice, as well as before replacing antibiotics with plant polyphenols in livestock production systems.

2 Materials and methods

The experimental protocol applied in this study followed the guidelines of the Animal Care and Use Committee of Guangdong Ocean University.

2.1 Lambs and experimental protocol

The tea polyphenols used in this experiment were provided by Xi'an Best Biotechnology Co., Ltd. (Shaanxi, China). Tea polyphenols were acquired in the form of brown powders with a special smell, in which the content of tea polyphenols is 98.1%, that of catechin is 86.6%, and that of epigallocatechin gallate is 54.2%.

Thirty healthy Leizhou black goats (about 2 months old) were selected as experimental animals, with an average weight of 9.32 ± 1.72 kg. All lambs were weaned at 2 months old, and 30 lambs were divided into 5 treatment groups with 6 replicates in each group. The experimental period was 42 days and the pre-feeding period was 7 days. The control group was fed with basic diet; the T1, T2, T3, groups were fed 2, 4, and 6 g/kg tea polyphenols, respectively; and the CTC group was fed 50 mg/kg chlortetracycline. Tea polyphenols and chlortetracycline fed every day were evenly mixed into the concentrated feed. Lambs were fed concentrate first and then roughage, and enough clean drinking water was provided every day during the experiment.

Before the experiment began, the lambs were vaccinated, deworming and numbered. Subsequently, Lamb houses, feeding pens, metabolic cages, water troughs, and feed troughs were thoroughly cleaned and sterilized. Finally, put three lambs in each pen and feed them according to the designed diet, three times a day in the morning, at noon and at night. During the feeding period, the feeding methods, experimental environment and management mode of all groups are the same. All experiments were designed by one-way random experiment. Daily teosinte was added into pellet feed by TMR, and the formulation of the basal diet (Table 1) was in accordance with the nutritional requirements of the Feeding standard of Goat, China (NY/T861-2004).

2.2 Sample collection

Blood was collected from four goat lambs in each group from the jugular vein on the day 42 of the experiment. A total of 10 mL of blood was collected from the jugular vein using a blood collection tube with coagulant, and it was then centrifuged at 3,000 rpm for 10 min. After centrifugation of the blood collection tube without anticoagulation, the supernatant was absorbed to prepare serum and then stored at −80°C for testing.

After the lamb was slaughtered, the abdominal cavity was quickly opened and duodenum, jejunum, and ileum tissue samples were collected and washed three times with pre-cooled phosphate buffer. The samples were subsequently divided into two parts. Some samples of duodenum, jejunum, and ileum samples were cut into 3–4 cm pieces, placed into an enzyme-free centrifuge tube, immediately transferred to liquid nitrogen, and stored at −80°C. In the other part, the intestinal tissues of the weaned lambs were preserved in 4%

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TABLE 1 Composition and level of basal diet (dry matter basis).

Items	Content
Ingredients (%)	
Pennisetum × sinese	50.00
Corn	29.00
Soybean meal	10.00
Wheat bran	7.50
NaCl	0.50
$CaHPO_4$	0.50
Limestone	0.50
Premix[1]	2.00
Total	100
Nutrient level	
DM (%)	90.80
ME[2] (MJ/kg)	10.43
Crude protein	14.69
Crude fat	2.84
ADF	26.23
NDF	39.90
Ca	0.54
P	1.10
Crude ash	7.90

[1]The premix provided the following per kg of diets: VA 17500 IU, VD6 200 IU, VE 50 IU, Cu 20 mg, Fe 75 mg, Se 0.4 mg, Mn 80 mg, Co 0.3 mg, I 1.2 mg, Zn 40 mg. DM, dry matter; ME, metabolic energy; ADF, acid detergent fiber; NDF, neutral detergent fiber. [2]ME is the calculated value.

paraformaldehyde for morphological analysis and detection of the apoptosis rate.

2.3 Growth performance and intestinal histomorphology

On day 42, body weight and feed intake were measured, and the average daily gain (ADG), average daily feed intake (ADFI), and feed conversion rate (FCR) were calculated.

To determine the morphology of the intestinal tissue, the tissue samples were dehydrated, made transparent, embedded in paraffin, cut into 5 μm thick sections, and stained with hematoxylin and eosin (HE). The steps of the determination were as follows: (1) the fixed intestinal tissue was removed and washed with ethanol and water; (2) the tissue was dehydrated with ethanol at different concentration gradients and made transparent with xylene twice after dehydration for 20 min; (3) the tissue was soaked in wax and embedded at 60°C; (4) the wax blocks were cut into 5 μm thick slices with a slicer; (5) the wax-embedded samples were baked at 60°C, dewaxed, and dyed with HE; (6) the samples were dehydrated with ethanol, made transparent with xylene, and sealed. The tissue sections were observed using an optical microscope. ImagePro Plus 6.0 was used to measure the height of the intestinal villi (from the top of the villi to the junction of villi and crypt) and the depth of the crypt (the vertical distance from the junction of intestinal villi to the bottom of the intestinal gland), and the villus height/crypt depth value was calculated.

2.4 Determination of serum cortisol and immune indexes

An enzyme-linked immunosorbent assay kit (Nanjing Jiancheng Bioengineering Institute, Jiangsu, China) was used to determine the levels of immunoglobulin (IgA and IgM), COR, and cytokines (IL-1β, IL-6, IL-10, TNF-α, and IFN-γ) in the serum, and the detection method was strictly in accordance with the manufacturer's instructions.

2.5 Effects of intestinal permeability and SlgA

D-lactic acid (D-LA), lipopolysaccharide (LPS), diamine oxidase (DAO), and SlgA were determined using enzyme-linked immunosorbent assay kits according to the manufacturer's guidelines (Jiangsu Meimian Industrial Co. Ltd., Jiangsu, China).

2.6 Intestinal antioxidant index

After the frozen intestinal mucosa samples were thawed on ice, intestinal mucosa samples (0.5 g) were weighed and added to pre-cooled saline at a mass-to-volume ratio of 1:9 (g/mL) and ultrasonically pulverized to prepare tissue homogenates. After centrifugation at 3,000 × *g* at 4°C for 15 min, the supernatant was collected and stored in a −80°C refrigerator, and the antioxidant indices of the intestine (GSH-Px, CAT, T-SOD, MDA, T-AOC) were determined using ELISA kits according to the manufacturer's guidelines (Nanjing Jiancheng Bioengineering Institute, Jiangsu, China).

2.7 Intestinal tight junction protein-related gene

Intestinal RNA was extracted using the FastPure Cell/Tissue Total RNA Isolation Kit V2 (Vazyme Biotech Co., Ltd., Nanjing, China), and then the purity and concentration of RNA were determined by spectrophotometer for subsequent experiments. cDNA synthesis was carried out through reverse transcription according to the instructions of the HiScript lI Q RT SuperMixfor qPCR (+ gDNA wiper) reverse transcription kit (Vazyme Biotech Co., Ltd., Nanjing, China). Primers were designed according to the mRNA sequences of goat target genes (ZO-1, occludin, and claudin-1) and the internal reference gene GAPDH on the NCBI official website and then passed on to Shenggong Biotechnology Co., Ltd. (Shanghai, China) for synthesis; the sequence is shown in Table 2. As shown in Table 2. The relative mRNA expression of ZO-1, occludin, and claudin-1 was determined using a real-time fluorescence quantitative PCR instrument according to the instructions of the ChamQ Universal SYBR qPCR Master Mix (Vazyme Biotech Co., Ltd., Nanjing, China), and the $2^{-\Delta\Delta Ct}$ method was used for calculation.

TABLE 2 Real-time PCR primer sequences.

Genes	Primer sequences (5′-3′)	GenBank accession No.	Length (bp)
Claudin-1	F: CCCCAGTCAATGCCAGGTATG	XM_005675123.3	169
	R: TCTTTCCCACTGGAAGGTGC		
ZO-1	F: TGGACAAAGAGAAGGGTGAGAC	XM_018066118.1	110
	R: TTTAGGATCACAGTGTGGTAGG		
Occludin	F: GGCCTCTGGGTCTCTCTACA	XM_018065677.1	154
	R: AACCATGAACCCCAGCACAA		
GAPDH	F: GATGCCCCCATGTTTGTGATG	XM_005680968.3	160
	R: CGTGGACAGTGGTCATAAGTC		

[1]F, forward primer; R: reversed primer. [2]*ZO-1*, Zonula Occludens; GADPH, glyceraldehyde-3-phosphate dehydrogenase.

2.8 Detection of intestinal cell apoptosis

Apoptosis was assessed via a terminal deoxynucleotidyl transferase (TdT)-mediated deoxyuridine triphosphate (dUTP) nick end labeling (TUNEL) assay using an Apoptosis Detection Kit (Servare Biotech Inc., Hubei, China). First, paraffin sections were deparaffinized, and proteinase K working solution was used to cover the tissue sections for repair. The tissue sections were then incubated at 37°C for 25 min and washed with PBS. Membrane-breaking permeabilization was then performed, and the tissue was covered with drops of membrane-breaking working solution, incubated for 20 min at 27°C, placed on a shaker, and washed with PBS. The TUNEL kit was then applied, and the reagent was added to the section according to the instructions and incubated for 3 h. The sections were again covered with PBS and washed, the PBS was removed, DAPI Ran staining solution was added dropwise, and the sections were incubated in the dark for 10 min. Finally, the sections were sealed with an anti-fluorescein quencher, and images were captured through microscopic examination.

2.9 Statistical analysis

The experimental data were collated using Excel 2019 to establish a database, and SPSS software (version 26.0) was used for one-way ANOVA. The data were analyzed by linear effect and quadratic effect, and then the differences between groups were analyzed by Tukey multiple comparison test. The column chart is made by using GraphPad Prism 8. Statistical significance was set at $p < 0.05$.

3 Results

3.1 Effects of tea polyphenols on growth performance of weaned lambs

As shown in Table 3, the final weight of lambs in the T2 and T3 groups was significantly higher than that of lambs in the CON group ($p < 0.05$). Compared with the CON group, the ADG and ADFI of lambs in the T2, T3, and CTC groups were significantly increased, while the FCR was significantly decreased ($p < 0.05$). There were no significant differences between the other groups ($p > 0.05$). In addition, there was a significant linear and quadratic relationship ($P_L < 0.05$, $P_Q < 0.05$) between the final weight, ADG and FCR and the level of dietary tea polyphenols.

3.2 Effect of tea polyphenols on intestinal histomorphology of weaned lambs

The effects of dietary tea polyphenols on intestinal morphology are shown in Figure 1 HE staining revealed that the intestinal villi in the duodenum, jejunum, and ileum were denser and longer than those in the CON group. As shown in Table 4, in the duodenum, compared with the control group, the duodenal villus height of lambs fed with tea polyphenols and CTC increased significantly, especially in T3 group of lambs ($p < 0.05$). Moreover, the duodenal CD values of lambs in T2 group were significantly lower than the other groups ($p < 0.05$), while the ratio of VH/CD was higher than the rest of the groups ($p < 0.05$). A significant linear relationship and quadratic effect between tea polyphenol concentration and duodenal intestinal histomorphology were demonstrated ($P_L < 0.05$, $P_Q < 0.05$). In addition, in the jejunum, the intestinal villi of lambs in the T3 group were significantly higher than those of the remaining groups ($p < 0.05$) and were linearly related ($P_L < 0.05$). There was no significant difference in jejunal CD values ($p > 0.05$), and the VH/CD ratio in the jejunum of lambs in the T2 group was significantly higher than that in the CON group ($p < 0.05$), but the addition of dietary tea polyphenols has no linear and quadratic effects on the CD value and VH/CD ratio of jejunum ($P_L > 0.05$, $P_Q > 0.05$). In the ileum, the VH values in the jejunum and ileum of the T2, T3 and CTC groups were significantly higher than those of the CON and T1 groups ($p < 0.05$), and the CD values in the ileum of the lambs in the T2 group were significantly lower than those of the CON group ($p < 0.05$), and there was a significant linear and quadratic effect between the VH and CD values and tea polyphenols ($P_L < 0.05$, $P_Q < 0.05$). The VH/CD ratios in the ileum of the lambs in the T2 group were significantly higher than those in the other groups ($p < 0.05$), but there was only a quadratic effect among the test treatment groups ($P_Q < 0.05$).

TABLE 3 Effect of dietary tea polyphenols on growth performance of weaned lambs.

Items	Groups					SEM	*p*-value		
	CON	T1	T2	T3	CTC		ANOVA	Linear	Quadratic
Initial weight (kg)	8.73	8.83	9.51	9.92	9.31	0.21	0.401	0.16	0.36
Final weight (kg)	10.24^{b}	10.78^{b}	12.62^{a}	12.63^{a}	11.38^{ab}	0.35	0.01	0.01	0.02
ADG (g/d)	35.83^{c}	39.88^{c}	78.17^{a}	69.44^{ab}	53.44^{b}	4.68	<0.01	<0.01	<0.01
ADFI (g/d)	524.09^{b}	502.22^{bc}	562.78^{b}	756.67^{a}	436.11^{c}	30.14	<0.01	0.29	<0.01
FCR (g)	14.91^{a}	12.84^{ab}	7.23^{d}	10.95^{bc}	8.10^{cd}	0.83	<0.01	<0.01	0.03

[1]CON: basal diet; T1: basal diet +2 g/kg tea polyphenols; T2: basal diet +4 g/kg tea polyphenols; T3: basal diet +6 g/kg tea polyphenols; CTC: basal diet +50 mg/kg chlortetracycline. ADG, average daily gain; ADFI, average daily feed intake; FCR, feed conversion rate. [2]SEM, standard error of the mean. [a–d]Values in the same row with different letters are significantly different ($p < 0.05$). Results are presented as the mean ± SEM ($n = 6$).

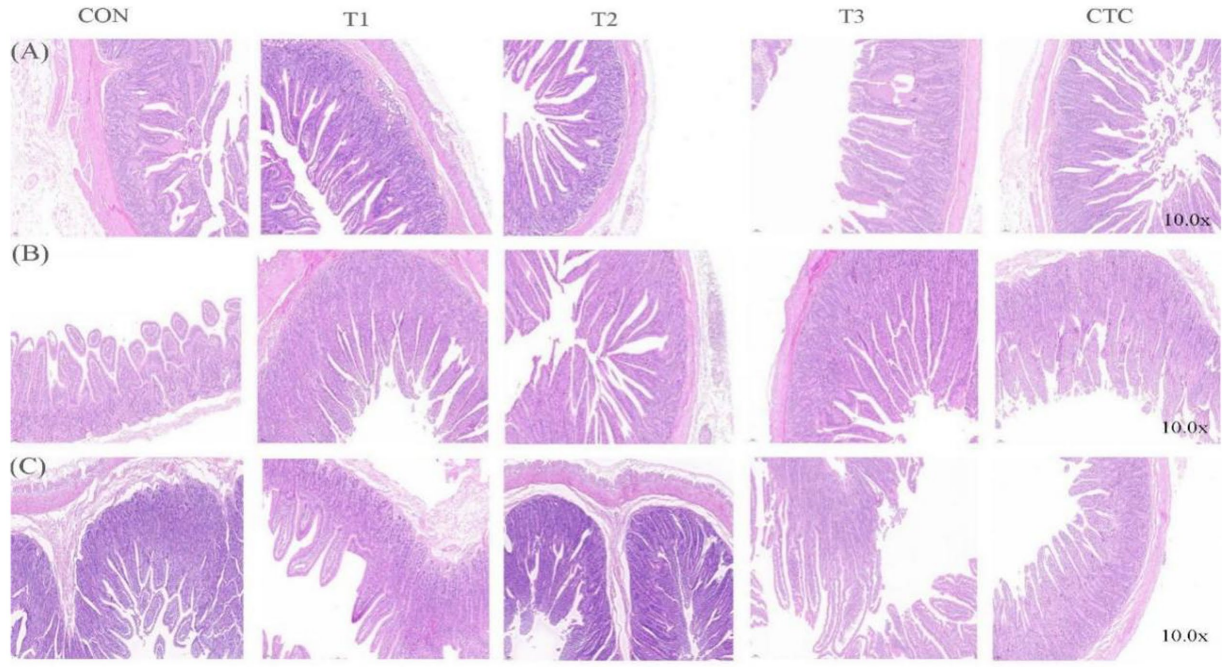

FIGURE 1
Micrographs of the duodenum, jejunum, and ileum of weaned lambs fed diets supplemented with teatoxin: **(A)** duodenum, **(B)** jejunum, and **(C)** ileum. CON: basal diet; T1: basal diet +2 g/kg tea polyphenols; T2: basal diet +4 g/kg tea polyphenols; T3: basal diet +6 g/kg tea polyphenols; CTC: basal diet +50 mg/kg chlortetracycline.

3.3 Effects of tea polyphenols on serum cortisol and immunoglobulin in weaned lambs

As shown in Figure 2, IgA and IgM in the serum of lambs in T1, T2, T3 and CTC groups were significantly higher than those in the control group ($p < 0.05$), and the immunoglobulin content in CTC group was higher than that in other groups ($p < 0.05$). Serum cortisol levels in lambs were significantly lower in all tea polyphenol groups, especially in the T2 group ($p < 0.05$). The serum cortisol content of lambs in the CTC group was significantly lower than that of the other groups ($p < 0.05$).

3.4 Effects of tea polyphenols on cytokines in serum of weaned lambs

In order to further evaluate the anti-inflammatory effect of tea polyphenols, we measured the cytokines which are very important to indicate the inflammatory state of animals, and studied whether adding tea polyphenols can improve the immune function of weaned lambs. The results are displayed in Figure 3. Compared with the control group, all groups of lambs supplemented with tea polyphenols and CTC significantly reduced the levels of cytokines IL-1β, IL-6, TNF-α and IFN-γ in serum ($p < 0.05$), and significantly increased the

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TABLE 4 Effect of dietary polyphenols on intestinal morphology of weaned lambs.

Items	Groups					SEM	*p*-value		
	CON	T1	T2	T3	CTC		ANOVA	Linear	Quadratic
Duodenum									
VH (μm)	479.13^{c}	619.55^{b}	652.87^{b}	827.63^{a}	711.65^{b}	28.70	<0.01	<0.01	0.04
CD (μm)	421.35^{a}	426.43^{a}	278.48^{b}	510.68^{a}	477.15^{a}	21.22	<0.01	0.04	0.01
VH/CD	1.14^{c}	1.51^{bc}	2.35^{a}	1.63^{b}	1.49^{bc}	0.10	<0.01	0.04	<0.01
Jejunum									
VH (μm)	495.75^{c}	583.85^{bc}	661.15^{ab}	816.23^{a}	696.08^{ab}	31.07	<0.01	<0.01	0.08
CD (μm)	393.80	482.8	375.35	514.23	489.25	19.92	0.142	0.128	0.912
VH/CD	1.28^{b}	1.22^{b}	1.87^{a}	1.61^{ab}	1.44^{ab}	0.08	0.07	1.83	0.08
Ileum									
VH (μm)	457.15^{b}	559.23^{b}	615.28^{ab}	778.68^{a}	535.70^{b}	31.24	<0.01	0.03	<0.01
CD (μm)	417.87^{ab}	460^{ab}	347.08^{b}	554.13^{a}	508.25^{a}	22.74	0.02	0.04	0.35
VH/CD	1.13^{b}	1.23^{b}	1.81^{a}	1.44^{ab}	1.08^{b}	0.09	0.03	0.80	<0.01

[1]CON: basal diet; T1: basal diet +2 g/kg tea polyphenols; T2: basal diet +4 g/kg tea polyphenols; T3: basal diet +6 g/kg tea polyphenols; CTC: basal diet +50 mg/kg chlortetracycline. VH, villus height; CD, crypt depth; VH/CD, ratio of villus height to crypt depth. [2]SEM, standard error of the mean. [a–c]Values in the same row with different letters are significantly different ($p < 0.05$). Results are presented as the mean ± SEM ($n = 6$).

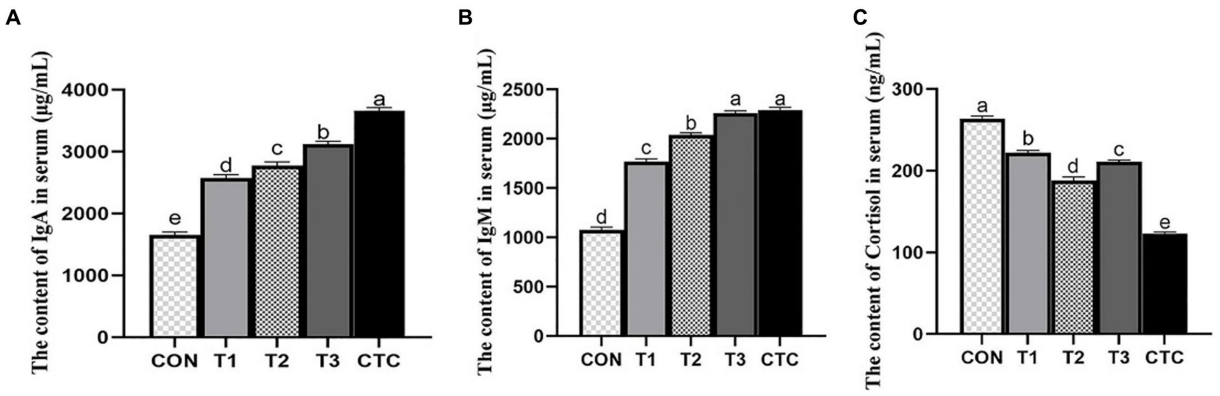

FIGURE 2
Effects of tea polyphenols on serum cortisol hormone and immune globulin of weaned lambs. [1]CON: basal diet; T1: basal diet +2 g/kg tea polyphenols; T2: basal diet +4 g/kg tea polyphenols; T3: basal diet +6 g/kg tea polyphenols; CTC: basal diet +50 mg/kg chlortetracycline. **(A–C)** The content of IgA, IgM, and cortisol in the serum, respectively. IgA, immunoglobulin A; IgM, immunoglobulin M. [2]SEM, standard error of the mean. [a–e]Values in the same row with different letters are significantly different ($p < 0.05$). Results are presented as the mean ± SEM ($n = 6$).

serum levels of IL-10 ($p < 0.05$), especially in lambs inT3 and CTC groups.

3.5 Effects of tea polyphenols on intestinal permeability of weaned lambs

The contents of D-LA, LPS and DAO in lamb serum were significantly decreased by adding different concentrations of tea polyphenols and antibiotics ($p < 0.05$), thus affecting intestinal permeability. Results As shown in Figure 4, the serum levels of d-lactic acid, endotoxin and DAO in the CON group were significantly higher than those in the other groups ($p < 0.05$). In the tea polyphenols group, the contents of D-LA, LPS and DAO in serum of lamb in T2 group were significantly lower than those in other tea polyphenols groups ($p < 0.05$). The contents of D-LA, endotoxin and DAO in serum of lambs in CTC group were significantly lower than those in other groups ($p < 0.05$).

3.6 Effect of tea polyphenols on the content of SIgA in intestine of weaned lambs

The effects of different concentrations of tea polyphenols on intestinal SIgA content are shown in Figure 5. In the duodenum, the SIgA content in lambs in the T3 and CTC groups was significantly higher than that in the other groups ($p < 0.05$). In the jejunum, the SIgA content in the other groups was significantly higher than that in the CON group ($p < 0.05$). In the ileum, compared with the CON group, the SIgA content in lambs in the T2, T3, and CTC groups was significantly higher ($p < 0.05$). In particular, the SIgA content of lambs

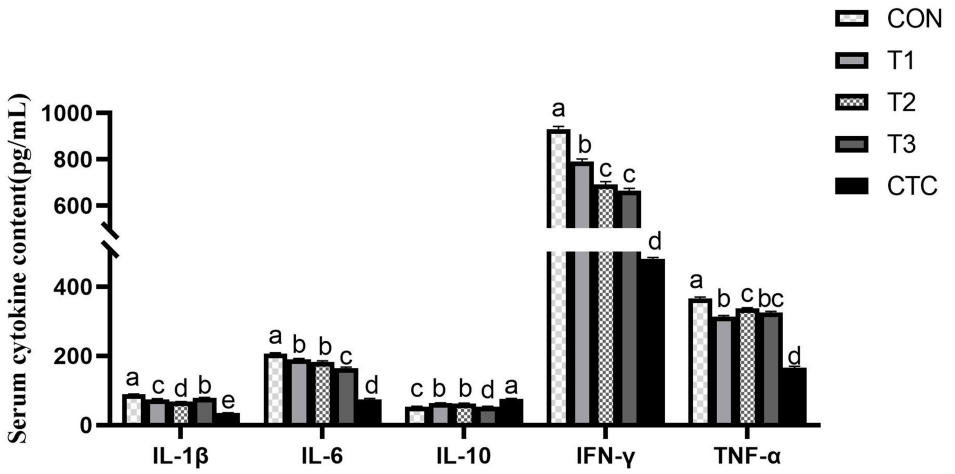

FIGURE 3
Effect of tea polyphenols on cytokines in serum. [1]CON: basal diet; T1: basal diet +2 g/kg tea polyphenols; T2: basal diet +4 g/kg tea polyphenols; T3: basal diet +6 g/kg tea polyphenols; CTC: basal diet +50 mg/kg chlortetracycline. IL-1β, interleukin-1β; IL-10, interleukin-10; IL-6, interleukin-6; TNF-α, tumor necrosis factor-α; IFN-γ, interferon-γ. [2]SEM, standard error of the mean. [a–d]Values in the same row with different letters are significantly different ($p < 0.05$). Results are presented as the mean ± SEM ($n = 6$).

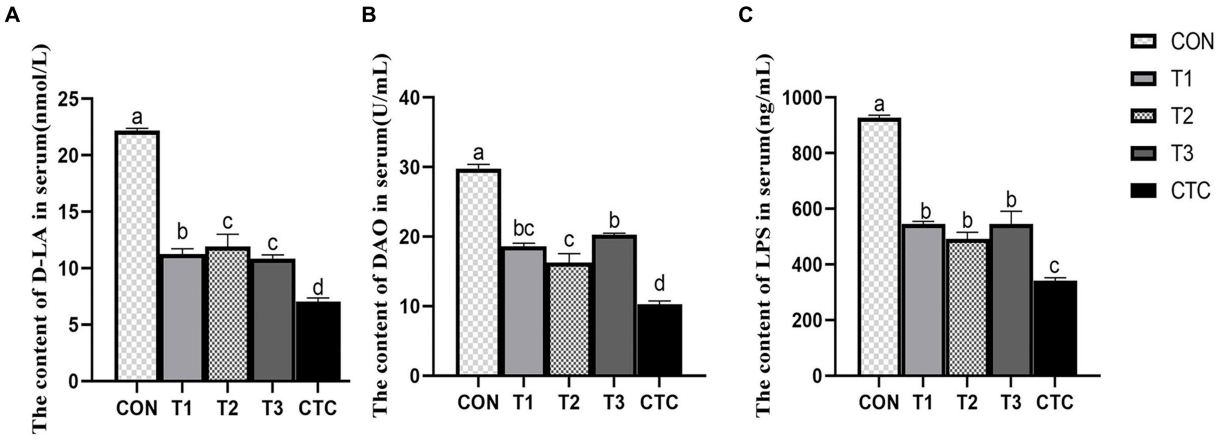

FIGURE 4
Effect of dietary tea polyphenols on intestinal permeability in weaned lambs. [1]CON: basal diet; T1: basal diet +2 g/kg tea polyphenols; T2: basal diet +4 g/kg tea polyphenols; T3: basal diet +6 g/kg tea polyphenols; CTC: basal diet +50 mg/kg chlortetracycline. **(A–C)** The content of D-LA, DAO, and LPS in the serum, respectively. D-LA, D-lactic acid; DAO, diamine oxidase; LPS, lipopolysaccharide. [2]SEM, standard error of the mean. [a–d]Values in the same row with different letters are significantly different ($p < 0.05$). Results are presented as the mean ± SEM ($n = 6$).

in the CTC group was higher than that of the remaining groups ($p < 0.05$).

3.7 Effect of tea polyphenols on intestinal antioxidant capacity of weaned lambs

Figure 6 shows that in the duodenum, compared with CON group, the activity of T-SOD in the intestines of lambs in all tea polyphenols groups and CTC groups increased significantly ($p < 0.05$), and the content of MDA in the intestines of lambs in T2, T3 and CTC groups decreased significantly ($p < 0.05$). The GSH-PX activity of lambs in T2 and CTC groups was significantly higher than that in other groups ($p < 0.05$), and the T-AOC activity of lambs in T1, T2 and CTC groups was significantly higher than that in CON and T1 groups ($p < 0.05$). In addition, the CAT content of lamb in CTC group was significantly higher than that in other groups ($p < 0.05$), and there was no significant difference among other groups ($p > 0.05$). In jejunum, the T-SOD activity of lambs in T2, T3 and CTC groups was significantly higher than that in CON and T1 groups ($p < 0.05$). In addition, the activities of GSH-PX and CAT in lamb intestines in T2 and CTC groups were significantly higher than those in other groups ($p < 0.05$), and the MDA content in lamb intestines in T3 and CTC groups was significantly lower than that in other groups ($p < 0.05$). Compared with the control group, there was no significant change in T-AOC ($p > 0.05$). In the ileum, the activity of T-SOD increased gradually with the increase of tea polyphenol concentration, especially in T3 and CTC groups were significantly

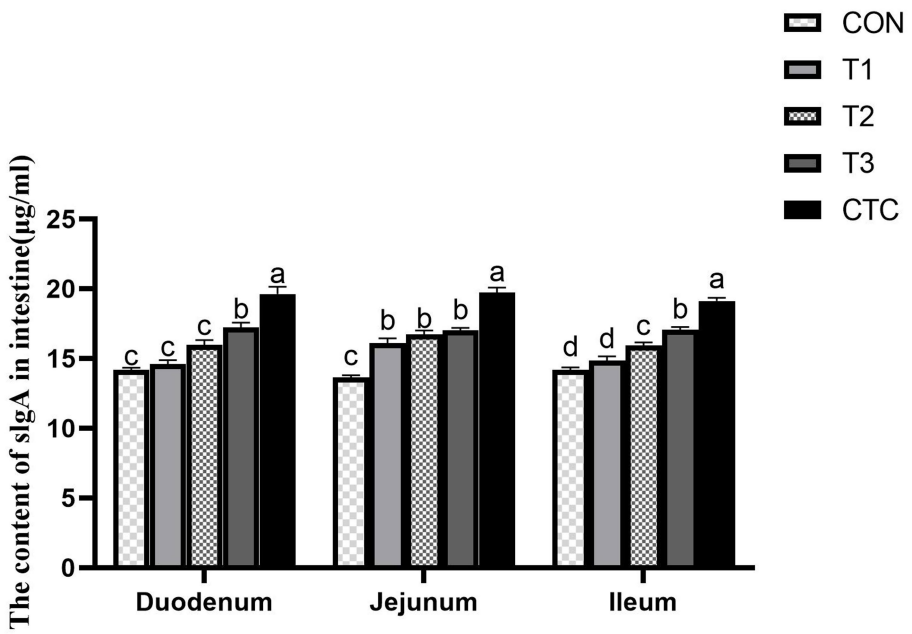

FIGURE 5
Effect of dietary tea polyphenols on intestinal SIgA content in weaned lambs. [1]CON: basal diet; T1: basal diet +2 g/kg tea polyphenols; T2: basal diet +4 g/kg tea polyphenols; T3: basal diet +6 g/kg tea polyphenols; CTC, basal diet +50 mg/kg chlortetracycline. SIgA, secretory immunoglobulin A. [2]Results are presented as the mean ± SEM ($n = 6$). [a–d]Means were significantly different ($p < 0.05$).

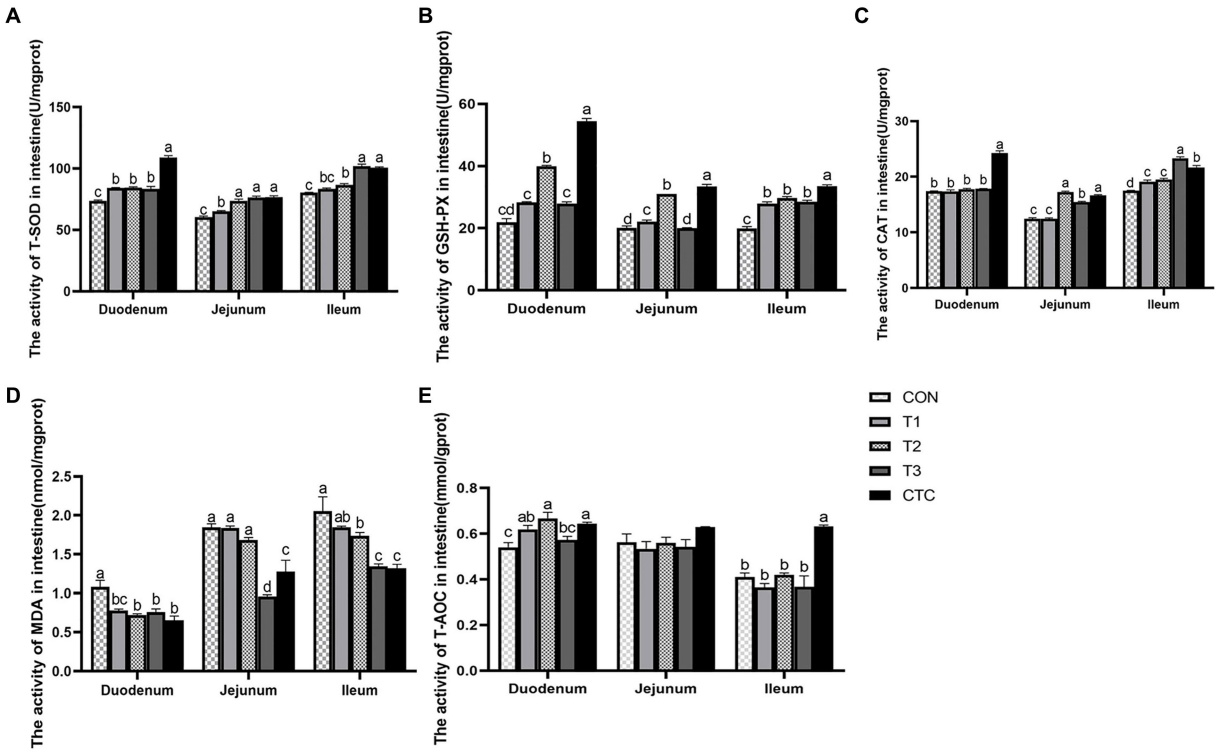

FIGURE 6
Effect of tea polyphenols on intestinal antioxidant power in weaned lambs. [1]CON: basal diet; T1: basal diet +2 g/kg tea polyphenols; T2: basal diet +4 g/kg tea polyphenols; T3: basal diet +6 g/kg tea polyphenols; CTC: basal diet +50 mg/kg chlortetracycline. **(A–E)** The activity of T-SOD, GSH-PX, CAT, MDA, and T-AOC in the duodenum, jejunum, and ileum, respectively. T-SOD, total superoxide dismutase; CAT, catalase; GSH-PX, glutathione peroxidase; MDA, malondialdehyde; T-AOC, total antioxidant capacity. [2]SEM, standard error of the mean. [a–d]Values in the same row with different letters are significantly different ($p < 0.05$). Results are presented as the mean ± SEM ($n = 6$).

higher than the rest of the groups ($p < 0.05$). The ileal GSH-PX activity of the lambs in CON group was significantly lower than the rest of the groups and the GSH-PX activity of the lambs in CTC group as significant ($p < 0.05$). The CAT activity of lambs was significantly higher in all tea polyphenol groups compared to CON group and was significantly higher in T3 group than the rest of the groups ($p < 0.05$). In addition, the intestinal MDA of lambs in T2, T3 and CTC groups was significantly lower than that of CON and T1 groups. The intestinal T-AOC of lambs in CTC group was higher than that of the remaining groups ($p < 0.05$), and the differences among the rest were not significant ($p > 0.05$).

3.8 Effect of tea polyphenols on genes related to intestinal tight junction protein in weaned lambs

Except for the histological observations, the integrity of intestinal epithelium is usually determined by the expression of key tight junction genes. Thus, we further measured the relative gene expression of ZO-1, Occludin, and Claudin-1 in in sheep intestine and the results were listed in Figure 7. In duodenum, the contents of *ZO-1*, *Claudin-1* and *Occludin* in intestines of lambs in T2 and T3 groups were significantly higher than those in CON group ($p < 0.05$), and the contents of *Occludin* in intestines of lambs in CTC group were also significantly higher than those in CON group ($p < 0.05$). In jejunum tissue, the contents of *ZO-1*, *Claudin-1* and *Occludin* in the intestines of lambs in T2 and T3 groups were significantly higher than those in CON group and F1 group, while the contents of *ZO-1* and *Occludin* in the intestines of lambs in CTC group were significantly higher than those in CON group ($p < 0.05$). In ileum tissue, the contents of *ZO-1*, *Claudin-1* and *Occludin* in lamb intestine in T2 and T3 groups were significantly higher than those in other groups ($p < 0.05$), but there was no significant difference between CTC group and other groups ($p > 0.05$).

3.9 Effect of tea polyphenols on apoptosis rate of intestinal cells in weaned lambs

As shown in Figure 8, the apoptosis rates of lambs in the duodenum, jejunum, and ileum in the T2, T3, and CTC groups were significantly lower than those in the CON group ($p < 0.05$). The differences between the remaining groups were not statistically significant ($p > 0.05$).

4 Discussion

4.1 Effects of tea polyphenols on growth performance and intestinal morphology of weaned lambs

It has been demonstrated that intestinal structure and development play a crucial role in the digestive and absorptive processes of the intestines and that intestinal villi have an absorptive function, which can be indicated by villus height and villus surface area (16). The depth of crypt determines the speed of epithelial cell formation by mitosis of intestinal villi, and reflects the colonization rate and maturity of crypt cells. Therefore, the value of villus height/crypt depth is an important index to measure intestinal development and function (17, 18). In addition, some studies have shown that there is a positive correlation between villi length, nutrient absorption and digestibility. The shorter the intestinal villi and the deeper the crypt, the weaker the digestibility, thus affecting the growth potential of animals (19). Early weaning induces villus atrophy and crypt hypertrophy, resulting in diminished nutrient digestion and absorption, which may precipitate diarrhea (20). Aziz-Aliabad et al. (21) found that adding green tea powder to broiler feed significantly increased villus height and villus height/crypt depth values, enhanced the digestion and absorption of nutrients, and improved growth performance. Another study found that feeding fermented tea dregs to juvenile largemouth bass resulted in higher intestinal villus heights

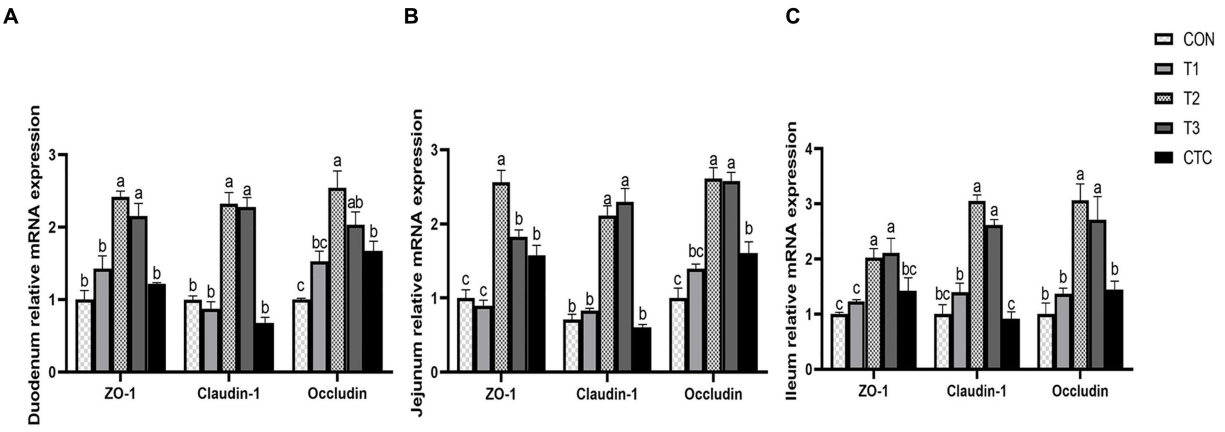

FIGURE 7
Effects of tea polyphenols on intestinal tight junction proteins in weaned lambs. [1]CON: basal diet; T1: basal diet +2 g/kg tea polyphenols; T2, basal diet +4 g/kg tea polyphenols; T3, basal diet +6 g/kg tea polyphenols; CTC, basal diet +50 mg/kg chlortetracycline. **(A–C)** The gene expression of ZO-1, claudin-1, and occludin in the duodenum, jejunum, and ileum, respectively. [2]The results are expressed as the mean ± SEM ($n = 6$). The mean values of [a–d] were significantly different ($p < 0.05$).

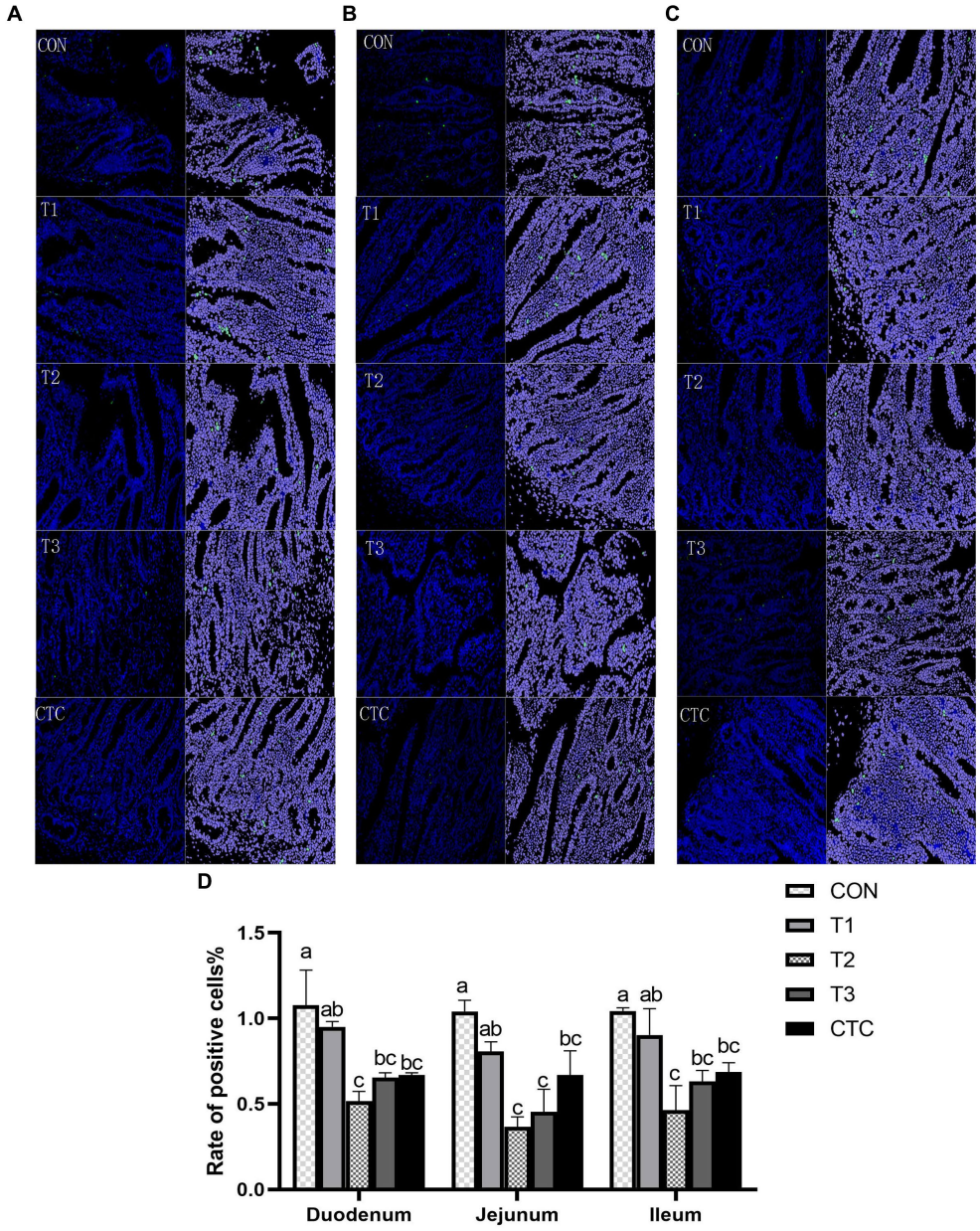

FIGURE 8
Effects of tea polyphenols on apoptosis in the intestines of lambs. Representative small intestine cross-sections (200×) of lambs from CON, T1, T2, T3, and CTC groups after TUNEL staining. Cells green in color are apoptotic cells, and those in blue are nonapoptotic cells. **(A)** Duodenum, **(B)** jejunum, and **(C)** ileum. **(D)** Apoptotic cell death rate and the results are expressed as SEM ± mean ($n = 6$); $^{a-c}$Means are significantly different ($p < 0.05$). CON: basal diet; T1: basal diet +2 g/kg tea polyphenols; T2, basal diet +4 g/kg tea polyphenols; T3, basal diet +6 g/kg tea polyphenols; CTC, basal diet +50 mg/kg chlortetracycline.

and widths, indicating, demonstrating, enhanced intestinal integrity and stability (22). Furthermore, Yang et al. (23) compared the effects of different levels of green tea by-products (0.5%, 1%, and 2%) with those of antibiotics and obtained significant results in terms of body weight gain. These results are similar to those of the present study. In this study, the addition of 4 g/kg tea polyphenols significantly reduced the recess depth of the duodenum and ileum and significantly improved the growth performance and intestinal villus height of the animals. Tea polyphenols can improve intestinal development by enhancing the integrity of the intestinal mucosa's morphology, thus improving animal feed conversion rates and the digestion and absorption of nutrients, which ultimately improves animal growth performance.

4.2 Effects of tea polyphenols on cortisol and immune function of weaned lambs

The immune function of the intestinal mucosa is mediated by immune cells and cytokines. When animals are stimulated by

weaning, macrophages can produce and secrete a large number of inflammatory cytokines, including IL-1β, IL-6, IFN-γ, and TNF-α, which mediate the inflammatory response in animals (24). Immunoglobulin is the main antibody involved in mucosal immunity; it can specifically bind antigens to exert immune effects and it plays an important role in humoral immunity. An increase in immunoglobulin secretion indicates an improvement in immune function (25). COR is typically used as an index to measure stress in animals. Related studies have shown that when lambs are in the weaning stage, the serum skin COR increases significantly over a short time (26), and LPS of Gram-negative bacteria can activate TLR4-mediated inflammatory pathways, such as NF-κB, and induce the production of inflammatory cytokines (IL-1β, IL-2, IL-6, and TNF-α) (27). The addition of green tea polyphenols to juvenile Wuchang bream under ammonia stress significantly reduced the cortisol concentration, and the IL-1B, TNF-α, and IgM levels in the spleen also significantly decreased, thus alleviating oxidative stress and ammonia damage (28), which was similar to the results of this study that tea polyphenols significantly reduced the levels of cytokines and cortisol in serum and increased the content of immunoglobulin. Song et al. (29) found that oolong tea polyphenols inhibited the activation of NF-κB in mice, downregulated the levels of pro-inflammatory cytokines (TNF-α, IL-1β, and IL-6), and significantly increased the levels of the anti-inflammatory factor IL-10. In addition, administering Chinese sweet tea to mice with induced allergies resulted in increased fecal IgA levels, indicating enhanced intestinal immunity (30). Studies have shown that 4–6 g/kg tea polyphenols can play an anti-inflammatory role by regulating the transduction of cytokines, reducing the content of pro-inflammatory cytokines, and maintaining the health of the body by increasing the immunoglobulin and IL-10 contents.

4.3 Effect of tea polyphenols on antioxidant capacity of weaned lambs

In the process of weaning, because the intestine is the organ with the largest contact area with food, it is stimulated by various stressors, such as foreign antigens, microorganisms, and their toxins in the diet, and excessive free radicals are produced in the body. When the antioxidant defense system of the body cannot remove excessive free radicals (ROS), the oxidation-reduction homeostasis of the body is unbalanced, leading to oxidative stress (31). When excessive oxygen free radicals are produced, they cause damage to lipids and cell membranes, proteins, and nucleic acids, resulting in intestinal damage (32, 33). The antioxidant activity of polyphenols depends on their ability to react with ROS to a great extent, and the o-quinone or p-quinone methylated substance derived from polyphenols can also upregulate the gene of the antioxidant reaction element, thus improving the enzyme activities of CAT, SOD and GPx, restoring redox stability, and preventing systemic or local inflammation (34). Ding et al. (35) found that adding tea residue to the basic diet can significantly improve the activities of GSH-Px, T-AOC, CAT, and SOD activities in the serum of growing pigs and reduce MDA content. MDA is the main product of lipid peroxidation, it can be used to monitor the lipid oxidation state. Studies have found that 300 mg/kg of tea polyphenols can reduce the MDA concentration in the intestines of young hybrid sturgeons (36). Similarly, it has been reported that catechin can alleviate the oxidative damage to chicken lymphocytes induced by hydrogen peroxide, increase the expression of antioxidant genes SOD and GSH-Px, and demonstrate the antioxidant ability of scavenging free radicals and reducing the formation of H_2O_2 and ROS (37). These experimental results are similar to the results of this study in that adding 4–6 g/kg tea polyphenols and antibiotics were found to improve the antioxidant activity in the intestines of weaned lambs, significantly reduce the MDA content, and improve the antioxidant capacity of the body. Therefore, tea polyphenols have antioxidant properties. In addition, the improvement in antioxidant capacity may be closely related to the improvement in intestinal integrity and immunity observed in this study.

4.4 Effect of tea polyphenols on intestinal permeability and barrier function of weaned lambs

Intestinal permeability refers to the ability of the intestinal mucosal epithelium to allow some types of molecules to pass through it in a passive diffusion mode that is not mediated by a carrier or channel. An increase in intestinal permeability inevitably leads to the invasion of bacteria and endotoxins from the intestinal cavity into the rest of the body through the intestine, potentially resulting in severe infections (38). In addition, when the intestinal tract is damaged, the D-LA produced by bacterial metabolism and cleavage accumulates excessively, and the intracellular enzyme DAO existing in intestinal villous epithelial cells crosses the intestinal mucosal barrier and enters the blood circulation together with D-LA (39). Therefore, its serum content can reflect the integrity of intestinal barrier function and the degree of injury (40). In addition, the intestinal epithelial tight junction structure composed of proteins such as Claudins, Occludin and ZO-1 forms a selectively permeable intercellular barrier by sealing the intercellular space between adjacent intestinal epithelial cells, preventing environmental toxins, intestinal cavity antigens, and microorganisms from entering the circulatory system (41). Studies have shown that green tea extract can improve the intestinal barrier function of high-fat mice, alleviate the decrease in tight junction protein *ZO-1* and *Claudins* genes, and reduce the endotoxin content in the serum, thus providing some protection against intestinal damage (42). The administration of catechin, the main component of tea polyphenols, to mice can also reduce the activity of serum DAO and the concentration of D-LA, while also increasing the abundance of Occludin and ZO-1 in the duodenum, jejunum, and ileum, which further indicates that catechin supplementation can maintain the integrity of the intestinal barrier in mice (43). In addition, adding L-theanine to the diet can significantly increase the mRNA and protein expression of *ZO-1*, *Claudin-1*, and *Occludin* in the jejunum, ileum and epithelial cells of piglets, and decreased their serum levels of D-LA and DAO (44). These results are similar to the results of this study, which demonstrated that adding 4 mg/kg of tea polyphenols can reduce intestinal permeability and increase the mRNA expression of tight junction proteins. This shows that tea polyphenols protect the integrity of the intestinal barrier in weaned lambs and are beneficial to their intestinal health.

4.5 Effects of tea polyphenols on apoptosis rate and SlgA of intestinal cells in weaned lambs

Apoptosis plays an important role in programmed cell death and cell division and is closely related to animal health. Many factors, such as inflammation, can induce the apoptosis of intestinal cells, leading to the destruction of mucosal integrity and changes in epithelial barrier function, which in turn can result in the invasion of bacterial pathogens (45, 46). Intestinal inflammation and injury caused by weaning stress trigger the release of reactive substances, such as nitric oxide and oxygen, into the intestinal cavity, leading to intestinal epithelial cell apoptosis and diarrhea (47). Tea polyphenols can inhibit exogenous cell injury, such as oxidative stress, reduce cell apoptosis in tissues, and ensure the health of the body (48). SlgAis the main component of the intestinal mucosal defense system, which can inhibit the attachment of microorganisms to the respiratory epithelium and the reproduction of pathogenic microorganisms, as well as maintain the internal environment of the intestinal mucosa (49). Studies have shown that feeding apple polyphenols to pigs can increase intestinal SlgA production to enhance intestinal immunity and improve body health (50). In addition, Yuan et al. (51) showed that feeding vanadium to chickens increases the apoptosis rate of chicken duodenal cells and causes gastrointestinal disorders, but adding tea polyphenols could inhibit this increase in the apoptosis rate, or even make it decline. Studies have shown that DSS can induce colitis, causing oxidative stress and apoptosis in mice. TUNEL analysis has further demonstrated that DSS can significantly increase the frequency of apoptotic cells in the colon mucosa, but green tea polyphenols have been found to greatly reverse this trend and improve mucosal barrier function (52) In this study, 4 and 6 g/kg of tea polyphenols and chlortetracycline had a good inhibitory effect on intestinal cell apoptosis in weaned lambs and increased the content of SlgA in the intestine, indicating that tea polyphenols could enhance the intestinal mucosal immunity of weaned lambs and inhibit the apoptosis caused by weaning stress, which was similar to the findings of Ma et al. (53) and Giakoustidis et al. (54) in mice. Therefore, tea polyphenols can reduce the oxidative stress of the intestine during weaning, reduce the damage caused by free radicals to epithelial cells, and thus protect the morphology of the intestine.

5 Conclusion

The results showed that the effect of adding 4–6 g/kg tea polyphenols to the diet was better, which could not only enhance the immunity and antioxidant capacity of lambs, but also improve the intestinal barrier function, reduce intestinal damage and protect intestinal health. The potential mechanism of tea polyphenols may be closely related to inhibiting intestinal inflammation, oxidative stress and reducing apoptosis. These findings provide theoretical support for developing tea polyphenols as feed additives to improve the intestinal health of livestock.

Data availability statement

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

Ethics statement

The animal studies were approved by the Animal Care and Use Committee of Guangdong Ocean University (SYXK-2018-0147, 2018). 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

YuX: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Software, Visualization, Writing – original draft. FY: Funding acquisition, Methodology, Resources, Validation, Writing – review & editing. JW: Software, Writing – review & editing. PW: Software, Writing – review & editing. XQ: Investigation, Writing – review & editing. XH: Formal analysis, Writing – review & editing. YiX: Formal analysis, Writing – review & editing. SG: Conceptualization, Methodology, Project administration, Resources, Validation, Writing – review & editing.

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Conflict of interest

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

Publisher's note

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Factors affecting the rumen fluid foaming performance in goat fed high concentrate diet

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Feeding high concentrate diets is highly prone to rumen bloat in ruminants, which is very common in production. This study explored the factors responsible for the occurrence of foamy rumen bloat. The experiment was conducted using goats as test animals, fed high concentrate diets and scored for rumen distension into high, medium and low bloat score groups. Rumen fluid was collected from 6 goats in each group separately. The foaming production, foam persistence, pH value, viscosity and the content of protein, total saccharide and mineral elements in rumen original fluid (ROL) were measured, and the protein and total saccharide content in rumen foam liquid (RFL) and rumen residual liquid (RRL) were determined. The results showed that the protein content in rumen original fluid and rumen foam liquid was significantly higher than that in rumen residual liquid ($p < 0.05$), and the protein content in rumen foam liquid was 10.81% higher than that in rumen original fluid. The higher the rumen bloat score, the higher the foam production, foam persistence, viscosity, protein, Ni, Mg, Ca, and K concentrations of the rumen original fluid, and the lower the PH and Na concentrations of the rumen original fluid; correlation analysis showed that the viscosity of the rumen original fluid was significantly and positively correlated with the foam production and foam persistence ($p < 0.05$). Foaming production and foam persistence of rumen original fluid were significantly and positively correlated with the contents of protein, total saccharide, K, Ca, Mg and Ni ($p < 0.05$). and negatively correlated with the content of Na ($p < 0.05$); after controlling other components those were significantly related to the foaming performance of rumen original fluid only protein still was significantly positively correlated with the foam persistence of rumen original fluid ($P<0.05$). In summary, the contents of protein, total saccharide and mineral elements in the rumen fluid had a significant effect on the foaming performance of rumen in ruminants, with protein playing a decisive role and the other components playing a supporting role. Reducing the content of protein in the diet in production is beneficial to reduce the occurrence of rumen bloat in ruminants.

KEYWORDS

goat, high concentrate diet, rumen bloat, foam, protein

1 Introduction

In the modern intensive farming model, the use of high concentrate diet (HCD) is often increased to improve production performance (1, 2). Horse used for meat production are often fed a starch-based concentrate feed in many European countries to shorten the fattening period. However, long-term feeding HCD tends to increase the total amount of volatile fatty acids and valeric acid in the horse's intestinal tract, which reduces the integrity of the intestinal mucosa, thus leading to gastrointestinal inflammation (3, 4). Offering total mixed rations

(TMR) to ruminants, promote synchronized intake of concentrate and roughage can reduce the risk of rumen toxicity and promote animal health and welfare (5). However, long-term feeding of HCD can lead to digestive diseases, rumen bloat is one of the common digestive diseases. One study found that digestive-related mortality on rangelands accounted for 19.5–28.4% of all mortality compared to mortality from other causes, with 96.3% of digestive mortality diagnosed as rumen bloat (6). In most cases, rumen bloat leads to the death of ruminants due to digestive diseases. Early studies believed that too much and too fast gas produced by rumen fermentation was the main reason of rumen bloat induced by HCD. Rumen microorganisms decompose starch to produce low-grade fatty acids, carbon dioxide and methane (7), and use monosaccharides and disaccharides produced by feed decomposition to synthesize glycogen for storage in microorganisms. Therefore, compared with fiber substances, equivalent amount of starch produces more gas in the rumen, so the rumen is prone to bloat (8). The starch content in ruminants rises in accordance with the increased proportion of concentrate in their diet, leading to an elevated incidence of rumen bloat. However, studies have found that although the digestion rate and gas production rate of pressed barley in the rumen are faster than that of whole barley, the incidence of rumen bloat of pressed barley diet is significantly lower than that of whole barley diet (9, 10). In addition, studies have shown that rumen fermentation rate and degree of wheat is higher than that of barley, sorghum or corn (11). However, recent studies reported that rumen bloat is caused by foam-encased gases produced by rumen fermentation that cannot be properly expelled (12–14). Protein (15), polysaccharide (16), mineral ions (17), etc. coming from diet, rumen microbial synthesis and rumen microbial fermentation diet in rumen fluid may act as the foaming agent or foam stabilizer. But up to now, the key components in rumen fluid that affect the foaming production or foam persistence have not been determined. Our study hypothesized that factors affecting rumen foam production and foam stability may be related to the nutrient composition in high concentrate. In this study, the components affecting the foaming performance of rumen fluid in goats fed with HCD were analyzed. The results can lay a foundation for the rational application of HCD in production.

2 Materials and methods

For The research procedure used in current study was approved by Animals policy and welfare committee of Agricultural research organization of Sichuan province China and in agreement with rules of the Animal Care and Ethical Committee of the Sichuan Agricultural University (Ethics Approval Code: SCAUAC201608-5).

2.1 Experimental animals and management

The animal experiments were conducted at the farm of the Animal Nutrition Institute, Sichuan Agricultural University, Ya'an, Sichuan Province, China. The experimental animals were 26 healthy Jianzhou big-eared goats, aged 8–10 months, weighing about 30 kg, which were immunized and dewormed before the experiment. During the experiment, the goats were kept in a single pen and feed twice a day (feeding at 9:00 in the morning and at 17:00 in the afternoon, respectively) to ensure that the goats eat freely, and enable the goats to drink freely.

2.2 Experimental diet

The experimental diet were configured in according with the to the Chinese Feeding Standard of Meat-producing Sheep and Goats (NY/T816-2004). Before the start of the experiment, the goats were fed with oat hay, and the diet was gradually transitioned to a total mixed ration with a concentrate-roughage ratio of 80:20 in 14 days (see Table 1 for the composition and nutrient level of the diet).

2.3 Chemical analysis

Chemical analysis was carried out according to the methodology described in the literature (18, 19): Diet samples were dried in an oven at 65°C and passed through a 1-mm sieve before determining the DM was determined. Crude protein (CP) content was determined by Kjeldahl method. Ether extract (EE) was determined by Soxhlet extraction method as described by AOAC (20). Ash was determined by scorching in a muffle furnace at 550°C. Neutral detergent fiber (NDF) and acid detergent fiber (ADF) were determined using the method of Van Soest et al. (21). In order to avoid the interference of aflatoxin (AFB1) on the experimental results, the content of aflatoxin in feed ingredients was effectively controlled in this study. Control of aflatoxins in feed at levels well below the established safety limits for animal feed. This precaution was taken to safeguard the health and welfare of the animals. Aflatoxin contamination in animal feed can cause serious health hazards, including effects on growth and damage to the liver by maintaining feed quality within safe limits (22), we aim

TABLE 1 Composition and nutrient levels of experimental diet (basis on DM).

Ingredients	%	Nutrients	%
Corn	48.25	CP	15.93
Wheat bran	7.30	EE	5.23
Soybean meal	10.50	NDF	19.55
Rapeseed meal	2.00	ADF	7.34
Cottonseed cake	8.00	Ca	0.76
Oat grass	4.00	P	0.49
Wheat straw	2.00	Starch	36.18
Alfalfa hay	14.00	DE (Mcal.kg^{-1})	2.88
Calcium carbonate	1.00		
Calcium hydrophosphate	0.45		
NaCl	0.50		
$NaHCO_3$	1.00		
Premix[1]	1.00		
Total	100.00		

[1]The premix provided the following per kg of diet: vitamin A 2200 IU; vitamin D 250 IU; vitamin E 20 IU; Fe 40 mg; Cu 10 mg; Zn 30 mg; Mn 40 mg; I 0.8 mg; Se 0.2 mg; Co 0.11 mg.

to minimize the potential impact of aflatoxins on the results of research.

2.4 Bloat scoring and sample collection

A 20-day feeding trial was performed. The 14th-19th days of the feeding trial, animals were scored for bloat 2-3 h after morning feeding daily, three raters scored the animals' bloat on a 0–5 severity scale (From 0 to 5, the severity of bloat increased): 0 = no foam; 1 = slight foam, but no pressure and abdominal bloat; 2 = some foam, enough pressure to expel foam, but no abdominal bloat; 3 = some foam, enough pressure to cause abdominal bloat on one side; 4 = some foam, enough pressure to cause abdominal bloat on right and left sides; 5 = some foam, severe abdominal bloat, and in a state of severe compression (23). The average of the three scorers' scores was taken as the bloat score for each goat. The mean and standard deviation (SD) of bloat score (BS) was calculated for each goat. Then, the SD values above and below the mean were used to group animals into high-bloat-score (HBS, BS > mean + 0.5 × SD) group, middle-bloat-score (MBS, mean 0.5 × SD < BS < mean + 0.5 × SD) group and low-bloat-score (LBS, BS < mean − 0.5 × SD) group according to the literature described method (24, 25). Rumen contents was collected from six goat in each group separately after 3 h after the 20th day morning feeding: The selected goats were immobilized and the rumen contents were collected using a stomach tube with a vacuum pump, the first 30 mL of contents pumped were discarded. Afterwards, 50 mL of rumen contents were collected and the collected samples were photographed and observed. The pH value was immediately measured using a portable magnetic-thunder pH meter (PHBJ-260, China), were filtered through four layers of gauze to obtain the rumen fluid and after centrifugation (3,000 g, 2 min), the rumen supernatant was collected and defined as the rumen original liquid (ROL) in this experiment. The ROL was stored at - 20°C for subsequent analysis.

2.5 Determination of foaming performance

The improved Roche method combined with the Rudin method was used to measure the foamability and foam stability of ROL (14, 26). The foaming power measured by the improved Roche method is expressed in terms of the volume of foam obtained under specific experimental conditions. The ROL was first treated in a constant temperature water bath at 39°C for half an hour, introduce 30 mL of ROL into a 100 mL airtight separatory funnel tube, slowly inject CO2 gas into the ROL, transforms ROL into a massive foam and continue ventilation at a pressure of 1 Pascal (Pa) for 60 s. Foaming power is related to the decay of foam volume within 5 min after foam formation. At the end of inflation, record the number of milliliters of foam formed at 30 s, 1 min, and 5 min after stopping the liquid flow, and the average value of the three time points is taken as the foaming production (mL) of the ROL. Open the sampling valve of the separatory funnel to let the rumen fluid flow out slowly, and collect it with a 50 mL beaker. When the foam is about to flow out, close the sampling valve immediately, and the fluid collected at this time is defined as rumen residual liquid (RRL). The Rudin method measures foam stability with attention to the effects of temperature, ventilation rate, gas type, and other factors. After ventilating to convert ROL to foam, The time required for the foam column to collapse by itself until all the foam completely disappears is used as an indicator of foam stability, expressed in foam persistence (min), each sample was measured 3 times and the average value was taken. The liquid formed after the collapse of the foam column was collected and defined as rumen foam liquid (RFL).

2.6 Determination of protein content

Coomassie blue staining method (14) was used to determine the protein content in the ROL, RRL and RFL, respectively. The brief process is: take 1 mL of liquid, centrifuge (10,000 g, 10 min) and take the supernatant and dilute it with normal saline at a ratio of 1:3. Dilute the Coomassie Brilliant Blue storage solution with distilled water at a ratio of 1:4. Add 1.5 mL of diluted Coomassie Brilliant Blue working solution to 25 μL of sample supernatant., shake and mix. After standing at room temperature for 10 min, the OD595nm value was measured under a microplate reader.

2.7 Determination of viscosity

The dynamic viscosity of ROL was determined by capillary viscometer (GB/T 22235–2008) (27). The process is: take 5 mL of ROL and add it to the inlet of the viscometer, and let it stand in a water bath at (39 ± 0.1) °C for 5 min. Use the ear washing ball to suck the liquid, and let the liquid flow down naturally under the action of gravity after the rumen fluid is sucked up to the scale line. Use a stopwatch to record the time t1 when the ROL flows through the upper and lower scale lines of the viscometer, and repeat 3 times to get the average value. Finally, take 5 mL of absolute ethanol and repeat the above steps to obtain the time t2, and finally calculate the liquid viscosity according to the formula.

2.8 Determination of saccharide content

The phenol-sulfuric acid method (14) was used to measure the total saccharide content in ROL, RRL and RFL. The process is: take the glucose standard solution and configure the concentration of the standard curve according to the instructions. Take 1 mL of liquid, centrifuge for 10 min to get supernatant, dilute 1,000 times with normal saline for later use. Take 2 mL of the standard substance of each concentration and the sample to be tested, add 1 mL of the pre-prepared 5% phenol solution, shake and mix. Slowly add 5 mL of concentrated sulfuric acid along the tube wall, shake well, and let stand at room temperature for 30 min. A microplate reader (SpectraMax-190, Molecular Devices, United States) was used to measure the OD490nm value of the standard and samples, and calculate the total saccharide concentration of the samples.

TABLE 2 The foaming performance, pH value and viscosity of rumen original liquid ($n = 6$).

Items	Group[1]		
	LBS[2]	MBS[2]	HBS[2]
Foaming production (mL)	11.97 ± 3.33^{c}	17.31 ± 0.70^{b}	23.76 ± 4.60^{a}
Foam persistence (min)	15.45 ± 7.78^{b}	29.91 ± 5.53^{a}	32.76 ± 5.96^{a}
pH value	6.36 ± 0.06^{c}	6.16 ± 0.03^{b}	6.00 ± 0.16^{a}
Viscosity (mpa.s)	52.74 ± 5.66^{c}	73.30 ± 8.48^{b}	97.67 ± 11.09^{a}

[1]In the same row, values with same or no small letter superscripts meaning no significant difference ($p > 0.05$), at the same time different small letter superscripts meaning significant difference ($p < 0.05$). [2]LBS, low rumen bloat score; MBS, medium rumen bloat score; HBS, high rumen bloat score.

TABLE 3 The comparison of protein and total saccharide content in the rumen liquid among groups ($n = 6$).

Items	Group[1]		
	LBS[2]	MBS[2]	HBS[2]
Protein content (g/L)			
Rumen original liquid	4.4 ± 0.47^{c}	5.15 ± 0.35^{b}	6.25 ± 0.69^{a}
Rumen foam liquid	5.09 ± 0.41^{b}	5.66 ± 0.37^{b}	6.78 ± 0.65^{a}
Rumen residual liquid	2.97 ± 0.43	2.87 ± 0.78	3.43 ± 0.97
Total saccharide content (g/L)			
Rumen original liquid	5.51 ± 0.99^{b}	8.75 ± 2.17^{b}	18.14 ± 4.88^{a}
Rumen foam liquid	5.65 ± 0.99^{b}	8.74 ± 1.9^{b}	17.97 ± 5.08^{a}
Rumen residual liquid	5.33 ± 0.74^{b}	8.43 ± 1.88^{b}	18.05 ± 4.85^{a}

[1]In the same row, values with same or no small letter superscripts meaning no significant difference ($P > 0.05$), at the same time different small letter superscripts meaning significant difference ($P < 0.05$). [2]LBS, low rumen bloat score; MBS, medium rumen bloat score; HBS, high rumen bloat score.

2.9 Determination of mineral element content

The concentrations of main mineral elements (Na, K, Ca, Mg, S, P, Cl) and trace mineral elements (Fe, Cu, Mn, Zn, Ni) in ROL were determined by an Agilent Technologies 7500c inductively coupled plasma mass spectrometry (28) (ICP-Ms) system (Agilent Technologies, Santa Clara, CA). According to the test results, the anion and cation balance value (CAD), the sum of the concentration of cations (Na, K, Ca, Mg, Fe, Cu, Mn, Zn, Ni) and the sum of the concentration of anions (S, P, Cl) was calculated, respectively. The CAD unit is mEq/Kg, and the calculation formula is CAD = (Na/23+ K/39) - (Cl/35 + S/16) (29).

2.10 Calculations and statistical analyses

All data were first preliminarily organized using Excel 2016. Using SPSS 23.0 statistical software, the Shapiro–Wilk test and Levene's test were first performed for normality and chi-square test (30). A one-way ANOVA was performed on the differences between groups. Duncan's multiple comparisons were then performed. And the Pearson method in SPSS 23.0 was used to perform correlation analysis and partial correlation analysis between foaming performance and components of rumen original fluid. The differences were considered statistically significant when $p < 0.05$. All data were presented as mean ± standard deviation.

3 Results

3.1 Comparison of foaming characteristics

Compared to the LBS group,the HBS and MBS groups exhibited significantly higher foaming production,and viscosity and significantly lower PH values. Moreover, the HBS group demonstrated notably higher foaming production, pH values was significantly lower than that of the MBS group. Additionally, the foam persistence of the LBS group was significantly lower than both the HBS and MBS groups ($p < 0.05$) (Table 2).

3.2 Comparison of protein and total saccharide content

The protein content in the ROL, the difference among the three groups all reached a significant level ($P < 0.05$), and the higher the bloat score, the higher the protein content in the ROL (Table 3). The protein content in the RFL was significantly lower in the MBS group and in the LBS group than in the HBS group ($P < 0.05$), but there was no significant difference between the MBS group and the LBS group ($P > 0.05$). The protein content in RRL had no significant difference among the three groups ($P > 0.05$). The total saccharide content in ROL, RFL and RRL was significantly lower in MBS group and LBS group than in HBS group ($P < 0.05$), but there was no significant difference between MBS group and LBS group ($P > 0.05$).

Regardless of grouping effect, the comparison of protein and total saccharide content among ROL, RRL and RFL was presented in Table 4. The protein content, ROL and RFL were significantly higher than that of RRL ($P < 0.05$), although the difference between ROL and RFL was not significant ($P > 0.05$), but in value, RFL was 10.81% higher than ROL. There was no significant difference in total saccharide content among ROL, RRL and RFL ($P > 0.05$) (Table 4).

3.3 Foaming performance and content of mineral elements

The content of Ni in HBS group and MBS group was significantly higher than that in LBS group ($P < 0.05$); the content of Na in HBS group and MBS group was significantly lower than that in LBS group ($P < 0.05$); The content of Ca in the HBS group was significantly higher than that in the MBS group and the LBS group ($P < 0.05$); the content

TABLE 4 The comparison of protein and total saccharide content among rumen original liquid, rumen foam liquid and rumen residual liquid ($n = 18$).

Items	Protein content (g/L)[1]	Total saccharide content (g/L)[1]
Rumen original liquid	5.27 ± 0.92[a,1]	10.80 ± 6.26
Rumen foam liquid	5.84 ± 0.86[a]	10.79 ± 6.16
Rumen residual liquid	3.09 ± 0.75[b]	10.61 ± 6.27

[1]The same column of data with the same or no letter indicates that the difference is not significant ($p > 0.05$), and the same column of data with different shoulder letters indicates that the difference is significant ($p < 0.05$).

TABLE 5 Contents of mineral elements in rumen fluid ($n = 6$).

Item	Group		
	LBS[2]	MBS[2]	HBS[2]
Fe (mg/Kg)	40.24 ± 10.27[1]	32.98 ± 6.92	44.84 ± 11.63
Cu (mg/Kg)	2.208 ± 0.67	1.596 ± 0.41	2.26 ± 0.29
Mn (mg/Kg)	16.7 ± 4.20	14.72 ± 2.61	15.56 ± 4.51
Zn (mg/Kg)	1.25 ± 0.38	0.906 ± 0.08	1.304 ± 0.35
Ni (mg/Kg)	0.154 ± 0.05[b]	0.236 ± 0.02[a]	0.28 ± 0.08[a]
Na (mg/Kg)	3,484 ± 41.59[b]	3,482 ± 8.36[a]	3,362 ± 61.81[a]
K (mg/Kg)	2090 ± 22.36[b]	2,142 ± 13.04[b]	2,272 ± 69.79[a]
Ca (mg/Kg)	351.2 ± 93.22[b]	418 ± 50.77[b]	509.2 ± 24.68[a]
Mg (mg/Kg)	78.84 ± 20.69[b]	136.6 ± 17.99[a]	151.8 ± 40.75[a]
S (mg/Kg)	306.4 ± 98.54	235.2 ± 22.84	278.6 ± 45.44
P (mg/Kg)	1966 ± 243.88	1940 ± 266.08	1984 ± 291.08
Cl (mg/Kg)	286.89 ± 53.76	297.16 ± 58.87	316.48 ± 63.37
CAD (mEq/Kg)	4980.71 ± 132.34	5091.64 ± 50.99	5038.92 ± 88.53
Sum of Cation (mg/Kg)	490.59 ± 119.55[c]	605.04 ± 65.78[b]	725.24 ± 38.31[a]
Sum of Anion (mg/Kg)	8133.29 ± 311.57	8096.36 ± 287.72	8213.08 ± 323.19

[1]In the same row, values with same or no small letter superscripts meaning no significant difference ($P > 0.05$), at the same time different small letter superscripts meaning significant difference ($P < 0.05$). [2]LBS, low rumen bloat score; MBS, medium rumen bloat score; HBS, high rumen bloat score.

of Mg in the HBS group and the MBS group significantly higher than the LBS group ($P < 0.05$); the sum of the cation concentrations in the HBS group and the MBS group was significantly higher than that in the LBS group ($P < 0.05$), and the sum of the cation concentrations in the HBS group was significantly higher than that in the MBS group ($P < 0.05$) (Table 5).

3.4 Correlation analysis between foam properties and rumen fluid properties

The pH value of rumen fluid was significantly negatively correlated with its foaming production and foam persistence ($P < 0.05$), and the viscosity was significantly positively correlated with foaming production and foam persistence ($P < 0.05$) (Table 6).

TABLE 6 Correlation analysis of pH value and viscosity with foaming production and foam persistence of rumen fluid ($n = 18$).

Items	Foaming production (mL)	Foam persistence (min)
pH value	−0.70**[1]	−0.82**
Viscosity (mpa.s)	0.96**	0.76**

[1]Pearson correlation analysis was used for correlation analysis, *$P<0.05$, **$P<0.01$.

3.5 Correlation analysis of foaming performance and components

There was a significant positive correlation between the protein concentration in the ROL and the foaming production and foam persistence of ROL ($P < 0.05$), which were also significant positive correlation ($P < 0.05$) with the total saccharide concentration of rumen fluid (Table 7).

The content of Ni in trace mineral elements was significantly positively correlated with the foaming production and foam persistence of rumen fluid ($P < 0.05$); the content of Na in main mineral elements was significantly Negatively correlated with the foaming production and foam persistence of rumen fluid ($P < 0.05$); the content of K, Ca, Mg in the main mineral elements and the foaming production and foam persistence of the rumen fluid were significantly positively correlated ($P < 0.05$); There was a significant positive correlation between the sum of cations and the foam production and foam persistence of rumen fluid ($P < 0.05$) (Table 7).

3.6 Analysis of partial correlation between foaming production performance and rumen fluid components

Correlation analysis was performed on the components in Table 7 those were significantly correlated with the foaming performance of the rumen fluid. The results showed that after controlling the influence of other components, only the protein and the foam persistence was significantly positively correlated ($p < 0.05$). The correlation between other components and rumen foaming production and foam persistence, and the correlation between protein and foaming production were no longer significant ($p > 0.05$). And compared with the correlation analysis, the correlation between the protein and the foam persistence in the partial correlation analysis decreased (Table 8).

4 Discussion

The current study shows that HCD leads to swelling and enlargement of the foam in the rumen, which hinders the expulsion of rumen fermentation gases, thus leading to rumen bloat (31). In the present experiment, a large amount of foam was also observed during the collection of rumen fluid from highly grouped goats. There is a lack of research on the causes of rumen foam formation, our study examines factors affecting rumen foam formation and foam stability with the aim of reducing rumen bloat in ruminant production through nutritional modulation.

TABLE 7 Correlation analysis between foaming performance and components of rumen original fluid ($n = 18$).

Items	Foaming performance of rumen original fluid	
	Foaming production (mL)	Foam persistence (min)
Protein (g/L)	0.90**	0.79**
Total saccharide (g/)	0.73**	0.56*
Fe (mg/Kg)	0.36	0.19
Cu (mg/Kg)	0.24	0.01
Mn (mg/Kg)	0.17	−0.16
Zn (mg/Kg)	0.38	0.04
Ni (mg/Kg)	0.81**	0.58*
Na (mg/Kg)	−0.90**	−0.52*
K (mg/Kg)	0.96**	0.62*
Ca (mg/Kg)	0.88**	0.68**
Mg (mg/Kg)	0.55*	0.76**
S (mg/Kg)	0.15	−0.13
P (mg/Kg)	0.17	0.24
Cl (mg/Kg)	0.40	0.3
CAD 2 (mEq/Kg)	−0.467	−0.168
The sum of the cations (mg/Kg)	0.84**	0.75**
The sum of the anions (mg/Kg)	0.33	0.29

[1]Pearson correlation analysis was used for correlation analysis, *P<0.05, **P<0.01.2. CAD, anion and cation balance value.

TABLE 8 Partial correlation analysis between foaming performance and components of rumen fluid.

Items	Foaming performance of rumen fluid	
	Foaming production (mL)	Foam persistence (min)
Protein (g/L)	0.49	0.75*
Total saccharide (g/L)	−0.69	−0.33
Ni (mg/Kg)	0.47	0.10
Na (mg/Kg)	−0.51	−0.45
K (mg/Kg)	0.43	−0.56
Ca (mg/Kg)	0.63	0.52
Mg (mg/Kg)	0.58	0.62
The sum of the cations (mg/Kg)	−0.55	0.50

[1]Pearson correlation analysis was used for correlation analysis, *P<0.05, **P<0.01.

4.1 PH value and rumen foam

Previous studies have shown that when ruminants were fed HCD, the pH value in the rumen usually decreases (32–35) due to the accumulation of VFAs produced by fermentation of rumen microorganisms (36, 37). This was also verified in the present experiment. The pH value of all the samples tested in this study was below 6.5, and it was also found that the pH value of the rumen fluid was significantly negatively correlated with the foaming production and foam persistence ($P < 0.05$) (Table 2), which may be due to the fact that pH affects the hydrophilicity of the headgroups of the solution, leading to structural changes in the film of the foam fluid (38). This result suggests that the lower the rumen pH value, the higher risk of developing foamy rumen bloat, which is consistent with the higher incidence of rumen bloat in production with higher ratio of dietary concentrate.

4.2 Viscosity and rumen foam

The viscosity of the rumen fluid was significantly positively correlated with the foaming production and foam persistence of the goats fed the HCD ($P < 0.05$) (Table 7). Previous studies have shown that the higher the viscosity of a liquid, the more stable the foam it produces (39). Lewis et al. (40) also showed that improving liquid viscosity has a positive impact on the stability of liquid foam, which is consistent with the results of this study. Cheng et al. (41) believed that a soluble mucopolysaccharide secreted by bacteria in the rumen increased the viscosity of rumen fluid. This soluble mucopolysaccharide was an exopolysaccharide produced by microbial fermentation. However, in this study, the high viscosity of rumen fluid may also be related to the high proportion of corn, meal and cake material in the diet, because these feedstuffs also contain a high proportion of soluble non-starch polysaccharides (such as β-glucan, arabinoxylan, etc). Dissolution of endosperm cell wall macromolecules NSP in high concentrate feeds alters the molecular chain length, leading to an increase in viscosity (42). The viscosity of rumen fluid is elevated by the increase in NSP content in HCD, resulting in a large amount of stable foam in the rumen. Previous studies have shown that NSP enzymes are able to cleave the long chain structure of NSP (43), which reduces the viscosity of chowders in the gastrointestinal tract of animals. This provides a new idea to reduce the occurrence of rumen bloat by altering the viscosity of rumen fluid.

4.3 Protein and rumen foam

The present study found that the protein content in the RFL was much higher than that in the ROL and RRL (P<0.05), which indicated that the protein could be enriched in the rumen foam, which was consistent with the study of Ying et al. (44). As the skeleton component of foam, protein plays a vital role in maintaining the stability of rumen foam. Proteins are low-activity surfactants that can be adsorbed at the air-liquid interface and be carried into the foam, where they are finally enriched in the foam liquid. When its peptide chain is stretched on the foam liquid surface, it will form a two-dimensional protective network through the interaction of intramolecular and intermolecular forces, which can maintain the stability of the foam. Since true protein in ruminant saliva is almost negligible, rumen protein can only be derived from dietary and microbial sources. Although rumen microorganisms can synthesize a large number of bacterial proteins every day, there is no clue that rumen foam formation is related to bacterial proteins so far. Rumen

microorganisms can also synthesize a large number of secreted proteins. If the formation of rumen foams is related to the secreted proteins of microorganisms, and only high-concentrate diet can cause the formation of a large number of rumen foams, it can be inferred that high-concentrate diet may affect the microbial community structure of the type or quantity of secreted proteins by changing the structure and composition of rumen microorganisms, and ultimately induce rumen foams. Whether this conjecture is correct remains to be proved. However, previous studies have shown that soluble protein level in feed plays a role in rumen bloating, and reducing soluble protein level in feed can reduce the risk of rumen bloating (45). The application of some antinutritional factors can reduce soluble proteins in the rumen, for example, water-soluble condensed tannins can bind to soluble proteins and convert them into condensed tannin-bound forms of proteins, thus reducing the amount of soluble proteins in the rumen and reducing the production of rumen foam (46). The key to reducing the incidence of rumen bloat is to control the amount of soluble protein in the rumen, which requires research into better nutritional strategies to counteract it.

4.4 Total saccharides and rumen foam

In this experiment, although the total saccharide content in the rumen fluid has a certain facilitating effect on improving the foaming production and foam persistence of the rumen fluid, the total saccharide is not significantly enriched in the foam fluid, but is more uniformly dispersed in the RFL, RRL and ROL, this is the same result as Michael et al. (44). Generally speaking, during the formation of foam, substances in the liquid that are conducive to foaming will be adsorbed on the surface of the foam, such as protein, which will change the surface activity of the foam and affect the ability of the foam to drain liquid, thus affecting the foam stability. The reason why carbohydrates could not be enriched in foam fluid may be that they are low polar molecules and thus cannot be adsorbed. Soluble polysaccharides have a high characteristic viscosity, which when dissolved in water leads to an increase in solution viscosity (47), and this increase in viscosity slows down the liquid flow rate at the surface of the foam, thus contributing to the stabilization of the foam. Previous studies have found that rumen microbial mucopolysaccharides produced by rumen bacterial fermentation are the main substances leading to the increase in rumen viscosity, and that such mucopolysaccharides may contribute to the generation of rumen foams, but this role has not been studied yet (31).

4.5 Minerals and rumen foam

Mineral elements play a very important role in maintaining the normal physiological activities of animals (48–51). For example, the distribution of Ca in extracellular fluid and soft tissue is related to the blood agglutination, membrane permeability, muscle contraction, nerve impulse conduction, secretion of some hormones, and activity and stability of some enzymes (49). K is the main cation in the intracellular fluid, which participates in the physiological activities of maintaining osmotic pressure to regulate acid–base and water balance. Fe is an essential component of many proteins related to the transport and utilization of oxygen. These proteins include hemoglobin, myoglobin, and many cytochromes and iron sulfur proteins in the electron transport chain. Fe is also a component or activator of several mammalian enzymes (49–51) Therefore, macro and trace mineral elements are generally supplemented in ruminant feed. This experiment found that mineral elements also have a significant impact on the foaming performance of rumen fluid. Compared with anions, mineral cations have a greater impact on the foaming performance of rumen fluid. The contents of Ni, Mg, Ca and K in the rumen fluid detected in this test, as well as the sum of cations, were significantly positively correlated with the foaming production and foam persistence of the rumen fluid ($P<0.05$) (Table 7). Harris et al. (17) reported that trace amounts of mineral cations and mineral hydroxides would affect the foam stability in some cases, the concentration of Ni^{2+} would affect the stability of rumen foam, and the reduction of foam stability was accompanied by the decrease of Ni^{2+} concentration in the foam. Smith et al. (52) found that Ca^{2+} or Mg^{2+} solution was used to spray alfalfa foliage. After eating the alfalfa, the incidence of rumen bloat of lambs increased significantly, and the concentrations of Ca^{2+} and Mg^{2+} of animals with bloat increased significantly increased. Majak et al. (53) found that the occurrence of bloat in cattle was related to the increase and decrease of K^+ and Na^+ concentrations in the rumen. The above research results were consistent with the results of this experiment. It is worth noting that, unlike other cations, Na^+ showed a significant negative correlation with the foaming performance of rumen fluid in this study, suggesting that increasing the concentration of Na in HCD may significantly reduce the occurrence of rumen bloat Rate. This finding can be used in the development of technologies to prevent high concentrate diet rumen bloat.

Rumen bloat can have a direct impact on animal health, which in turn can lead to economic losses. In studies by Rumbaugh (54) and Tanner et al. (55), the average annual loss due to rumen bloat in Australia was estimated at $180 million, while in the United States it was estimated at more than $310 million per year. These figures underscore the financial strain imposed on livestock production due to rumen bloat. It is vital to find out what is causing the bloat. In this study, the influence of various components of rumen fluid on the interrelationship of foam formation was analyzed in depth. Correlation analyses emphasized the important relationship between protein, total sugars and mineral elements and rumen fluid foaming characteristics, but the partial correlation analysis showed that after controlling the influence of other factors, only protein was still related to the foam persistence of rumen foam. This suggests that, although a variety of components contribute to foaming, the production and stability of foam in rumen fluid is primarily influenced by the presence and impact of proteins. This finding confirms the conclusions of Isabel et al. (56). This demonstrates the decisive role of proteins in all the factors affecting the foaming properties of rumen liquor. Tannin is a secondary metabolite in plants. Tannin can promote protein metabolism in the rumen of ruminants and increase the absorption of amino acids in the small intestine of animals. Some studies have shown that adding tannins to diets can eliminate bloat, due to the fact that tannins can destroy protein foam (57). However, tannins are poorly palatable and have a bitter taste that animals do not like to feed on. In one study, goats and sheep preferred poorly palatable hay when fed high grain concentrates, and the addition of plant odors to feed increased ruminant intake (58). This is feasible for the addition of

moderate amounts of tannins to feeds in production to control rumen bloat. In addition, the defoamer's help to break the tension on the surface of the bubbles, thus making it easier for the bubbles to burst. Defoamers accelerate the fusion of bubbles, making it easier for gases to escape. Such additives require further research.

This study highlights the importance of proteins in influencing rumen foam performance, but further research is needed to more fully understand the role of other factors in rumen distension. For example, total saccharide, its contribution to the foaming performance of rumen fluid may be because it increases the viscosity of the whole rumen fluid system, enriches more protein into the foam of rumen fluid, and helps to improve the stability of rumen foam. Sodium bicarbonate was added to the diets in this study, which is commonly used in production to increase rumen pH and reduce the risk of rumen acidosis, but sodium bicarbonate and other inorganic salts in the feeds react with fatty acids to form carboxylates (59), which have a better foaming function. Does this have an effect on rumen foam formation, Whether this could have an effect on rumen foam formation needs further investigation. Additionally, although this study showed that proteins are the main factor influencing rumen foam production and foam stability, no in-depth study was conducted to investigate the source of the proteins. A deeper understanding of the function of proteins in the rumen and their association with foam formation can help improve feed formulation and management practices to reduce the incidence of rumen foamy bloat.

5 Conclusion

This study found that protein content in rumen fluid was significantly and positively correlated with foam production and foam stability under high concentrate feeding conditions in goats and played a decisive role. Reducing the protein content of the diet during production is essential for rumen bloat prevention. Application of low protein diets is an effective measure to prevent rumen bloat.

Data availability statement

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

Author contributions

ZT: Writing – original draft, Writing – review & editing. LW: Formal analysis, Funding acquisition, Supervision, Writing – review & editing. JFL: Project administration.

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Conflict of interest

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

Publisher's note

Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2024.1299404/full#supplementary-material

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Effects of adding *Allium mongolicum* Regel powder and yeast cultures to diet on rumen microbial flora of Tibetan sheep (*Ovis aries*)

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The purpose of this experiment was to study the effect of *Allium mongolicum* Regel powder (AMR) and yeast cultures (YC) on rumen microbial diversity in Tibetan sheep in different Ecological niches. A total of 40 male Tibetan lambs with an initial weight of 18.56 ± 1.49 kg (6 months old) were selected and divided into four groups (10 sheep/pen; $n = 10$). In the Control Group, each animal was grazed for 8 h per day, in Group I, each animal was supplemented with 200 g of concentrate per day, in Group II, each animal was supplemented with 200 g of concentrate and 10 g of AMR per day, in Group III, each animal was supplemented with 200 g of concentrate and 20 g of YC per day. The experiment lasted 82 days and consisted of a 7-day per-feeding period and a 75-day formal period. The results indicated that at the phylum level, the abundance of Bacteroidota and Verrucomimicrobiota in L-Group II and L-Group III was increased, while the abundance of Proteobacteria was decreased in the LA (Liquid-Associated) groups. The proportion of F/B in S-Group II and S-Group III was increased compared to S-Group I and S-CON in the SA (Soild-Associated) group. At the genus level, the abundance of uncultured_rumen_bacterium and Eubacterium_ruminantium_group in L-Group II and L-Group III was increased. Furthermore, while the abundance of Rikenellaceae_RC9_gut_group was decreased in the LA, the abundance of Prevotella and Eubacterium_ruminantium_group was increased in the S-Group II and S-Group III compared to S-Group I and S-CON. The abundance of probable_genus_10 was the highest in S-Group II in the SA group. After the addition of YC and AMR, there was an increase in rumen microbial abundance, which was found to be beneficial for the stability of rumen flora and had a positive impact on rumen health.

KEYWORDS

Tibetan sheep, *Allium mongolicum* Regel powder, yeast cultures, rumen microbiota, 16S

Introduction

In pursuit of higher breeding returns, antibiotics and antigenicity animal preparations are widely used in livestock and poultry production to improve feed conversion rate, anti-stress ability, and animal production efficiency (1). Resistance to bacterial pathogens is a result of the long-term use of antibiotic additives (2). Except for a small amount that can break down

into non-toxic and harmless substances, 30–90% of antibiotics will enter the environment with excreta (3). Reaching a certain level of antibiotic residues can affect the structure of the microbial community, resulting in serious ecological imbalances that ultimately threaten human public health. Therefore, adding green additives such as plants, enzymes, and probiotics with regulatory activity to replace antibiotics is of great significance for improving animal productivity and rumen (4). At present, green additives that have been developed to replace antibiotics include microecological preparations, feed enzyme preparations, Chinese herbal medicine additives, and oligosaccharides (5, 6). *Allium mongolicum* Regel powder (AMR) has been widely studied for its safe and non-toxic side effects, improved feed palatability, and improved mutton flavor, which have led to increased productivity in ruminants (1). Research has found that providing garlic and its organic components to ruminants can help improve rumen fermentation and digestive capacity (7). Other research found that Allium plants can affect rumen fermentation (8). Previous research has shown that adding *Allium mongolicum* essential oil to the diet can effectively reduce the abundance of Bacteroidota flora, while simultaneously increasing the relative abundance of Firmicutes. This dietary modification also leads to an elevated Bacteroidota to Firmicutes ratio, which is known to promote improved energy utilization and facilitate animal growth and development (9, 10). Ethanol extract of *Allium mongolicum* was added to the diet and it was found that the abundance of Bacteroidota in the AME group was lower, while the abundance of Firmicutes, Proteobacteria, and Cyanobacterium was higher. At the genus level, it can reduce the abundance of bacterial genera related to the production of propionic acid, such as *Prevotella, Succinniclassicum, Selenomonas*, etc. In summary, *Allium mongolicum* and its extract have a certain regulatory effect on rumen fermentation and microbial flora. As a green additive for ruminants, *Allium mongolicum* and its extract have broad market prospects and huge potential value.

There has been a significant increase in concern over the effects of products containing active yeast and yeast cultures on rumen fermentation over the past few decades. Adding YC to feed can promote the digestion and absorption of nutrients by animals, reduce feed costs, and play an important role in maintaining animal health. It is increasingly being used to improve animal production performance and breeding efficiency (11). This study reveals that yeast cultures (YC) treated with high pressure (inactivated) do not have an effect on increasing the number of effective rumen bacteria (12, 13). However, YC that still have metabolic activity can successfully improve the activity of rumen microorganisms and increase the number of beneficial bacteria. Studies have shown that adding active dry yeast can improve the diversity and relative abundance of rumen microorganisms in fattening bulls (14). There are also studies showing that YC can increase microbial species abundance in the large intestine of calves, stimulate the colonization of fibrinolytic bacteria, and thus increase the production of butyrate (15).

Currently, the mechanism of the effects of YC and AMR on the production performance and rumen microbiota of ruminants is not clear, and there are differences in their application effects in production. Therefore, in-depth research on the effects of YC and AMR on the metabolism and physiological characteristics of gastrointestinal flora will contribute to a better understanding of the interaction between YC, *Allium mongolicum*, and gastrointestinal microorganisms in future research. In addition, it also helps us to develop green additives that can better improve the nutrition of anti-animals.

Materials and methods

Test materials

Yeast Culture (YC) was provided by an animal health limited company. The nutritional components of the product are as follows: Crude protein (CP) $\geq$ 12%, water $\leq$ 10%, Crude fiber (*CF*) $\leq$ 5%, Crude ash $\leq$ 3%, Ether extract (EE) $\geq$ 3%, *Saccharomyces cerevisiae* $\geq 2.0 \times 108$ CFU/g.

The preparation of Mongolian shallot Regel powder (AMR) involves drying and crushing fresh AMR purchased from the market. The preparation process is as follows. Place the fresh AMR flat on the clean and dry enamel plate and put it in the dry oven, set the temperature to 65°C, heat at a constant temperature for 12 h, treat with a 60–120 mesh crusher to form a powder, pass through an 80 mesh sieve, pack in plastic bags, and store at room temperature for future use. The nutritional content of AMR is shown in Table 1.

Animals and experimental design

A total of 40 healthy male Tibetan sheep by Shangchuang Tibetan Sheep Husbandry Cooperative in Haidong, Qinghai Province were used as experimental animals. This study adopted a single-factor design for the experiments; forty male Tibetan sheep lambs with an initial weight of 18.56 ± 1.49 kg (6 months old) were selected and divided into four groups (10 sheep/pen; $n = 10$). Each animal in the control group was grazed for 8 h per day. The other groups were grazed for 8 h every day. In Experiment Group I, each animal was supplemented with 200 g of concentrate per day. In Experiment Group II, each animal was supplemented with 200 g of concentrate and 10 g of AMR per day (the amount of AMR added was based on the experimental results of Zhang Xiuyuan) (16). In Experiment Group III, each animal was supplemented with 200 g of concentrate and 20 g of YC per day (the amount of YC added was recommended in the instructions for use). The pre-trial period was 7 days, and the trial period was 75 days. Supplementary feed concentrate is produced by Lanzhou Zhengda Co., Ltd., and its composition and nutritional levels are shown in Table 2.

TABLE 1 The nutritional content of *Allium mongolicum* Regel powder.

Items	Content
DE/(MJ/kg)	12.71
ME/(MJ/kg)	10.47
Dry matter	93.40
Crude protein	22.34
Ether extract	4.68
Neutral detergent fiber	33.31
Acid detergent fiber	24.61
Calcium	1.40
Phosphorus	0.47

TABLE 2 Composition and nutrient levels of the supplemented concentrate after grazing (DM basis) %.

Ingredients	Content	Nutrient levels2)	Content
Soybean meal	5.00	DE/(MJ/kg)	12.04
Corn	50.00	ME/(MJ/kg)	9.87
Wheat bran	5.00	Dry matter	86.84
Cottonseed meal	3.00	Crude protein	14.89
Alfalfa hay	35.00	Ether extract	2.19
Limestone	0.50	Neutral detergent fiber	19.23
CaHPO4	0.60	Acid detergent fiber	14.26
NaCl	0.50	Calcium	1.08
Premix[1])	0.40	Phosphorus	0.46
Total	100.00		

Breeding management

Between October 2021 and January 2022, the experiment was conducted at the Shangchuang Tibetan Sheep Husbandry Cooperative in Haidong, Qinghai Province. Before the start of the experiment, the sheep shed was uniformly cleaned and disinfected. After deworming the sheep, the vaccine was injected according to the normal immune procedure, and the ear labels of the test sheep were registered. The sheep were divided into four columns according to their groups, with 10 males in each column for feeding. Daily environmental management of the sheep shed was strengthened. During the test, in addition to carrying out regular feeding, it was important to carefully observe the test sheep's daily feeding and water intake to ensure normalcy. Additionally, it is crucial to monitor rumination patterns, fecal consistency, and the mental well-being of the test subjects. Keep the feeding environment and conditions of each group consistent during the test period. To ensure that the test sheep could receive a certain amount of AMR and YC, the AMR or YC was mixed with a small amount of concentrate before the sheep could freely feed. To ensure free drinking during this time, it is recommended to add extra supplements after testing the sheep.

Sample collection and index determination

Fresh rumen contents from slaughtered Tibetan sheep were collected following the sampling requirements of microbial testing after the experiment. We mainly collected samples from the rumen and transferred them to sterile frozen storage tubes. We immediately placed them in liquid nitrogen and transferred them to −80°C ultra-low temperature refrigerators for analysis of rumen microbiota.

Sample pretreatment

To pretreat the rumen liquid-associated (LA) microbiota, the filtered rumen fluid has been centrifuged at 10,000 rpm for 20 min at room temperature. After discarding the supernatant, the precipitate was collected. A small amount of physiological saline was used to suspend the precipitate, which served as a rumen liquid phase microorganism. Finally, the suspension was stored at −80°C.

To pretreat the rumen liquid-associated (LA) microbiota, 50 g (based on actual weight) of solid sample was transferred to a centrifuge tube and 150 mL (based on actual weight) of sterilized physiological saline was added to resuspend plant particles. The mixture was shaken and mixed well for 30 s and then centrifuged at 350 rpm for 15 min at room temperature to precipitate plant particles. The supernatant was carefully removed, transferred to a new tube (marked as S), and then placed at 4°C or on ice. The recovered sediment particles were suspended again in 25 mL (depending on the actual weight) of anaerobic paint thinner containing 0.15% (v /v) Tween-80. The mixture was shaken and mixed for 30 s, then put on ice for 2.5 h to elute closely attached bacteria. The eluted mixture system was centrifuged at 350 rpm for 15 min at room temperature to remove plant particles. The filtrate was mixed with the supernatant in the S bottle and centrifuged at 4°C, at 10000 rpm for 20 min. The precipitated portion was the recovered solid microbial cells; the sediment was resuspended in the smallest volume of sterilized physiological saline, which was the rumen solid-phase microorganism, and stored for analysis at −80°C.

In order to distinguish the groups after different treatments, the LA test groups were named separately as L-CON, L-Group I, L-Group II, and L-Group III, and the SA test groups were named separately as S-CON, S-Group I, S-Group II, and S-Group III.

Measurement indicators and methods

The TGuide S96 magnetic bead method soil /fecal genome DNA extraction kit (Tiangen Biotechnology (Beijing, China) Co., Ltd.) was used to extract total microbial DNA from Tibetan sheep rumen samples. After extraction, the microplate reader (manufacturer: Gene Company Limited, model synergy HTX) was used to detect the concentration of nucleic acid, according to the detection and amplification, and 1.8% agarose was used to detect the integrity of the PCR products after amplification. Following extraction, the concentration of nucleic acid was determined using a microplate reader (manufacturer: Gene Company Limited, model: synergy HTX). The integrity of the PCR products obtained from the amplification was assessed using 1.8% agarose gel electrophoresis. Specific primers containing barcodes for the V1–V9 variable regions of 16S rRNA genes of bacteria that meet both total and quality standards were designed. Veriti96well9902 (Applied Biosystems, Foster City, CA, United States) PCR instruments and a 16S full-length reaction system (30 μL system) for PCR amplification were used (Table 3). The specific primer sequence was 27F_(16S-F) (5'-AGRGTTTGATYNTGGCTCAG-3′) and1492R_(16S-R) (5'-TASGGHTACCTTGTTASGACTT-3′).

TABLE 3 16S full-length reaction synthesis (30 μL synthesis).

	Dosage (μl)
Genomic DNA	1.5
NFW	10.5
KOD ONE MM	15
Barcode primer pair	3
Overall system	30

Illumina MiSeq (Illumina Inc. San Diego, CA, United States) sequencing and data analysis were completed by Biotech Co., Ltd. (Beijing, China). After extracting the total DNA samples, specific primers with barcodes were synthesized based on the full-length primer sequences for PCR amplification. The resulting products were purified, quantified, and normalized to form sequencing libraries (SMRT Bell). The constructed libraries underwent quality inspection, and those that passed the quality check were sequenced using PacBio Sequel II. The data generated by PacBio Sequel II was in BAM format, and the CCS files were exported using the SMRT Link analysis software. The data was then identified based on the barcode sequences to differentiate the samples and transformed into FASTQ format. The sequenced sequences were subjected to OTU (Operational Taxonomic Units) clustering and bioinformatics statistical analyses using software such as Mothur (version v.1.30.1) and Usearch (version 7.0), with a similarity of 97%.

Statistical analysis

Taxonomy annotation of the OTUs/ASVs was performed based on the Naive Bayes classifier in QIIME2 (17) using the SILVA database (release 138.1) with a confidence threshold of 70%. The Alpha diversity was calculated and displayed using QIIME2 and R software. Beta diversity was determined to evaluate the degree of similarity of microbial communities from different samples using QIIME2. Principal coordinate analysis (PCoA), heatmaps, UPGMA, and nonmetric multidimensional scaling (NMDS) were used to analyze the beta diversity. Furthermore, we employed Linear Discriminant Analysis (LDA) effect size (LEfSe) (18) to test the significant taxonomic differences between groups. A logarithmic LDA score of 4.0 was set as the threshold for discriminative features. In addition, at the phylum, class, order, family, genus, and specifications levels, the composition of each sample community was statistically analyzed. Meanwhile, the charting was performed using the online platform.

Results

In the LA and SA groups, a total of 370,848 CCS sequences were obtained after 48 samples were sequenced and identified by barcode. Each sample produced at least 6,760 CCS sequences, with an average of 7,726 CCS sequences (Supplementary Table S1). The effective tags of all samples were clustered with 97% identity, and a total of 1,488 OTUs were obtained. The representative sequences of OTUs were selected for species annotation, and a total of 19 phyla, 32 classes, 55 orders, 108 families, 209 genera, and 341 species were annotated in all samples (Supplementary Table S2).

Effects of AMR and YC on Alpha diversity of liquid-associated and solid-associated microbiota in rumen

According to the Alpha diversity analysis of OTU based on the 16s results of rumen microorganisms, In the rumen LA microorganisms, significant differences ($p < 0.05$) were observed in the ACE and Chao1 indexes among the four experimental groups. The ACE index and Chao1 index of the L-CON, L-Group I, and L-Group II experimental groups were significantly higher than those of L-Group III. The Simpson index and Shannon index did not show significant differences between the groups (Figure 1). As shown in Figure 2, according to the OTU analysis of 16s rumen microbial detection results, the Alpha diversity analysis of rumen SA microorganisms revealed no significant differences in the ACE index, Chao1 index, and Shannon index among the four experimental groups. However, there were significant differences observed in the Simpson index between the groups. The Simpson index of the S-CON, S-Group II, and S-Group III was significantly higher than that of S-Group I.

As shown in Figure 3A, there were 338 OTUs in the rumen LA flora of the four experimental groups, of which 160 were L-CON specific OTUs, 164 were L-Group I specific OTUs, 99 were L-Group II specific OUTs, and 59 were L-Group III specific OTUs. The principal coordinate analysis of species abundance data at the OTU level shows that the coefficients of PCoA plot samples PC1 and PC2 were, respectively, 19.68 and 15.75%. It was observed that there was a higher degree of individual aggregation between L-CON and L-Group III, suggesting a higher similarity in microbial community structure in this group compared to other sample types. Additionally, the samples within this group exhibited close proximity to each other. The microbial community structure of L-Group I and L-Group II was relatively independent of L-CON; in addition, a significance test ($R2 = 0.381$, $p = 0.001$) was conducted in conjunction with PERMANOVA, indicating differences in the structure of rumen LA microbial communities (Figure 3B). According to Figure 3C, there were 351 OTUs in the rumen SA flora of the four experimental groups, of which 148 were S-CON specific OTUs, 130 were S-Group I specific OTUs, 79 were S-Group II specific OTUs, and 77 were S-Group III specific OTUs. The principal coordinate analysis was based on the species abundance data at the OTU level. The PCoA plot samples exhibited coefficients of 25.50 and 16.57% for PC1 and PC2, respectively. The bacterial communities in S-CON, S-Group I, S-Group II, and S-Group III appear to be relatively independent. A significance test ($R2 = 0.457$, $p = 0.001$) was conducted in conjunction with PERMANOVA, indicating differences in the structure of rumen liquid phase microbial communities (Figure 3D).

Effects of AMR and YC on beta diversity of liquid-associated and solid-associated microbiota in rumen

The main differences in the phylum level of rumen bacteria between the four groups are shown in Figure 4A. Bacteroidota and Firmicutes are the two dominant bacteria in Phylum with microbial abundance higher than 1% at the level of rumen LA microbiota. The

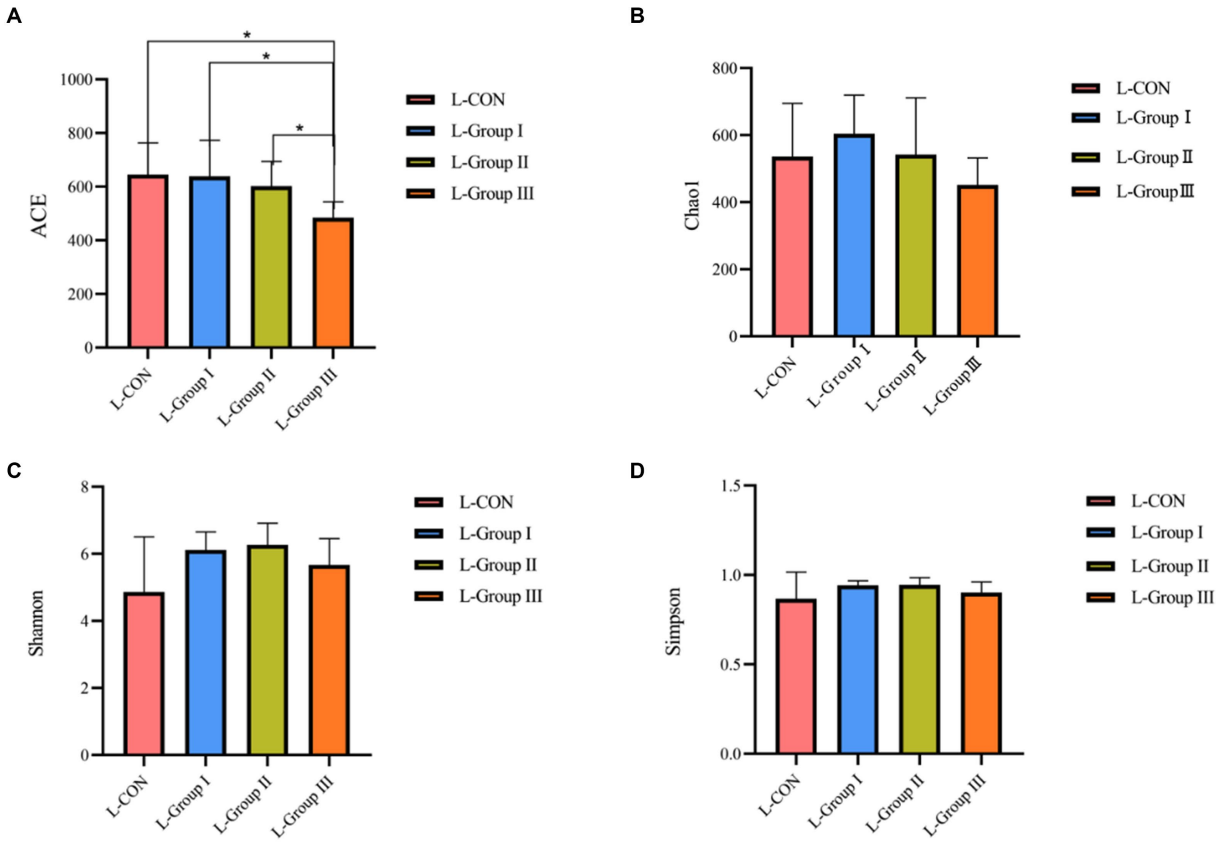

FIGURE 1
Effects of AMR and YC on Alpha diversity index of rumen LA microorganisms. ACE **(A)**, Chao1 **(B)**, Shannon **(C)**, and Simpson **(D)**, respectively.

relative abundance of Bacteroidota and Firmicutes in the L-CON, L-Group I, L-Group II, and L-Group III were 58.23, 38.61, 44.71, and 55.49% and 36.75, 54.57, 50.22, and 38.62%, respectively, with significant differences between the groups ($p < 0.05$). The abundance of Verrucomimicrobiota significantly increased while Proteobacteria decreased in L-Group II. In SA rumen microorganisms, the two dominant strains are Bacteroidota and Firmicutes. The relative abundance ratio of Firmicutes in the four experimental groups was as follows: S-CON>S-Group II > S-Group III > S-Group I, with values of 58.66, 50.53, 49.75, and 42.72%, respectively. On the other hand, the relative abundance ratio of Bacteroidota in the four experimental groups was as follows: S-Group I > S-Group III > S-Group II > S-CON, with values of 54.14, 47.23, 46.50, and 38.23%, respectively. The relative abundance of the two dominant Phylum in this experiment showed a significant negative correlation (Figure 4A).

At the genus level, in LA rumen microorganisms, the abundance of *Prevotella* and *Lachnospiraceae_XPB1014_group* in L-CON was significantly higher than in L-Group I, L-Group II, and L-Group III ($p < 0.05$). Additionally, the abundance of *probable_genus_10* in L-Group I was significantly higher than in L-CON, L-Group II, and L-Group III ($p < 0.05$). Moreover, the abundance of *Prevotellaceae_UCG_001* in L-Group II was significantly higher than in L-CON, L-Group I, and L-Group III ($p < 0.05$). Furthermore, the abundance of *uncultured_rumen_bacterium* in L-Group II and L-Group III was significantly higher than in L-CON and L-Group I ($p < 0.05$) (Figure 4B). In SA rumen microorganisms, the abundance of *Christensenellaceae_R_7_group* and *Lachnospiraceae_XPB1014_group* in S-CON was significantly higher than in S-Group I, S-Group II, and S-Group III ($p < 0.05$). The abundance of *Dialisrer* and *uncultured_rumen_bacterium* in S-Group I was significantly higher than in S-CON, S-Group II, and S-Group III ($p < 0.05$). Furthermore, the abundance of *probable_genus_10* in S-Group II was significantly higher than in S-CON, S-Group I, and S-Group III ($p < 0.05$). Lastly, the abundance of *Eubacterium_ruminantium_group* in S-Group III was significantly higher than in S-CON, S-Group I, and S-Group II ($p < 0.05$; Figure 4B).

At the genus level, the abundance of *Prevotella* was significantly higher in L-CON than in S-CON; the abundance of *uncultured_rumen_bacterium*, *Lachnospiraceae_XPB1014_group,* and *Succiniclasticum* was higher in S-CON than in L-CON; the abundance of *Prevotella, Rikenellaceae_RC9_gut_group,* and *Dialister* was higher in L-Group I than in S-Group I; the abundance of *uncultured_rumen_bacterium* was higher in S-Group I than in L-Group I; the abundance of *Prevotella* and *Succiniclasticum* was higher in L-Group II than in S-Group II, but the abundance of *probable_genus_10* was higher in S-Group II than in L-Group II; the abundance of *Prevotella* and *uncultured_rumen_bacterium* was higher in L-Group III than in S-Group III; and the abundance of *Rikenellaceae_RC9_gut_group* and *Eubacterium_ruminantium_group* was higher in S-Group III than in L-Group III. Interestingly, *Prevotella* was higher in the LA groups than in the SA groups (Figure 4B). The abundance of *probable_genus_ 10* was highest in

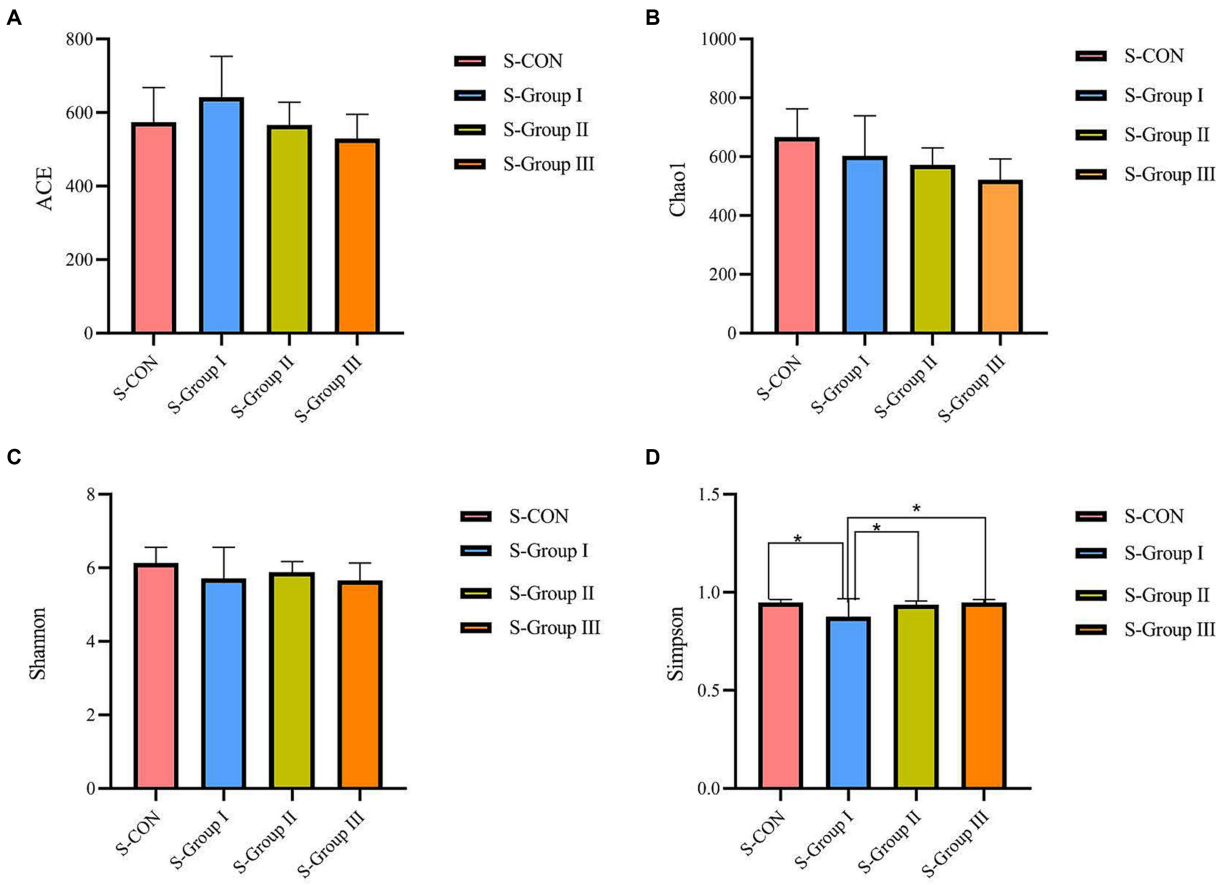

FIGURE 2
Effects of AMR and YC on Alpha diversity index of rumen SA microorganisms. ACE **(A)**, Chao1 **(B)**, Shannon **(C)**, and Simpson **(D)**, respectively.

S-Group II and the abundance of *Prevotelaceae_ UCG_ 001* was lowest in L-CON in the heat map (Figure 4C).

The LefSe Cladogram and LDA (Score > 4.0) in Figure 5 reveal significant differences in certain species between the L-CON and experimental groups. The use of concentrates and additives can cause changes in the microbiota in the rumen LA microbiota.

Setting the LDA threshold to 4 and comparing the differences between the four groups of microbial communities using LEfSe (Figure 6) shows that there are differences in the significantly enriched microbial genera between different experimental groups of SA rumen microbiota.

Discussion

Generally, the common method for studying rumen microorganisms is to collect rumen fluid. However, different rumen sites, including rumen epithelium, rumen juice, and solid rumen chyme, may have different microorganisms (19, 20). A previous study based on 16 s Ribosome RNA (rRNA) gene sequencing showed that there was no difference in the classification composition between the solid adhesion environment and the liquid environment in the rumen, which was mainly distinguished by the relative abundance of species (21).

In this study, the ACE index and the Chao1 index in the Alpha diversity analysis were significantly different among the LA groups. The ACE Index of L-CON and L-Group I was significantly higher than that of the experimental L-Group III group, and the Chao1 Index of L-CON, L-Group I, and L-Group II was significantly higher than that of L-Group III. The Simpson Index and Shannon Index showed no significant difference, indicating that the addition of AMR and YC had a significant impact on the abundance of rumen LA microorganisms. The ACE and Chao1 indexes in the SA groups were not significant in the test group, indicating that the addition of AMR and YC had little effect on the richness of SA microorganisms in the rumen of Tibetan sheep. In comparison, the Simpson index of S-CON, S-Group II, and S-Group III was 1.13, 1.12, and 1.08 times that of S-Group I, respectively. The Simpson index of S-Group I is the smallest, indicating that the effectiveness of the two additives is to some extent consistent with the grazing feeding mode on microbial diversity in the rumen. At the same time, it was found that L-Group I, which originally had the highest Simpson index among rumen LA microorganisms, showed the lowest Alpha diversity analysis among SA microorganisms. This phenomenon was also observed in the SA microorganisms of other test groups when compared with the LA microorganisms. This observation suggests that the rumen is an important factor in maintaining gut microbiota stability. To adapt to the changes in the environment and feed, the microbial abundance in different Ecological niches has not changed. However, the diversity of its flora will always remain in a certain dynamic equilibrium to ensure the normal operation of the rumen of animals.

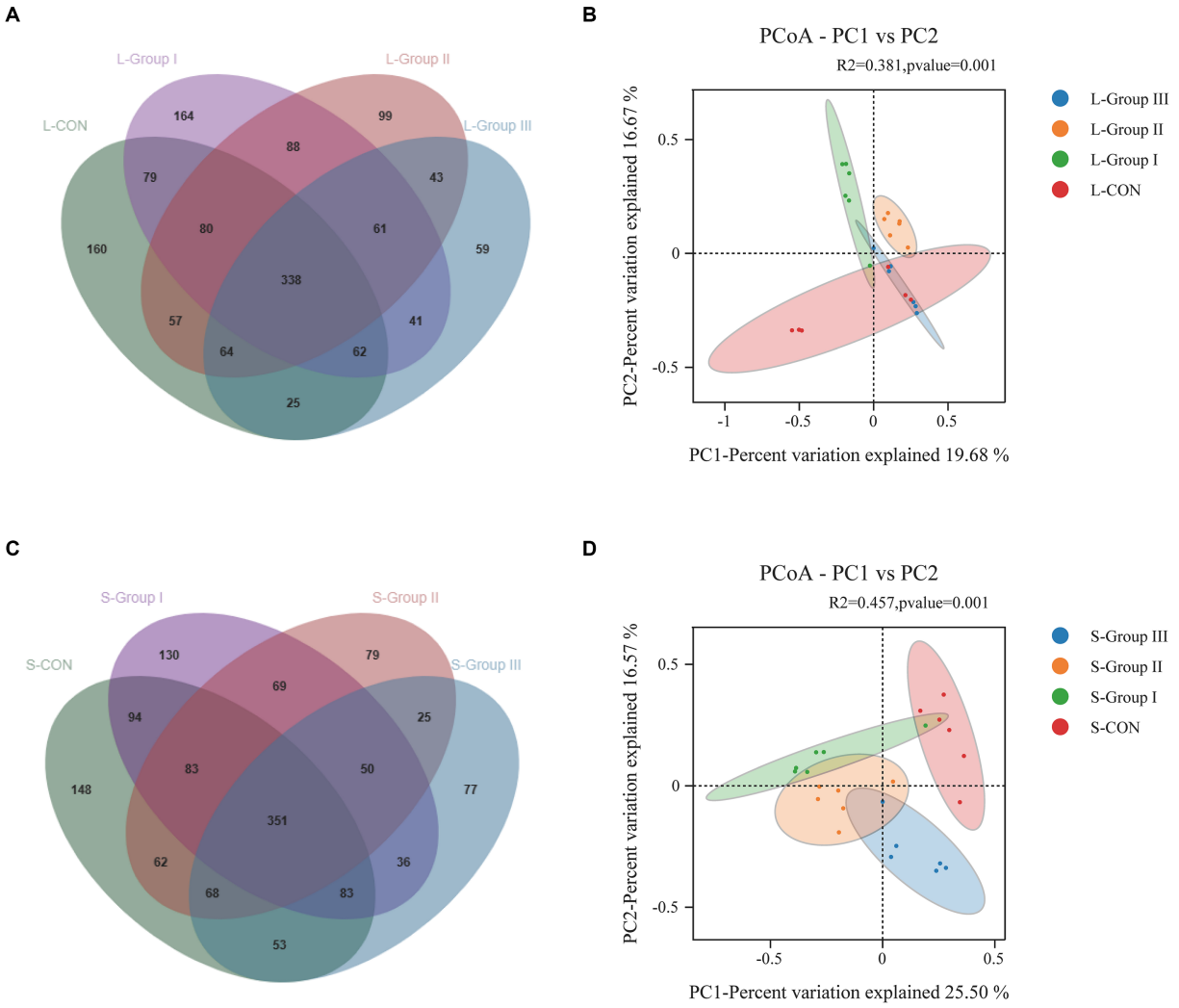

FIGURE 3
Rumen LA microorganisms Venn **(A)**, PCoA **(B)**, and rumen SA microorganisms Venn **(C)**, PCoA **(D)**. Different colors of Venn and PCoA represent different sample groups.

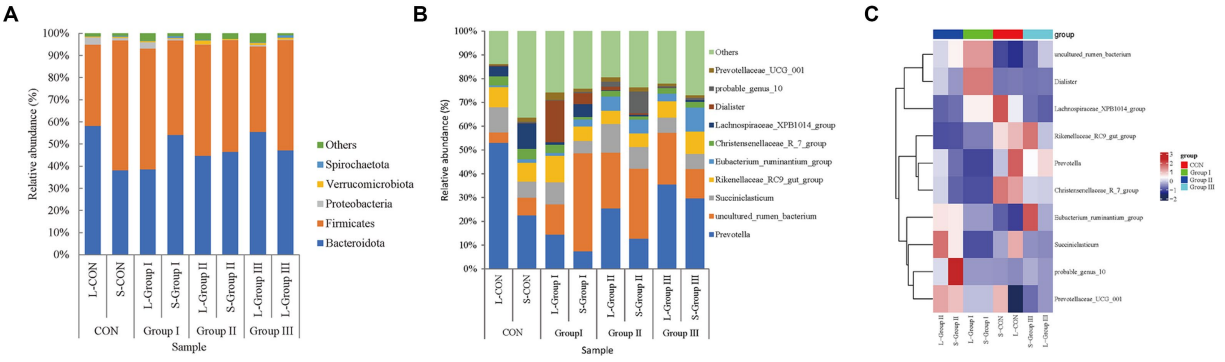

FIGURE 4
Bar chart of relative abundance at the phylum level **(A)**, genus level **(B)**, and Rumen LA and SA microbial heatmap **(C)**. Different colors in the bar chart represent species abundance.

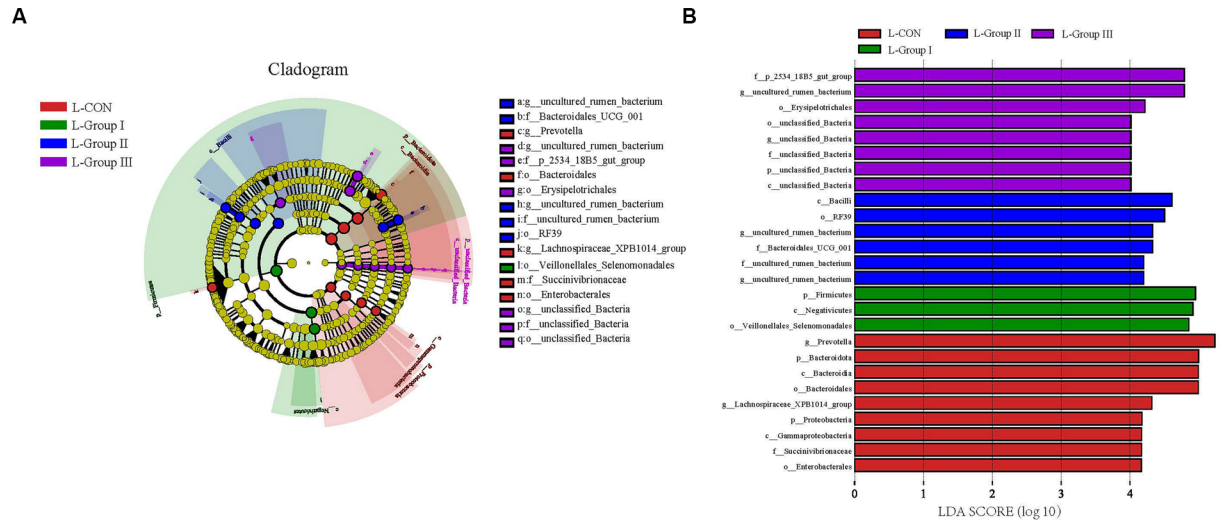

FIGURE 5
Microbial communities of different groups. The LEfSe analysis cladogram of LA microorganisms **(A)**. Linear discriminant analysis (LDA) was used to estimate the effect of each component (species) abundance on the difference **(B)**. Species with an LDA Score greater than a set value (set to 4.0 by default) are shown, with the length of the bars representing the effect size of the differing species (i.e., as an LDA logarithmic score), and the different colors of the bar graph indicate the sample group corresponding to the taxon with higher abundance.

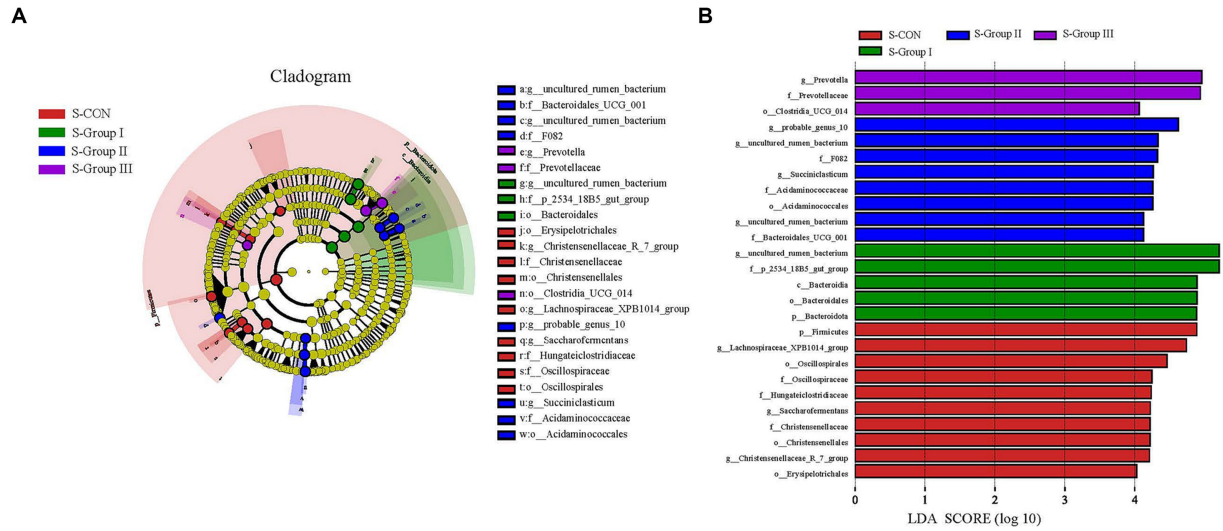

FIGURE 6
Microbial communities of different groups. The LEfSe analysis cladogram of SA microorganisms **(A)**. Linear discriminant analysis (LDA) was used to estimate the effect of each component (species) abundance on the difference **(B)**. Species with an LDA Score greater than a set value (set to 4.0 by default) are shown, with the length of the bars representing the effect size of the differing species (i.e., as an LDA logarithmic score), and the different colors of the bar graph indicate the sample group corresponding to the taxon with higher abundance.

In this experiment, PCoA analysis of rumen microbiota in the LA groups showed that the coordinates of L-Group II and L-Group III microbiota were closer, while L-CON and L-Group I showed more dispersion. The results demonstrate that the addition of AMR and YC to the diet increased the similarity of microbiota among Tibetan sheep samples. This suggests that AMR and YC are conducive to the stability of rumen microbial communities in Tibetan sheep. Bacteroidota is directly related to the degradation of non-fibrous nutrients, and its main function is to improve the utilization rate of carbohydrates. It can also improve immune function and inhibit inflammatory reaction occurrence (22), Firmicutes mainly degrade fibrous substances and promote the fermentation function of the rumen on feed (23). Bacteroidota and Firmicutes as dominant microbiota widely present in ruminants (24, 25). The two dominant phyla in this experiment are consistent with the results of this study. The abundance of Bacteroidota in L-Group II and L-Group III, with the addition of AMR and YC was higher than in L-Group I in the LA group. This indicates that the addition of AMR and YC contributes to the decomposition of non-fibrous substances in the rumen of Tibetan sheep fed in captivity. This result is consistent with the study on the effect of adding scallions

to the diet on the rumen microbiota of cattle (26). The study on adding YC to sheep feed showed that it can increase the relative abundance of Bacteroidota without having a significant impact on other phylum. This is consistent with the results of this experiment. The experimental group with added YC showed an increase in the abundance of Bacteroidota in the LA groups (27). The microbial abundance of Bacteroidota in L-CON was significantly higher than that of other test groups, while the abundance of Firmicutes was significantly lower. This suggests that grazing can improve the growth and proliferation of non-fiber-degrading bacteria in the rumen. This result is similar to other studies (28). With the addition of concentrate, AMR and YC resulted in a gradual increase in the abundance of Bacteroidota in L-Group I, L-Group II, L-Group III, S-Group I, S-Group II, and S-Group III. It may be that after feeding the concentrate to Tibetan sheep, the non-fibrous nutrients such as soybean meal, corn, and wheat bran in the concentrate increased the number of Bacteroidota. At the same time, the abundance of Spirochaete in the rumen of L-Group II and L-Group III was higher compared to L-Group I, and the abundance of Proteobacteria flora was reduced. Numerous studies have revealed that the Proteobacteria in the intestine can reflect the imbalance or unstable structure of the microecology, and the metabolic disorder of the microecology is often accompanied by the increase of the Proteobacteria. The use of two green additives, AMR and YC, improves the changes in rumen microbiota at the phylum level while contributing to maintaining the homeostasis of rumen microorganisms. An increasing number of studies have shown that different nutrients affect the digestive capacity of rumen microorganisms (29). Bacteroidota and Firmicutes are the dominant bacteria in the rumen, and their ratios (F/B ratio for short) may better reflect the physiological state of rumen flora (30). Compared to other groups, the addition of AMR did not improve the abundance of Spirochaete and Fibrobacterota (31). Spirochaete in S-Group II had more significant effects on the SA microorganisms than the other test groups, which might be based on different Ecological niches in the rumen in this experiment. Adding AMR to the diet can provide growth nutrients for these two bacteria. Verrucomimicrobia is a vital factor affecting mammalian immune function, and the decrease in its abundance is concerned with the decline in the immune function of the body (32). This indicates that the addition of YC can not only maintain the stable structure of the microbiota but also improve the immune ability of Tibetan sheep.

At the genus level, *Prevotella*, which belongs to *Bacteroidota*, is a widely existing bacterium in the rumen. Its main role is to participate in semi-fiber degradation and other components in the diet and various metabolic activities in the rumen. *Prevotella* produces a large number of complex enzymes, facilitating starch decomposition and some cell wall polysaccharides (33, 34). In the LA groups, the abundance of *Prevotella* in the L-CON was significantly higher than in other test groups. The abundance of *Prevotella* in L-Group I, L-Group II, and L-Group III showed a gradually increasing trend, which was similar to the Species distribution trend of *Bacteroidota* at the phylum level. Studies have also found that compared to calves fed with concentrate, calves fed with forage had a higher abundance of *Prevotella* in the rumen (35). This suggests that traditional grazing feeding models are more conducive to the growth and proliferation of LA microorganisms in the rumen compared to those fed with concentrate after grazing. The study found that differences in crude protein levels, neutral detergent fibers, and acidic detergent fibers in the diet can all cause differences in the species and abundance of *Prevotella* in the rumen of ruminants (36). Compared with L-Group II and L-Group III, the content of *Prevotella* in L-Group I increased, possibly due to the nutritional components of additives causing changes in the abundance of LA microbiota in the rumen. In the SA groups, the addition of YC resulted in an increase in the abundance of *Prevotella* and *Eubacterium ruminantium*, both of which belong to Firmicutes' S-Group III. This indicates that YC can improve the degradation of rumen cellulose in the SA groups (37). However, the specific functions of YC in this regard require further investigation. *Prevotelaceae_ UCG_ 001* as the main bacterial branch of *Bacteroidota*, is mainly responsible for the decomposition of Hemicellulose, carbohydrate, and protein. It exhibits the highest abundance in L-Group II, indicating that the addition of AMR can improve the efficiency of Tibetan sheep in the decomposition of hemicellulose to a certain extent. In addition, the inclusion of AMR increased the abundance of *problem_ genus_10* in the SA system, indicating that the use of additives in feed can effectively improve the abundance of SA system microorganisms in the rumen. *Christenselelaceae_ R_ 7_ Group* belonging to Firmicutes, is positively related to the generation and transport of volatile acids in the rumen. It also promotes the degradation of fiber materials and improves the utilization rate of forage. Adding AMR to the diet of Tibetan sheep resulted in an improvement in the levels of *Prevotella* and *uncultured_ rumen_bacterium* in the rumen microbiota. However, it also caused a decrease in the abundance of *Rikenellaceae_RC9_gut_group*. The decrease could be attributed to the high content of crude protein and fat in scallions, which enhances the degradation of hemifiber and other dietary components, as well as various metabolic activities in the rumen. These processes lead to the production of complex enzymes, starch, and some cell wall polysaccharides (33, 34), ultimately resulting in an increase in the abundance of LA microbial *uncultured_ rumen_bacterium.*

Furthermore, the combination of LEfSe analysis and the comparison between groups revealed notable changes in the structure and abundance of the core rumen flora in Tibetan sheep when concentrate, AMR, and YC were added to the LA groups. These findings have important implications for regulating the rumen flora environment in production, as they highlight the potential benefits of using effective additives and concentrate in this experiment. In addition, the analysis of LEfSe and LDA column plots in the SA system showed that the addition of AMR and YC had an impact on the core microbial community in the rumen. Specific bacterial genera showed noteworthy differences compared to other groups. These findings suggest that supplementary feeding alone may not fully account for these results, as factors such as animal species and levels of added concentrate may contribute to variations in the core microbiota and its similarity.

Conclusion

In both the LA and SA systems, the addition of AMR to the diet resulted in an improvement in the abundance of beneficial flora, such as Succiniclasticum, which aids in cellulose decomposition. This addition also increased the abundance of immune regulation and inflammatory suppression bacteria, such as *Verrucomicrobiota* and *Eubacterium_ruminantium_group*, while reducing the presence of unfavorable microbiota, including *Proteobacteria* and *Rikenellaceae_RC9_gut_group*. These changes ultimately promoted the stability of rumen microbiota. Similarly, the addition of YC increased the abundance of non-fiber degrading bacteria, such as

Bacteroidota and Spirochaetota, while reducing the presence of pathogenic bacteria, such as *Proteobacteria* and *Rikenellaceae_RC9_gut_group*. This supplementation not only enhanced the immunity of Tibetan sheep but also contributed to the stability of the rumen microbiota structure. Based on these results, we concluded that adding AMR and YC to the diet had a beneficial effect on rumen health. This provides a new perspective on the stability of feed additives on rumen microbial flora and provides new insights into the role of AMR and YC in diets.

Data availability statement

The original contributions presented in the study are publicly available. This data can be found at: https://www.ncbi.nlm.nih.gov/; PRJNA1018211.

Ethics statement

The animal study was approved by Animal Care Committee of Gansu Agricultural University (GSAU-AEW-2020-0057). The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

CW: Conceptualization, Data curation, Formal analysis, Investigation, Software, Writing – original draft, Writing – review & editing. JF: Formal analysis, Investigation, Methodology, Writing – review & editing. KM: Writing – review & editing. HW: Writing – review & editing. DL: Writing – review & editing. TL: Writing – review & editing. YM: Funding acquisition, Supervision, Writing – review & editing.

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Conflict of interest

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

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Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2024.1283437/full#supplementary-material

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Optimizing dietary rumen-degradable starch to rumen-degradable protein ratio improves lactation performance and nitrogen utilization efficiency in mid-lactating Holstein dairy cows

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The dietary rumen-degradable starch (RDS) to rumen-degradable protein (RDP) ratio, denoted as the RDS-to-RDP ratio (SPR), has been proven to enhance *in vitro* rumen fermentation. However, the effects of dietary SPR *in vivo* remain largely unexplored. This study was conducted to investigate the effect of dietary SPR on lactation performance, nutrient digestibility, rumen fermentation patterns, blood indicators, and nitrogen (N) partitioning in mid-lactating Holstein cows. Seventy-two Holstein dairy cows were randomly assigned to three groups (24 head/group), balanced for (mean±standard deviation) days in milk (116±21.5), parity (2.1±0.8), milk production (42±2.1kg/d), and body weight (705±52.5kg). The cows were fed diets with low (2.1, control), medium (2.3), or high (2.5) SPR, formulated to be isoenergetic, isonitrogenous, and iso-starch. The study consisted of a one-week adaptation phase followed by an eight-week experimental period. The results indicated that the high SPR group had a lower dry matter intake compared to the other groups ($p<0.05$). A quadratic increase in milk yield and feed efficiency was observed with increasing dietary SPR ($p<0.05$), peaking in the medium SPR group. The medium SPR group exhibited a lower milk somatic cell count and a higher blood total antioxidant capacity compared to other groups ($p<0.05$). With increasing dietary SPR, there was a quadratic improvement ($p<0.05$) in the total tract apparent digestibility of crude protein, ether extract, starch, neutral detergent fiber, and acid detergent fiber. Although no treatment effect was observed in rumen pH, the rumen total volatile fatty acids concentration and microbial crude protein synthesis increased quadratically ($p<0.05$) as dietary SPR increased. The molar proportion of propionate linearly increased ($p=0.01$), while branched-chain volatile fatty acids linearly decreased ($p=0.01$) with increasing dietary SPR. The low SPR group (control) exhibited higher concentration of milk

urea N, rumen ammonia N, and blood urea N than other groups ($p<0.05$). Despite a linear decrease ($p<0.05$) in the proportion of urinary N to N intake, increasing dietary SPR led to a quadratic increase ($p=0.01$) in N utilization efficiency and a quadratic decrease ($p<0.05$) in the proportion of fecal N to N intake. In conclusion, optimizing dietary SPR has the potential to enhance lactation performance and N utilization efficiency. Based on our findings, a medium dietary SPR (with SPR=2.3) is recommended for mid-lactating Holstein dairy cows. Nevertheless, further research on rumen microbial composition and metabolites is warranted to elucidate the underlying mechanisms of the observed effects.

KEYWORDS

rumen-degradable starch, rumen-degradable protein, lactation performance, nitrogen utilization efficiency, Holstein dairy cows

1 Introduction

Ruminants rely critically on rumen microbes to digest plant feed (1). The rumen microbial activity is closely linked to feed efficiency (2), and their microbial crude protein (MCP) supplies over half of the metabolizable protein reaching the small intestine for ruminants (3). Synchronizing the supply of energy and nitrogen (N) in the rumen was suggested as one effective strategy to maximize the capture of rumen degradable protein (RDP) and enhance rumen microbial activity and growth (4). The effects of synchronous diets have been widely explored *in vitro* and *in vivo* studies (5). However, the results were reported to be inconsistent. One potential reason for this discrepancy is the failure to accurately match the form of energy carrier substances. While current research has considered the degradation characteristics of dietary proteins in the rumen, the energy evaluation system for ruminant feed predominantly relies on chemical analysis, which does not accurately reflect the energy supply within the rumen (6).

The availability of carbohydrates serves as the primary factor controlling the energy supply for rumen microbes (7). These carbohydrates were categorized into four fractions (8): neutral detergent fiber (NDF), starch, neutral detergent soluble fiber (NDSF), and water-soluble carbohydrates (WSC). Compared to NDF, starch exhibits a faster fermentation rate in the rumen. This made the supply of energy at a rate closer to the ammonia released, thereby promoting rumen microbial activity and growth (5). The rumen-degradable starch (RDS), representing the extent and rate of starch degradation in the rumen, had been reported to be more effective than rumen-degradable NDF in promoting MCP synthesis (9). Additionally, in comparison to NDSF (e.g., pectin) and WSC (e.g., sucrose), starch could provide more carbon skeletons for rumen microbes (10). The starch-based diet led to more MCP synthesis *in vitro* culture (10) and milk protein yield in dairy cows (11) compared to a sucrose- (or pectin-) based diet. Therefore, the starch, especially RDS, might be more effective in regulating the rumen microbial growth and fermentation than other carbohydrates. Furthermore, the RDP function as the primary N source for rumen microbes, significantly influencing the composition of rumen microbes (12) and MCP synthesis (13). Davies et al. (14) reported that a judicious combination of RDS and RDP in low crude protein (CP) diets had the potential to improve MCP synthesis efficiency and animal productivity. Martins et al. (15) suggested that dietary recommendations for RDP should consider RDS for a more precise level to enhance MCP synthesis and minimize N excretion. Considering these study results, the dietary RDS to RDP ratio (SPR) might be an effective indicator of optimizing rumen microbial growth and rumen fermentation.

Our previous *in vitro* study showed a quadratic response in MCP synthesis and total volatile fatty acids (TVFA) concentration with increasing dietary SPR (16). However, the effect of dietary SPR *in vivo* has yet to be entirely determined. Therefore, we hypothesize that dietary SPR has the potential to optimize rumen microbial growth and fermentation, subsequently modulating lactation performance and nitrogen use efficiency (NUE) in dairy cows. Our objective was to evaluate the effects of dietary SPR on lactation performance, nutrient digestibility, rumen fermentation patterns, and N partitioning in mid-lactating Holstein cows.

2 Materials and methods

2.1 Ethics statement

This study was conducted between March 2021 and June 2021 at Hongda Commercial Dairy Farm in Baoding, China. The experiment was approved by the Institutional of Animal Care and Use Committee at Hebei Agricultural University, Baoding, China (with protocol JGL 2103; approval date: March 1, 2021). The Hongda Commercial Dairy Farm provided the necessary approvals and cooperation for the research.

2.2 Animals and experimental design

Seventy-two Holstein dairy cows (24 head/group) were used in a complete randomized design. Cows balanced for (mean ± standard deviation) days in milk (116 ± 21.5), parity (2.1 ± 0.8), milk production (42 ± 2.1 kg/d), and body weight (705 ± 52.5 kg) were assigned to one of three treatments. Three distinct diets were formulated with different SPR levels, which were low SPR (L-SPR, RDS/RDP = 2.1), medium SPR (M-SPR, RDS/RDP = 2.3), or high SPR (H-SPR, RDS/RDP = 2.5), respectively. The L-SPR treatment was designed as the control group

based on the observed dietary SPR value in farms exhibiting low NUE. The M-SPR treatment was derived from the optimal rumen fermentation performance observed in previous *in vitro* experiment (16). The H-SPR treatment was derived from earlier studies that integrated the maximum levels of RDS without observing adverse effects on rumen function (17). Cows were individually housed in tie stalls equipped with automatic drinking bowls. Cows had free access to water and were fed a total mixed ration (TMR; Table 1) twice daily at 0800 and 1,600 h. Cows were fed *ad libitum* throughout the study, ensuring at least 5% feed refusals. Cows were milked thrice daily at 0730, 1530, and 2,300 h. The study was conducted with 1-week to adapt to the experimental conditions, followed by an 8-week for sampling and data collection.

2.3 Diets and feed ingredients

The experimental diets were formulated to meet the recommendation of NRC (2001) (18) using the AMTS software platform (Agricultural Modeling & Training Systems, Groton, NY, United States) for a 700 kg cow producing 40 kg/d of milk containing 4.0% fat and 3.0% true protein. The diets contained 49% forage (forage sources and proportions were consistent across all diets) and 51% concentrate on a dry matter (DM) basis. All forages and concentrates were weighed and mixed thoroughly each morning using a mixer wagon (9SJW-500, Goke Agriculture Machinary Co. Ltd., Beijing, China). Diets maintained consistent levels of net energy for milk, CP, starch, neutral detergent fiber (NDF), and acid detergent fiber (ADF; Table 2). Differences in levels of RDS and RDP arose from varying concentrate mixtures. Specifically, RDS levels were adjusted by varying the proportions of ground corn and ground wheat in the diet, and the proportions of solvent-extract soybean meal (SSBM) and heat-treated soybean meal (HSBM) were regulated to maintain a consistent RDP level. A single source of wheat and corn was processed using a roller mill (model SSLG-15, Shuanghe Machinery Manufacturing Co. LTD, Shandong, China) to obtain ground wheat and ground corn. The particle size analysis, performed by the ASAE (2003, method S319.3) (19), revealed that the geometric mean particle sizes were 845 μm for ground wheat and 829 μm for ground corn, respectively. The HSBM used in this study were commercially manufactured using the same production process and batch (Xingpu Feed Co. LTD, Harbin, China). The *in situ* residues of SSBM and HSBM after 16 h of ruminal incubation were employed to determine the small intestinal digestibility of rumen-undegraded protein (20), with respective digestibility of 82.7 and 75.2%.

The dietary SPR was calculated as the ratio of dietary RDS to RDP. The dietary RDS or RDP content was calculated based on the actual measured values of the ingredients applied in the formulation, as determined by Equations (1,2) (21):

$$RDS = \sum_{i=1}^{n} PST_i \times ERDST_i \quad (1)$$

$$RDP = \sum_{i=1}^{n} PCP_i \times ERDCP_i \quad (2)$$

TABLE 1 Ingredients of experimental diet fed as a TMR (% of DM).

Ingredients, % of DM	Treatments (SPR)[1]		
	L-SPR (Control)	M-SPR	H-SPR
Corn silage	31.4	31.5	31.3
Alfalfa hay	4.93	4.94	4.92
Oat hay	12.4	12.4	12.4
Ground corn	15.7	7.9	0.9
Ground wheat	4.0	11.9	19.6
Solvent-extract soybean meal	11.6	8.3	5.7
Heat-treated soybean meal[2]	1.37	3.34	5.09
Wheat bran	2.16	2.95	2.55
Beet pellets	11.1	11.5	12.1
Fat powder	2.36	2.36	2.43
Premix[3]	3.0	3.0	3.0

[1]SPR, dietary rumen-degradable starch to rumen-degradable protein ratio; L-SPR, low SPR; M-SPR, medium SPR; H-SPR, high SPR.
[2]Xingpu Feed Co. LTD, Harbin, China, the intestinal digestibility of rumen-undegradable protein was 75.2%.
[3]Premix contained (per kg of DM): 2600 mg of $NaHCO_3$, 2,300 mg of $CaHPO_4$, 1,300 mg of $CaCO_3$, 700 mg of NaCl, 650 mg of Zn, 500 mg of Mn, 350 mg of MgO, 350 mg of Fe, 180 mg of Cu, 6 mg of Co, 8 mg of Se, 10 mg of I, 240,000 IU of vitamin A, 53,000 IU of vitamin D_3, 2,000 mg of vitamin E, 13 mg of biotin, and 90 mg of β-carotene.

TABLE 2 Chemical composition of experimental diet fed as a TMR (% of DM, unless noted).

Composition	Treatments (SPR)[1]		
	L-SPR (Control)	M-SPR	H-SPR
NE_L, Mcal / kg[2]	1.7	1.7	1.7
CP	15.6	15.6	15.7
RDP[3]	9.3	9.3	9.3
Starch	26.4	26.5	26.7
RDS[3]	19.4	21.1	22.9
SPR	2.1	2.3	2.5
Ether extract	4.5	4.6	4.8
NDF	31.6	31.7	31.7
ADF	17.5	17.6	17.7
Forage NDF	20.3	20.4	20.3
$peNDF_{8.0}$[4]	15.2	15.2	15.3
ERDST[5]	73.5	79.6	85.9
ERDCP[5]	59.6	59.6	59.2

[1]SPR, dietary rumen-degradable starch to rumen-degradable protein ratio; L-SPR, low SPR; M-SPR, medium SPR; H-SPR, high SPR.
[2]NE_L, net energy for lactation, estimated by NRC (2001).
[3]RDP (rumen degradable protein, %) = CP (%) × ERDCP (%); RDS (rumen degradable starch, %) = Starch (%) × ERDST (%).
[4]$peNDF_{8.0}$, physically effective NDF inclusive of particles > 8 mm, measured as the NDF content of diet multiplied by $pef_{8.0}$.
[5]ERDS, effective ruminal degradability for starch; ERDCP, effective ruminal degradability for protein.

Where n is the number of ingredients containing starch or CP in the diet; PST_i represents the dietary starch proportion of feed i in the diet; PCP_i represents the dietary CP proportion of feed i in the diet, $ERDST_i$ represents the effective rumen starch degradability

of feed i; $ERDCP_i$ represents the effective rumen CP degradability of feed i.

The ERDST and ERDCP in the feed ingredients employed in this study were assessed through the *in situ* nylon bags technique. The nylon bags with a pore size of 50 μm were used, and the rumen outflow rate was set at 0.06/h according to Offner et al. (6). A detailed method of *in situ* ruminal degradation was referred to Li et al. (22). Degradation parameters and effective ruminal degradability (ERD) were determined using Equations (3,4) from Ørskov and McDonald (23):

$$Y_t = a + b \times \left(1 - e^{-kt}\right) \quad (3)$$

$$ERD = a + b \times k / \left(k + k_p\right) \quad (4)$$

where Y_t = disappearance proportion at time t; a = rapidly degradable fraction; b = slowly degradable fraction; k = constant rate of degradation of fraction b; t = time of incubation (h); kp = passage rate, the rumen outflow rate was set at 0.06/h. The nutrient content, ERDCP, and ERDST of the primary ingredients used in this study are listed in Supplementary Table S1.

2.4 Sampling and data collection

2.4.1 Collection of feed samples

Feed offered and refused were recorded daily. The TMR, feed ingredients, and feed refusals were collected weekly. The collected samples were immediately oven-dried at 55°C for 48 h to measure DM content. Subsequently, the samples were ground using a 1-mm screen (stand model 4 Wiley Mill, Arthur H. Thomas, Philadelphia, PA, United States) for chemical analyses. Daily DMI was calculated by subtracting the DM refusals from the DM offered. Weekly averages of DMI data were used for statistical analysis (24).

2.4.2 Collection of milk samples

Milk yield was recorded daily using the DeLaval milking system at milking time, and those records were used to calculate weekly averages for each cow for statistical analysis. Milk samples were obtained on d 1 weekly by mixing proportional aliquots from each milking and immediately transported to the laboratory for analysis. According to International Dairy Federation (25), the fat- and protein-corrected milk (FPCM) yield was computed as milk yield (kg/d) × (0.1226 × fat % + 0.0776 × protein % + 0.2534). Feed efficiency was determined as FPCM yield divided by DMI. The NUE was calculated as milk N divided by N intake.

2.4.3 Collection of feces and urine samples

Due to animal welfare, eight cows from each treatment (balanced for parity, milk production, and days in milk) were selected for feces, urine, rumen fluid, and blood sampling. Spot feces and urine samples were collected from each cow during weeks 0, 4, and 8. The samples cover 3 days (0200, 1100, and 2000 h on d 2; 0500, 1400, and 2300 h on d 3; 0800 and 1700 h on d 4 of each sampling week), representing 3, 6, 9, 12, 15, 18, 21, and 24 h after morning feeding. Approximately 100 g of fresh feces were collected from the rectum, pooled by each cow, dried at 55°C for 48 h, and ground through a 1-mm screen (stand model 4 Wiley Mill; Arthur H. Thomas, Philadelphia, PA, United States). Urine was collected by stimulating the vulva. Subsequently, the urine samples were acidified using a 4:1 volumetric ratio of 0.072 mol/L H_2SO_4 to urine. The acidified samples were then frozen at −20°C for further analysis.

2.4.4 Collection of rumen fluid samples

Rumen fluid samples were collected using an oral stomach tube during week 0, 4, and 8 (26). Five sampling times over 3 d were selected (1100 and 2000 h on d 5; 0800 and 1700 h on d 6; 1400 h on d 7 of each sampling week), representing 0, 3, 6, 9, and 12 h after morning feeding. To secure representative rumen samples, the oral stomach tube was inserted approximately 200 cm deep, reaching the central rumen. Additionally, to mitigate the influence of extraneous factors, rumen fluid sampling was consistently carried out by a single individual. The initial 100 mL of rumen fluid was discarded to prevent contamination from saliva or mucus. Subsequently, another 100 mL of rumen fluid was collected and filtered through four layers of cheesecloth. The rumen liquid pH was immediately measured using a portable pH meter (DENVER UB-7, Denver Instrument, Denver, United States). Two subsamples (5 mL) of rumen fluid were, respectively, preserved with 1 mL of 25% (wt/vol) HPO_3 and 1 mL of 1% (wt/vol) H_2SO4 and stored at −20°C until the determination of VFAs and ammonia N (NH_3-N).

2.4.5 Collection of blood samples

Blood samples were collected from the coccygeal vein before morning feeding on d 7 of week 0, 4, and 8. Approximately 20 mL of blood samples were collected into two 10 mL vacuum tubes (Vacutainer, Becton Dickinson, Franklin Lakes, NJ, United States) with no additives. Then, according to Shen et al. (27), serum samples were prepared and stored at −20°C for later analyses.

2.4.6 Measurement of body weight

Cows were weighed before morning feeding on the last day of week 0, 4, 8. Body weights were adjusted by subtracting the morning milking weights to accurately represent the cows' actual weight.

2.5 Samples analysis

2.5.1 Analysis of feed and fecal samples

The DM (method 930.15), ash (method 942.05), CP (method 968.06), ether extract (EE, method 920.39), and starch (method 996.11) contents in TMR, feed refusals, and feces were determined according to AOAC (28). The organic matter (OM) content was calculated as the difference between 100% and ash content. The content of NDF and ADF in TMR, feed refusals, and feces were analyzed using heat-stable α-amylase and sodium sulfite (29) and were expressed inclusive of residual ash (30). Acid detergent insoluble ash (ADIA) content in TMR, feed refusals, and feces was determined as outlined in Keulen and Young (31). The ADIA was used for calculating the apparent total-tract digestibility of nutrients (32), and the equation was:100 − (100 × (%ADIA in DM consumed/%ADIA in feces) × (%nutrient in feces/%nutrient in consumed DM)).

2.5.2 Analysis of urine samples

The weight of cows and the concentration of creatinine in urine were used to estimate total urine production. The estimation formula is as follows: Urine volume (L/d) = (Body weight (kg) × Creatinine excretion rate (mg/kg)) / Urine creatinine concentration (mg/L). The creatinine excretion rate referred to the amount of creatinine excreted per kilogram of body weight per day by cows, with a value of 29 mg/kg (33). The urinary allantoin and uric acid excretions were calculated by multiplying the concentrations by the respective daily urine volume. Daily urinary purine derivatives (PD) excretion was the sum of allantoin and uric acid excretion. The PD was used as an indicator of MCP synthesis based on the relationship derived by Chen and Gomes (34). Urinary urea-N, creatinine, allantoin, and uric acid concentrations were determined using commercial kits from Nanjing Jiancheng Bioengineering Institute, Nanjing, China. The inter-assay coefficients of variation were less than 10%, and the intra-assay coefficients of variation were less than 12%. Allantoin in urine was determined by the colorimetric method (34). Urinary N was determined according to AOAC (method 968.06) (28).

2.5.3 Analysis of milk samples

The percentage of milk fat, milk protein, lactose, milk urea nitrogen concentration (MUN), and somatic cell count (SCC) were determined using FOSS Milko Scan and Fossomatic FC (FOSS Food Technology Corp., Hillerød, Denmark). Yield of milk components was calculated by multiplying the daily milk yield by the percentage of a given milk component.

2.5.4 Analysis of rumen fluid samples

The concentration of rumen VFA was determined using gas chromatography (Agilent, 7890A, a fused silica column, 30 m × 0.32 mm × 0.25 mm; column temperature, 150°C; injector temperature, 200°C; and detector temperature, 250°C) as described by Shen et al. (35). The rumen fluid concentration of NH_3-N was measured using a phenol-hypochlorite assay (36).

2.5.5 Analysis of blood samples

Commercial kits (Jiancheng Bioengineering Inc., Nanjing, China) were used to determine the concentration of blood urea nitrogen (BUN) and glucose, as well as the activities of aspartate aminotransferase (AST) and alanine aminotransferase (ALT). Commercial ELISA kits (Huayuechang Biotechnology Co., Ltd., Beijing, China) were used to determine the total antioxidant capacity (T-AOC, kit no: DRE98021, detection range: 0–8 U/mL), insulin capacity (kit no: DRE98105, detection range: 0.8–30 mU/L), the insulin-like growth factor-1 concentration (IGF-1, kit no: DRE98016, detection range: 10–300 μg/L) and the nonesterified fatty acid concentration (NEFA, kit no: DRE98327, detection range: 20–560 μmol/L). The inter-assay coefficients of variation were lower than 10%, and the intra-assay coefficients of variation were lower than 12%.

2.6 Calculations and statistical analysis

Before analyses, all data were tested for normality using the Shapiro–Wilk test, and any parameter that was not normally distributed was normalized by Box-Cox transformation. The data were analyzed using the PROC MIXED procedure of SAS 9.4 (SAS Inst. Inc., Cary, NC). The statistical model was as follows:

$$Y_{ijk} = \mu + T_i + D_{j(i)} + cov_j + E_{ijk}$$

where Y_{ijk} is the dependent variable; μ is the overall mean; i is the SPR treatment, j is the cow; T_i is the effect of ith SPR treatment; $D_{j(i)}$ is the random effect of the jth cow within SPR treatment; Cov_j is the covariate effect, the data from week 0 were used for the covariate analysis; E_{ijk} is the residual error.

The model included diet as fixed effects, cow within SPR treatment, and residual error as random effects. The repeated measures analysis of results was subjected to five models (AR, UN, CS, SP, and VC). The weeks of data collection served as repeated measurements for DMI, milk production, milk composition, and feed efficiency. The days of sample collection function as repeated measurements for apparent nutrient digestibility, rumen fermentation parameters, nitrogen partitioning indicators, and blood indicators. The covariance structure with the smallest Schwarz-Bayesian criterion was used owing to the most desirable and reliable analysis (37). The PDIFF option of SAS was used to calculate and separate the least squares means. Orthogonal contrasts were conducted to determine linear and quadratic dose of dietary SPR responses. Treatment effects were declared significant at $p < 0.05$.

3 Results

3.1 Dry matter intake and milk performance

With increasing dietary SPR, there was a linear decrease in DMI ($p = 0.01$; Figure 1). The H-SPR group was reduced by 0.8 kg/d compared to the L-SPR group (control) and by 0.7 kg/d compared to the M-SPR group. Milk yield ($p = 0.01$), FPCM yield ($p = 0.01$), and feed efficiency ($p = 0.02$) exhibited a quadratic increase, with the M-SPR group demonstrating a 4.19% increase in milk yield, 4.63% in FPCM production, and 4.67% in feed efficiency compared to the L-SPR group (control). The SCC demonstrated a quadratic decrease as the dietary SPR increased ($p = 0.02$), with the M-SPR group showing a decrease of 31.04% compared to the L-SPR group (control). Different dietary SPRs did not influence the concentrations of milk fat, protein, and lactose, but the production of these milk components exhibited a quadratic increase ($p < 0.05$). Furthermore, in comparison to the L-SPR group (control), the concentrations of MUN in the M-SPR and H-SPR groups showed a linear decrease ($p < 0.05$), decreasing by 24.4 and 26.9%, respectively.

3.2 Total-tract apparent nutrient digestibility

As shown in Figure 2, the digestibility of DM ($p = 0.01$), OM ($p = 0.01$), CP ($p = 0.03$), EE ($p = 0.03$), starch ($p = 0.03$), NDF ($p = 0.03$), and ADF ($p = 0.02$) exhibited a quadratic increase with increasing dietary SPR, peaking in the M-SPR group. Compared to the L-SPR group (control), the M-SPR group showed significant improvements in DM (4.81%), OM (5.64%), NDF (4.29%), and ADF (6.14%) digestibility.

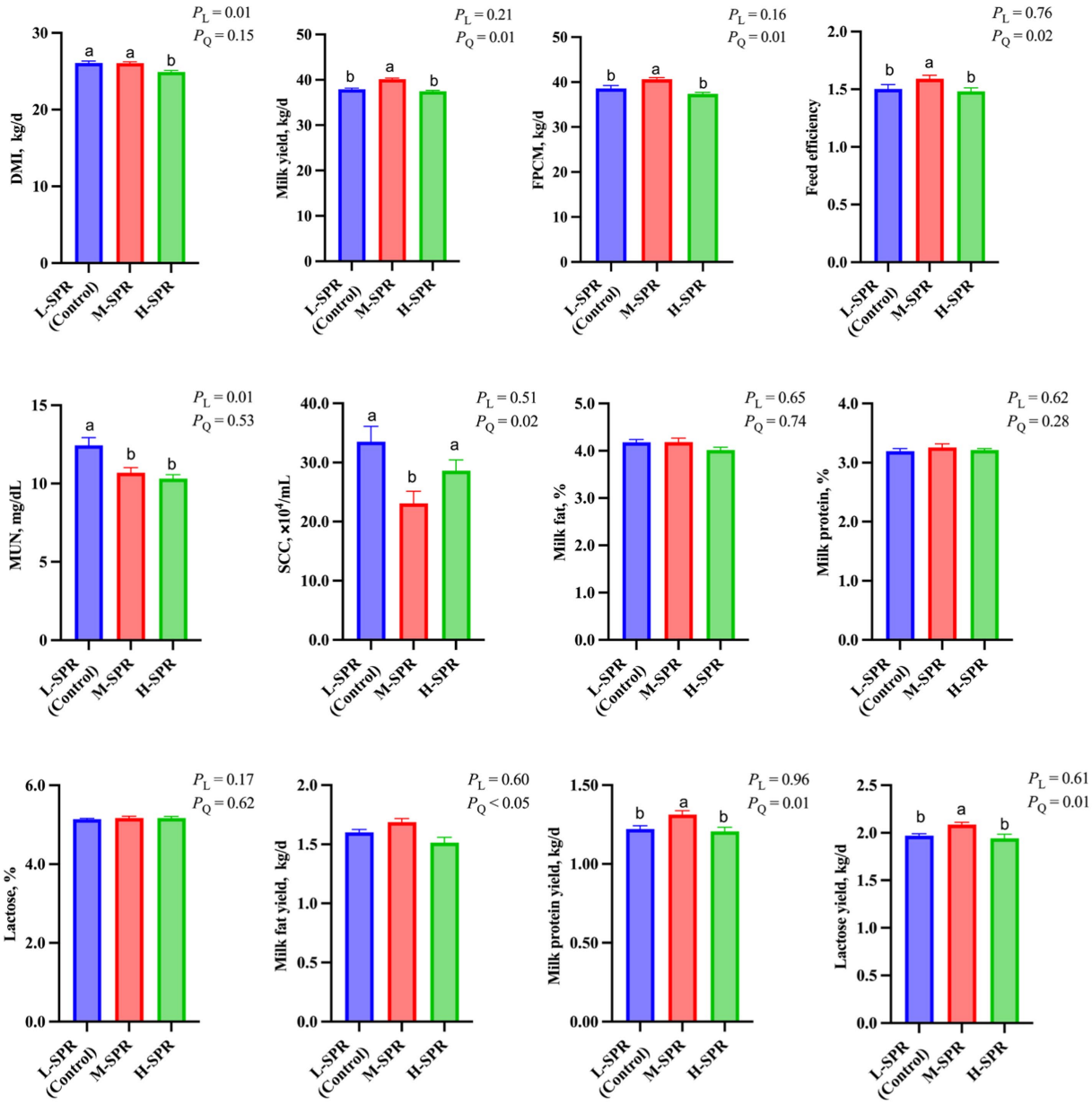

FIGURE 1
Effect of dietary rumen-degradable starch to rumen-degradable protein ratio (SPR) on dry matter intake and lactation performance in mid-lactating Holstein cows. Error bars indicate measure of variation within the dietary SPRs. Different letters (a–b) indicate statistically significant difference ($p < 0.05$). L is linear, and Q is quadratic effects for diet SPR; DMI, dry matter intake; MUN, milk urea nitrogen; FPCM, Fat- and protein-corrected milk; Feed efficiency, FPCM/DMI.

3.3 Rumen fermentation patterns

According to Figure 3, the dietary SPR did not affect rumen pH. However, as the dietary SPR increased, there was a quadratic increase in TVFA concentration ($p = 0.01$), with the M-SPR group showing a 5.36% rise compared to the L-SPR group (control). Although the molar proportion of acetate was not affected by dietary SPR, the molar proportion of propionate linearly increased ($p = 0.01$), and the ratio of acetate to propionate linearly decreased ($p = 0.01$) with increasing dietary SPR. Additionally, in comparison to the L-SPR group (control), the concentrations of NH_3-N in the M-SPR and H-SPR groups showed a linear decrease ($p = 0.01$), decreasing by 17.5 and 27.7%, respectively.

3.4 Microbial crude protein synthesis

In Figure 4, there was a quadratic response in the uric acid ($p = 0.03$), allantoin ($p = 0.01$), and total PD ($p = 0.01$) excretion, as dietary SPR increased, with the greatest amount reached at the M-SPR group. Accordingly, a quadratic increase ($p < 0.05$) was observed in MCP synthesis (g/d; g/kg of DMI; g/kg of digestible CP intake) with increasing dietary SPR. Compared to the L-SPR group (control), the M-SPR group

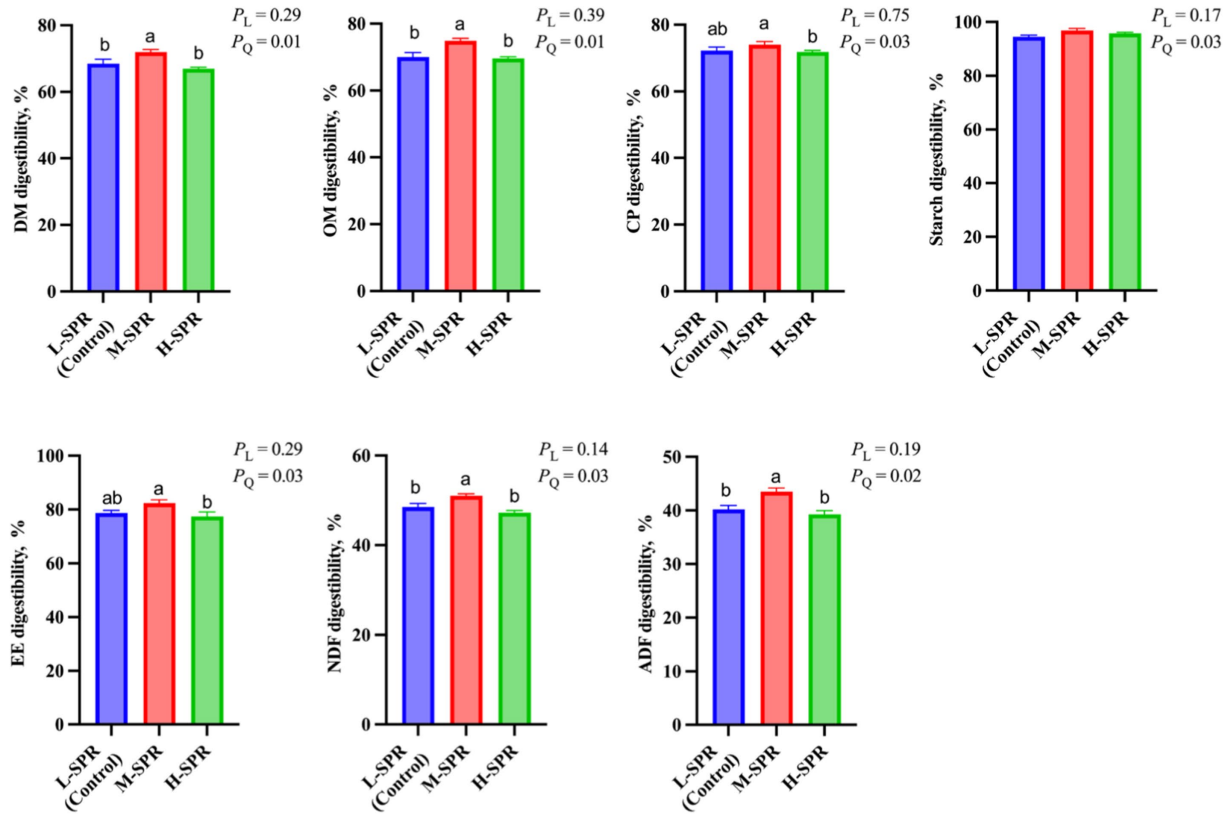

FIGURE 2
Effect of dietary rumen-degradable starch to rumen-degradable protein ratio (SPR) on nutrient apparent digestibility in mid-lactating Holstein cows. Error bars indicate measure of variation within the dietary SPRs. Different letters (a–b) indicate statistically significant difference ($p < 0.05$). L is linear, and Q is quadratic effects for diet SPR; DM, dry matter; OM, organic matter; CP, crude protein; EE, ether extract; NDF, neutral detergent fiber; ADF, acid detergent fiber.

exhibited a 9.16% increase in MCP (g/d), a 10.56% increase in MCP (g/kg of DMI), and a 9.02% increase in MCP (g/kg of digestible CP intake).

3.5 Blood indicators

As depicted in Figure 5, increasing the dietary SPR led to a quadratic rise in GLU concentration ($p = 0.01$), with the M-SPR group showing a significant 9.55% increase compared to the L-SPR group (control). The concentration of BUN displayed a linear decrease ($p < 0.05$), with reductions of 13.56 and 15.37% in the M-SPR and H-SPR groups, respectively, as compared to the L-SPR group (control). The T-AOC activity showed a quadratic rise with the increase of dietary SPR ($p = 0.04$), and the M-SPR group showed a notable 6.79% improvement compared to the L-SPR group (control). Furthermore, there were no significant differences among the three groups in the activity of AST, ALT, and insulin, as well as the concentration of NEFA and IGF-1.

3.6 Nitrogen partitioning

Although the N intake linearly decreased ($p = 0.01$; Figure 6), the milk N secretion ($p = 0.01$) and the proportion of milk N to N intake ($p = 0.01$) quadratically increased with increasing dietary SPR. Compared to the L-SPR group (control), the M-SPR group demonstrated a 6.47% increase in milk N secretion and a 7.00% increase in the proportion of milk N to N intake. With increasing dietary SPR, the proportion of fecal N to N intake exhibited a quadratic decrease ($p < 0.05$). Additionally, urinary N excretion ($p = 0.01$) and the proportion of urinary N to N intake ($p < 0.05$) showed a linear decrease. The N retention ($p = 0.03$) and the proportion of N retention to N intake ($p < 0.05$) displayed a linear increase. Total excretion N exhibited a quadratic decrease with the increase in dietary SPR ($p < 0.05$), resulting in a 6.15% reduction in the M-SPR group compared to the L-SPR group (control).

4 Discussion

In the previous *in vitro* experiment, dietary SPR had been established as an effective indicator for regulating rumen fermentation and MCP synthesis (16). Analyses of diets and milk from large-scale farms revealed a notable increase in MUN content when the dietary SPR fell below 2.1, which corresponded to the SPR value designated for the control group in this study. This increase in MUN was likely due to the surplus NH_3-N in the rumen, stemming from deficiencies in fermentable carbohydrates or excessive protein degradation (38). Therefore, optimizing dietary SPR by adjusting RDS or RDP levels could reduce rumen NH_3-N wastage, ultimately enhancing lactation performance.

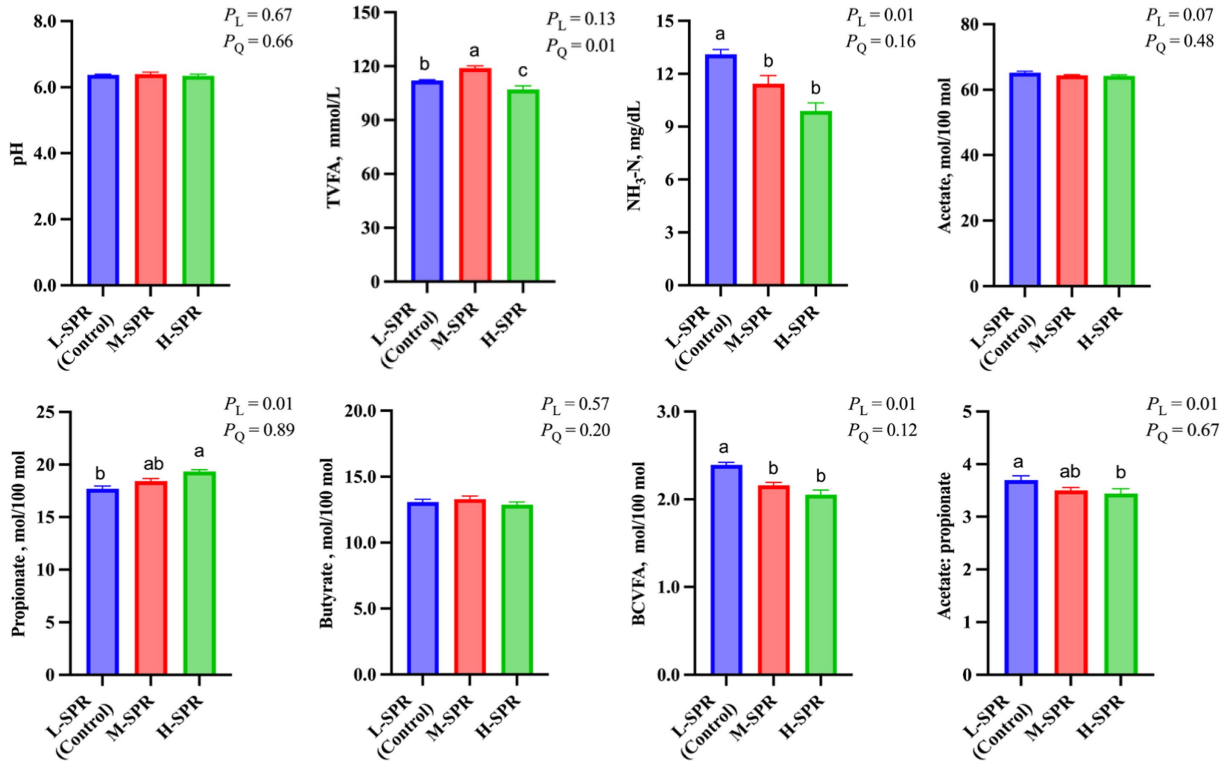

FIGURE 3
Effect of dietary SPR on rumen fermentation patterns in mid-lactating Holstein cows. Error bars indicate measure of variation within the dietary SPRs. Different letters (a–c) indicate statistically significant difference ($p < 0.05$). L is linear, and Q is quadratic effects for diet SPR. TVFA, total volatile fatty acid; Branched-chain volatile fatty acid = isobutyrate + isovalerate; NH_3-N, ammonia nitrogen.

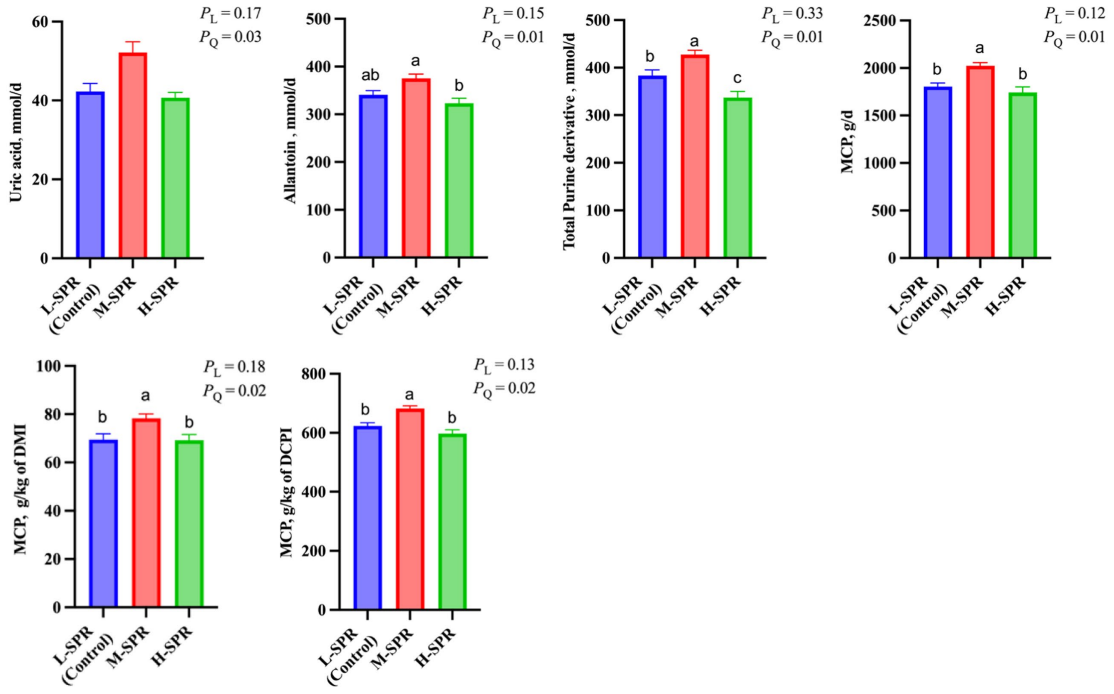

FIGURE 4
Effect of dietary rumen-degradable starch to rumen-degradable protein ratio (SPR) on urinary purine derivatives excretion in mid-lactating Holstein cows. Error bars indicate measure of variation within the dietary SPRs. Different letters (a–c) indicate statistically significant difference ($p < 0.05$). L is linear, and Q is quadratic effects for diet SPR. MCP, microbial crude protein; DMI, dry matter intake; DCPI, digestible CP intake.

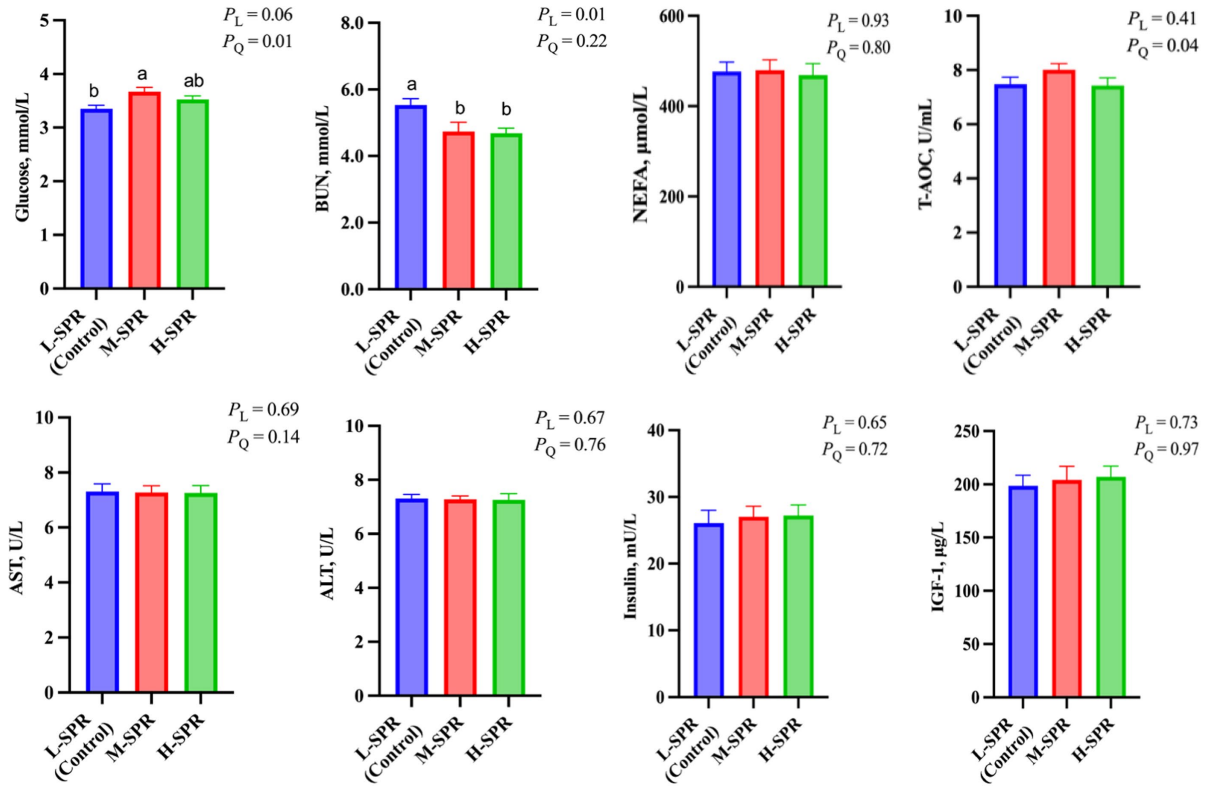

FIGURE 5
Effect of dietary rumen-degradable starch to rumen-degradable protein ratio (SPR) on blood indicators in mid-lactating Holstein cows. Error bars indicate measure of variation within the dietary SPRs. Different letters (a–b) indicate statistically significant difference ($p < 0.05$). L is linear, and Q is quadratic effects for diet SPR; BUN, blood urea nitrogen; NEFA, nonesterified fatty acid; T-AOC, total antioxidant capacity; AST, aspartate aminotransferase; ALT, alanine aminotransferase; IGF-1, insulin-like growth factor-1.

While current feeding standards recommend dietary RDP levels, less attention has been given to dietary RDS levels, which influence rumen energy and carbon skeleton supply (9, 10). This study maintained a consistent dietary RDP level while adjusting dietary SPR by altering RDS levels.

Although the diets were designed to have similar forage NDF and energy content, our study observed a linear decrease in DMI with increasing dietary SPR. Allen et al. (39) point out that the regulation of DMI is primarily achieved through metabolic signals rather than rumen fill effects. Miyaji et al. (40) and Savari et al. (41) reported that increasing RDS level could increase propionate production, leading to a lower DMI, which was consistent with our study. In the present study, the dietary RDS level elevated with increasing dietary SPR, which support more starch to be fermented in the rumen for more propionate release. In the liver, propionate is utilized for gluconeogenesis while also stimulating the oxidation of acetyl-CoA (42). In this process, acetyl-CoA is used for energy supply to increase ATP production, enhancing satiety, and stopping feed intake. Thus, the linear decrease in DMI might be explained by the linear increase in propionate proportion.

Cows in the M-SPR group produced 1.6 kg more milk compared to those in the L-SPR group (control), despite similar numerical values for DMI, which aligns with findings by Santos et al. (43). The improved digestibility of OM in the M-SPR group might elevate glucose concentration, potentially resulting in higher lactose yield. Lactose plays a pivotal role in regulating milk osmotic pressure and production (44). Therefore, the enhancement of lactose yield might positively influence milk production. Moreover, the M-SPR group exhibited enhanced serum T-AOC capacity and decreased milk SCC, indicating improved antioxidant capacity and reduced milk losses (45). Hence, the enhancement in antioxidant status is another factor contributing to the increase in milk production.

Milk fat is a critical indicator for assessing the production performance of dairy cows. Zheng et al. (17) discovered that elevating dietary RDS level in goat led to a reduction in *de novo* fatty acid synthesis and milk fat production. In our study, the M-SPR group exhibited a significant improvement in the digestibility of NDF and ADF, potentially providing more acetate as precursors for milk fat synthesis. Additionally, the M-SPR group had a higher apparent digestibility of EE, allowing them to acquire more exogenous fatty acids, which is another reason for the increased milk fat yield. A meta-analysis by Ferraretto et al. (46) observed that a one-unit increase in RDS resulted in a corresponding 0.02-unit increase in milk protein content. Zhong et al. (47) reported a quadratic relationship in milk protein content and yields with the increasing proportion of rumen-fermentable carbohydrates. The MCP, comprising over half of the metabolizable protein in dairy cows, has a similar essential amino acid composition to milk (3). The elevated milk protein yield and milk N secretion within the M-SPR group might be partly attributed to

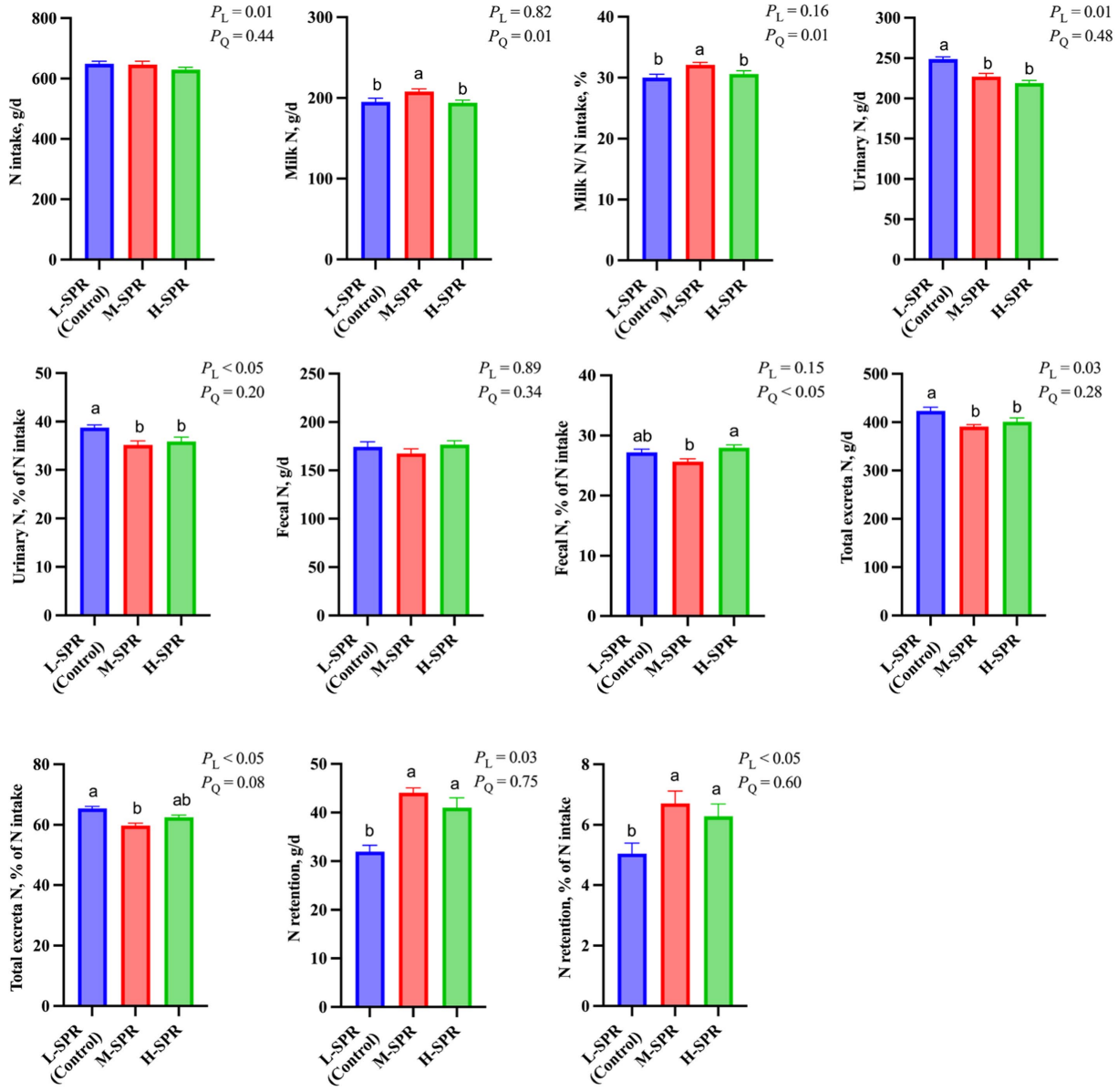

FIGURE 6
Effect of dietary rumen-degradable starch to rumen-degradable protein ratio (SPR) on nitrogen partitioning in mid-lactating Holstein cows. Error bars indicate measure of variation within the dietary SPRs. Different letters (a–b) indicate statistically significant difference ($p < 0.05$). L is linear, and Q is quadratic effects for diet SPR; Milk N, milk crude protein ÷ 6.25; Total N excretion, fecal N + urinary N; N retention, N intake − milk N − urinary N − fecal N.

its increased MCP synthesis. Furthermore, the heightened glucose concentration in the M-SPR group might diminish the necessity for certain amino acids in gluconeogenesis. Consequently, this elevated glucose concentration might also account for the increased milk protein yield in the M-SPR group.

In our study, the higher MUN and BUN concentration in the L-SPR group (control) indicated a lower degree of synchronization in energy and N supply within their rumen (48). The increased proportion of BCVFA in the L-SPR group (control) suggested that more amino acids were degraded to provide energy, leading to a subsequent rise in NH_3-N concentration. Since the diets were designed to be similar RDP level in our study, the increased proportion of urinary nitrogen to N intake in L-SPR group (control) was likely due to inadequate ruminal energy supply stemming from a lower dietary RDS level. Therefore, the lower NUE in L-SPR group (control) was attributed to the increased proportion of urinary nitrogen to N intake. Similar results were also reported by Kand and Dickhoefer (49). Furthermore, despite having the highest RDS levels, the H-SPR group did not exhibit a high NUE as the M-SPR group. This might be due to the higher proportion of fecal N to N intake in this group. The H-SPR group had a diet with a higher level of HSBM, which had lower intestinal digestibility (50), consequently increasing fecal N excretion.

Although an increase in TVFA concentration was observed in our study, the dietary SPR did not impact rumen pH. This might be attributed to the adequate dietary forage NDF and peNDF among treatments (8, 51). Within the normal range of rumen pH, a moderate dietary RDS level was observed to enhance the proliferation of cellulolytic and amylolytic bacteria compared to diets with low or high RDS levels (52). Moreover, Zhang et al. (53) reported a positive association between the MCP synthesis and the relative abundance of cellulolytic and amylolytic bacteria. Consequently, the increased synthesis of MCP in the M-SPR group might enhance the abundance of cellulolytic and amylolytic bacteria in the rumen. This, in turn, could lead to improved digestibility of starch and fiber for dairy cows, accompanied by a corresponding increase in ruminal TVFA concentration.

5 Conclusion

The dietary SPR emerges as a novel indicator, offering insights into the availability of energy and nitrogen within the rumen. A lower dietary SPR correlated with an increase in the proportion of urinary nitrogen excretion, while a higher dietary SPR was associated with a reduction in dry matter intake. Balancing dietary SPR could boost lactation performance and nitrogen utilization efficiency by improving MCP synthesis and nutrient digestibility. Considering the economic benefits and environmental protection, this study recommends a medium dietary SPR (with SPR = 2.3) for mid-lactating Holstein dairy cows. Nevertheless, further investigations on rumen microbial composition and metabolites are required to elucidate the underlying mechanisms responsible for the observed effects.

Data availability statement

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

Ethics statement

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

Author contributions

PC: Data curation, Methodology, Software, Writing – original draft. YL: Methodology, Writing – review & editing. MW: Data curation, Writing – review & editing. YS: Formal analysis, Funding acquisition, Writing – review & editing. ML: Writing – review & editing. HX: Writing – review & editing. NM: Writing – review & editing. YC: Writing – review & editing. QL: Writing – review & editing. MA: Writing – review & editing. ZW: Validation, Writing – review & editing. ZH: Visualization, Writing – review & editing. SR: Visualization, Writing – review & editing. LH: Methodology, Writing – review & editing. JL: Software, Writing – review & editing. YG: Funding acquisition, Methodology, Software, Supervision, Writing – review & editing. JgL: Funding acquisition, Investigation, Project administration, Resources, Supervision, Writing – review & editing.

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Conflict of interest

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

Publisher's note

Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2024.1330876/full#supplementary-material

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Effect of heat stress on blood biochemistry and energy metabolite of the Dazu black goats

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The objective of this study was to determine the effects of heat stress (HS) on physiological, blood biochemical, and energy metabolism in Dazu black goats. Six wether adult Dazu black goats were subjected to 3 experimental periods: high HS (group H, temperature-humidity index [THI] > 88) for 15 d, moderate HS (group M, THI was 79-88) for 15 d, and no HS (group L, THI < 72) for 15 d. Rectal temperature (RT) and respiratory rate (RR) were determined on d 7 and 15 of each period, and blood samples were collected on d 15 of each period. All goats received glucose (GLU) tolerance test (GTT) and insulin (INS) tolerance test on d 7 and d 10 of each period. The results showed that HS decreased dry matter intake (DMI) and INS concentrations ($p<0.05$), and increased RT, RR, non-esterified fatty acid (NEFA), cortisol (COR), and total protein (TP) concentrations ($p<0.05$). Compared to group L, the urea nitrogen (BUN) concentration increased and GLU concentration decreased in group H ($p<0.05$).During the GTT, the area under the curve (AUC) of GLU concentrations increased by 12.26% ($p>0.05$) and 40.78% ($p<0.05$), and AUC of INS concentrations decreased by 26.04% and 14.41% ($p<0.05$) in groups H and M compared to group L, respectively. The INS concentrations were not significant among the three groups ($p>0.05$) during the ITT. A total of 60 differentially expressed metabolites were identified in response to groups H and M. In HS, changes in metabolites related to carbohydrate metabolism and glycolysis were identified ($p<0.05$). The metabolites related to fatty acid βoxidation accumulated, glycogenic and ketogenic amino acids were significantly increased, while glycerophospholipid metabolites were decreased in HS ($p<0.05$). HS significantly increased 1-methylhistidine, creatinine, betaine, taurine, taurolithocholic acid, inosine, and hypoxanthine, while decreasing vitamin E in blood metabolites ($p<0.05$). In summary, HS changed the metabolism of fat, protein, and energy, impaired GLU tolerance, and mainly increased amino acid metabolism to provide energy in Dazu black goats.

KEYWORDS

heat stress, Dazu black goat, physiological indicators, hormonal, amino acids

1 Introduction

Heat stress (HS) is the body's nonspecific response under high temperature environment to produce a reaction combined. Animals with HS may experience a range of physiological and behavioral abnormalities that impair their ability to reproduce, grow, and produce (1, 2). According to a study by Hashem et al. (3), HS reduced the pH, cooking loss, water holding capacity, and shear force of Black Bengal goats' meat. HS has been shown to decrease growth performance and milk production of dairy goats by 12 and 3–10%, respectively (4, 5). HS can result in total animal losses averaging $2.4 billion annually (6). In addition, HS may cause damage to animal proteins, fats, and carbohydrates metabolism (7). It was found that HS caused a significant decrease in the abundance of several polar lipids such as phosphatidylcholine, phosphatidylserine, lysophosphatidylcholine, and glucosylceramide, while significantly increased the activities of glycogen phosphorylase and pyruvate dehydrogenase in muscle, as well as increased protein degradation (8, 9). The temperature–humidity index (THI) is a common bioclimatic indicator to assess HS. Goats critical temperature of the HS in 35–40°C (5), and goats can occur when the THI exceeds 80 (10). Goats have a wide isothermal zone, are highly resistant to heat (11), and recover from HS via physiological, biochemical, and metabolic changes (12–14). However, HS and even death of goats may occur at high environmental temperature (ET) and relative humidity (RH), especially for goats with production needs. ET of 34–36°C are reported to reduce the conception rate of female goats (15) and HS was found to decrease the expression of genes related to reproductive efficiency in Malabari goats (16). The Dazu black goat is native to Chongqing, China, and is characterized by a short black coat. It has the characteristics of cold and drought tolerance, strong stress resistance, efficient disease resistance, and roughage-resistance, which is of great significance to the development of livestock farming. Notably, black coats absorb more solar radiation, and short-haired goats tolerate radiant heat less than hairy goats (5). In Chongqing, the ET exceeds 35°C for an average of 40.77 d during the summer months with an average annual RH of 80%, which belongs to the high humidity area. Moreover, the housing environments of goats can be even more humid, which is more likely to cause HS for Dazu black goats. To sum up, no HS occurs when THI is less than 77.33 (17), was alert and in danger between 80 and 90, with extreme danger beyond 90 for goats (10), which influenced the physiology, blood biochemical indices, metabolism, and even cause the death of goats. The performance of the Dazu black goat was greatly affected in the summer. Therefore, it is of great significance to study the changes in physiological indexes, blood biochemical indexes, and blood metabolites of Dazu black goats to improve the metabolic regulation, feed digestibility, and growth performance of goats during HS in order to prevent and control HS.

To date, relatively few studies have investigated the physiological and metabolic changes of Dazu black goats in response to high ET and RH. Therefore, the present study aimed to assess the effects of HS on the physiological and blood biochemical indices as well as endogenous metabolites of Dazu black goats. The results of this study will help to clarify the response mechanisms of goats to HS and provide basic parameters for efficient and healthy breeding under hot and humid conditions.

2 Materials and methods

2.1 Animals, diets, and experimental design

Six wether adult Dazu black goats (28.4 ± 3.2 kg of body weight) were subjected to 3 experimental periods with a single-factor self-controlled trial. The total length of the trial was 52 d, with the pre-feeding period was 7 d and the 3 experimental periods consisting of (1) 15 d of high HS (group H, THI > 88); (2) 15 d of moderate HS (group M, THI was 79–88); and (3) 15 d of no HS (group L, THI < 72). ET and RH were manually controlled for temperature and humidity with the use of 4 heaters, 2 humidifiers, and 2 air conditioners. Three temperature and humidity data logger devices (Testo, Inc., Sparta Township, NJ, United States), which located about 1.6 meters above the ground, were used to record the ET and RH every 30 min. The ET and RH were under control from 8:00 to 18:00 h and 18:01 to 7:59 h the next day during the three treatment phases. THI was calculated from ET and RH values which were recorded. Table 1 displays the ET, RH, and THI during the duration of the experiment. The THI was calculated according to the formula described in Hamzaoui et al. (18):

$$\mathrm{THI} = \left(1.8 \times \mathrm{T_{db}} + 32\right) - \left(0.55 - 0.0055 \times \mathrm{RH}\right)\left(1.8 \times \mathrm{T_{db}} - 26.8\right),$$

where T_{db} is the dry bulb temperature (°C) and RH is the relative humidity (%).

All Dazu black goats were housed in single pens located in a barn on the Rongchang Campus of Southwest University and fed a total mixed ration (TMR) twice daily at 08:30 and 18:00 h with feed intake *ad libitum* and had free access to clean drinking water. It had displayed the composition and nutrient content of the daily TMR in Table 2. The ME of each raw material was calculated according to the Feeding Standard of Meat-Producing Sheep and Goats of Chinese Agricultural Industry Standards (HB, NY/T 816–2004). The quality of the TMR was checked periodically during the experiment to ensure the absence of mold and spores. The level of aflatoxin in the maize used, as determined by Total aflatoxin detection kit, was below the maximum tolerance threshold in the European Union. The results of this trial were not related to aflatoxins.

2.2 Temperature–humidity index

As can be seen in Figure 1, during the first period of the experiment, the THI was >88 at 15:00 h, indicating high HS. The THI

TABLE 1 ET, RH, and THI measurements during the experimental period.

Parameter		H	M	L
ET (°C)	08:00–18:00	35	30	22
	18:00–08:00	30	28	20
RH (%)	08:00–18:00	80	85	90
	18:00–08:00	80	85	90
THI	08:00–18:00	91	84	71
	18:00–08:00	83	81	71

ET, Environmental temperature; RH, Relative humidity; THI, Temperature–humidity index.

varied daily from 79 to 88 throughout the experiment's second period, indicating moderate HS. The THI was <72 in the third experiment period, which meant there was no HS.

2.3 Sample collection and analysis

2.3.1 Dry matter intake, rectal temperature, and respiratory rate

The average daily dry matter intake (DMI) of Dazu black goats was calculated as the difference between the initial amount of feed and the amount of feed leftover the following morning.

In accordance with the method described by Tucker et al. (19), rectal temperature (RT; obtained with a GLA 525/550 Hi-Performance Digital Thermometer, San Luis Obispo, CA) was measured by inserting a disinfected thermometer into the rectum of each goat at 08:00, 14:00, and 18:00 h on d 7 and 15 of each experimental period. The respiratory rate (RR) of each goat was recorded by calculating as breaths/min using a stopwatch for 1 min and the average value of three consecutive measurements was recorded.

TABLE 2 Ingredient and nutrient composition of the TMR (dry matter basis) %.

Ingredients		Nutrient composition[2]	
Pennisetum sinese Roxb	50.00	DM[3]	87.75
Corn	32.27	ME[4] (MJ/kg)	10.71
Wheat bran	7.20	CP[5]	11.43
Soybean meal	6.93	NDF[6]	50.56
NaCl	0.50	ADF[7]	36.06
$CaHPO_3{\cdot}2H_2O$	1.30	Ash	11.05
Limestone	0.80	Ca[8]	0.87
Premix[1]	1.00	P[9]	0.36
Total	100.00		

[1]Per kg of concentrated premix: vitamin A, 45,000 IU; vitamin D, 4,000 IU; vitamin E, 55 mg; vitamin PP, 130 mg; vitamin B_1, 14 mg; vitamin B_2, 12.5 mg; vitamin B_6, 5 mg; vitamin B_{12}, 0.02 mg; folic acid, 0.2 mg; Co, 1 mg; Fe, 100 mg; I, 2.5 mg; Mn, 65 mg; Cu, 13 mg; Zn, 225 mg; Se, 0.14 mg.
[2]Metabolizable energy levels were predicted and the rest nutrient levels were measured.DM[3], Dry matter; ME[4], Metabolizable energy; CP[5], Crude protein; NDF[6], Neutral detergent fiber; ADF[7], Acid detergent fiber; Ca[8], Calcium; P[9], Phosphorus.

2.3.2 Blood biochemistry

Blood samples were collected on d 15 of each experimental period before feeding at 8:00 h and used to prepare serum and plasma samples. After centrifugation at 3,000 rmp for 15 min, the supernatant was aspirated, and aliquots were stored in 1.5-mL centrifuge tubes at −20°C until assayed. Blood glucose (GLU), blood urea nitrogen (BUN), triglycerides (TG), cholesterol (CHO), high-density lipoprotein (HDL-C), and low density-lipoprotein (LDL-C) were measured with an automatic biochemical analyzer (model AU5800; Beckman Coulter, Inc., Brea, CA, United States). Commercial kits were used for the measurement of Non-esterified fatty acid (NEFA; Wako Chemicals GmbH, Neuss, Germany). Serum levels of total protein (TP), cortisol (COR), triiodothyronine (T_3), thyroxin (T_4), and insulin (INS) were measured with enzyme-linked immunosorbent assay (ELISA) kits (Nanjing Jiang Cheng Bioengineering Institute, Nanjing, China).

2.3.3 GLU tolerance test and INS tolerance test

At 08:00 h on d 7 and 10 of each period, GLU tolerance test (GTT) and INS tolerance test (ITT) of all goats was conducted after fasting for 12 h, respectively. A 50% dextrose (0.5 g/kg) solution (AGRIpharm Products, Grapevine, TX) was administered via the jugular catheter and immediately chased with 12 mL of sterile saline. GLU concentration was measured before insulin injection, which was administered at 0.75 U/Kg body weight. Blood samples were collected at 5 min before, at the time of GLU administration (0 min was used as a baseline parameter), and at 5, 10, 15, 20, 30, 45, 60, and 90 min after GLU load. Samples were collected into disposable glass culture tubes containing 250 U of sodium heparin and were immediately placed on ice. And then centrifuged at 3,000 rmp for 15 min. The plasma was divided into 2 aliquots, which were both frozen at −20°C; one aliquot was later analyzed for plasma glucose levels and the other for plasma insulin concentrations.

The GLU and INS responses to the GTT were measured as area under the curve (AUC). Mean GLU values from the samples obtained before the start of a challenge were used as the baseline metabolite concentrations, and the 0 time point sample was used for the INS

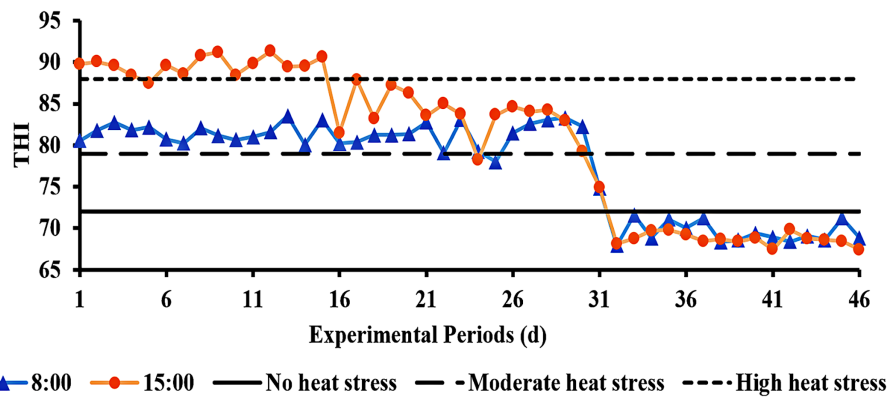

FIGURE 1
THI of the barn during the experimental period.

baseline value. The GLU and INS AUC were calculated through the 90 min sample during the GTT. The blood GLU AUC was calculated through the 90 min sample during the ITT.

2.3.4 Blood metabolites

At 15:00 h, on the last day of all three experimental stages, 3 mL of blood were collected from each goat into tubes coated with heparin sodium as an anticoagulant. Following a 15-min centrifugation at 3,000 rmp, the serum was moved to a fresh tube and kept at −80°C for non-targeted metabolomics examination. Blood samples stored at −80°C were thawed slowly at 4°C for sample pretreatment and analyzed by the Agilent 1,290 Infinity LC ultra-high performance liquid chromatography system (UHPLC). The samples were separated by UHPLC and analyzed by a Triple TOF 5600 mass spectrometer (AB SCIEX). The positive ion (ESI+) and negative ion (ESI-) modes were used for detection, and the raw data were converted into.MZXML format by ProteoWizard for data processing.

Non-targeted metabolomics analysis included ultra-high performance liquid chromatography with quadrupole time-of-flight mass spectrometry and pathway enrichment analysis in reference to the Kyoto Encyclopedia of Genes and Genomes (KEGG) database.[1] The KEGG database and MetPA software were used for pathway analysis of potential biomarkers to identify related metabolic pathways. The KEGG pathway enrichment analysis of differential metabolites was performed by Fisher's exact test.

2.3.5 Chemical analysis

The TMR samples were dried in an oven at 65°C until a constant weight was achieved. Upon drying, the samples were ground and passed through a 1-mm sieve for further analysis. The chemical composition of dry matter (DM), crude protein (CP), ether extract (EE), ash, calcium (Ca), and phosphorus (P) were measured according to the Association of Official Analytical Chemists [(20); AOAC]. Neutral detergent fiber (NDF) and acid detergent fiber (ADF) were determined according to Vansoest et al. (21). The ingredients and chemical composition of the Dazu black goats' TMR are presented in Table 2.

2.3.6 Statistical analysis

Excel 2016 (Microsoft Corporation, Redmond, WA, United States) was used to record the initial DMI, RT, RR, and blood biochemical data. IBM SPSS Statistics for Windows, version 26.0, was used to analyze the data using one-way analysis of variance or the paired sample t-test (IBM Corporation, Armonk, NY, United States). Graphpad Prism 8 software was used to plot DMI, RT, RR, and tolerance texts graphs (San Diego, CA, United States). Statistical analysis between multiple groups of repeated measurement samples was performed by Repeat ANOVA Measurement of variance and p value was calculated. Results were shown as the Mean ± SD. Probability (p) value <0.05 was considered significant. Metabolites with VIP > 1 and $p < 0.05$ that were applied to Student's t-test at univariate level among the groups were considered statistically significant.

1 https://www.genome.jp/kegg/

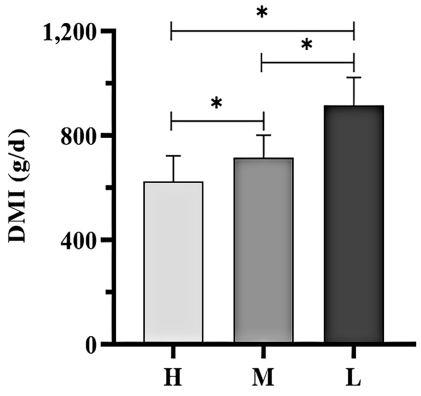

FIGURE 2
DMI of Dazu black goats of all experimental groups. *indicated significant difference ($p < 0.05$).

3 Results

3.1 Dry matter intake

The average DMI of Dazu black goats significantly increased as the THI decreased. Notably, DMI was significantly lower in groups H and M as compared to group L (Figure 2; $p < 0.05$).

3.2 Rectal temperature and respiratory rate measurements

Table 3 displays the statistical data on RT and RR. Compared to group L HS increased the RT of Dazu black goats at all three time points in groups H and M ($p < 0.05$). But there was no significant difference in RT between groups H and M at 08:00 and 18:00 h ($p > 0.05$). HS significantly increased the RR of Dazu black goats at all three time points ($p < 0.05$). The highest measurements of both RT and RR occurred at 14:00 h.

3.3 Blood biochemical indices

3.3.1 Blood indices

Compared to group L, HS increased NEFA, COR, and TP concentrations and decreased INS concentration ($p < 0.05$). The BUN concentration increased and the GLU concentration decreased in group H compared to group L ($p < 0.05$). There were no significant differences in CHO, HDL-C, LDL-C, TG, T_3, and T_4 contents among the three groups ($p > 0.05$; Table 4).

3.3.2 GLU tolerance test

As shown in Figure 3A, during the GTT, the GLU concentration in group H was higher than that in group L (20.25–58.00%) during the 0–20 min period. The GLU concentration in group M was higher than that in group L (23.89–77.47%) during the 0–90 min period. Figure 3B shows that the INS concentrations in group H (18.19–42.73%) and group M (8.46–34.17%) were lower than those in group L during 5–60 min.

Figure 4A shows that the AUC of GLU concentrations in groups H and M were 12.26% ($p > 0.05$) and 40.78% ($p < 0.05$) higher than that in group L, respectively. Figure 4B shows that the AUC of INS concentrations in groups H and M were 26.04 and 14.41% ($p < 0.05$) lower than that in group L, respectively.

3.3.3 INS tolerance test

As shown in Figures 5, 6, the AUC of GLU concentrations were not significant among the three groups ($p > 0.05$).

3.4 The blood metabolome

In total, there were 75 differential metabolites between groups H and L, 77 between groups M and L, and 26 between groups H and M. As compared to group L, 60 significantly different metabolites were identified in groups H and M, which mainly included amino acids, organic amines, carbohydrates, organic acids, and esters (Table 5). As shown in Figure 7, serum levels of glycogenic amino acids and ketogenic amino acids were significantly increased in groups H and M compared to group L ($p < 0.05$). In addition, serum levels of taurine, creatinine, choline, indole-2-carboxylic acid, L-carnosine, diethanolamine, inosine, creatine, and carbohydrates were also significantly increased ($p < 0.05$), while the serum levels of phenylacetyl glycine, erucamide, alpha-tocopherol, 1-palmitoyl-sn-glycero-3-phosphocholine, creatine, PC (16:0/16:0), 1,2-dioleoyl-sn-glycero-3-phosphatidylcholine, glycerophosphocholine, thioetheramide-PC, indoxyl sulfate, prostaglandin F3α, arachidic acid, and perindopril were significantly decreased ($p < 0.05$) in groups H and M compared to group L.

Comprehensive analysis of the differential metabolites obtained by comparisons of two pairs using KEGG metabolic pathways. The results showed that the differential metabolites in plasma were associated with "amino acid metabolism," "lipid metabolism," "carbohydrate metabolism," "nucleotide metabolism," "central carbon metabolism," "aminoacyl-tRNA biosynthesis," "ABC transporter" as well as digestion and absorption of proteins and minerals (Table 6).

TABLE 3 RT and RR of all experimental groups.

Times	Groups	RT (°C)	RR (breaths/min)
08:00	H	38.39 ± 0.17[a]	68.17 ± 9.46[a]
	M	38.37 ± 0.21[a]	60.35 ± 7.31[b]
	L	38.10 ± 0.30[b]	14.50 ± 2.78[c]
14:00	H	39.21 ± 0.29[a]	119.17 ± 21.80[a]
	M	38.64 ± 0.19[b]	78.85 ± 11.34[b]
	L	38.38 ± 0.25[c]	14.22 ± 2.10[c]
18:00	H	38.71 ± 0.14[a]	93.83 ± 20.21[a]
	M	38.67 ± 0.16[a]	74.65 ± 7.81[b]
	L	38.32 ± 0.22[b]	14.24 ± 2.51[c]

RT, Rectal temperature; RR, Respiratory rate. [a–c]Demonstrate a statistically significant distinction, $p < 0.05$.

4 Discussion

4.1 Physiological indicators

In this experiment, DMI increased as the THI decreased. In response to HS, animal secretion and expression of adiponectin and leptin will increase, adiponectin regulates feeding behavior by stimulating peripheral receptors to transmit nerve impulses to the hypothalamus, while leptin stimulates the hypothalamic appetitive center to reduce food intake (22–25), ultimately reducing metabolism and heat production. Reduced DMI and nutrient digestibility by HS can lead to undernutrition in animals and adversely affect their health (26). HS is reported to decrease DMI in sheep (27), dairy goats (18), and dairy cows (28).

RR and RT are the most common physiological indices of HS. Exposure to high ET inhibits the ability to dissipate heat, resulting in increased RT. Meanwhile, the RR is increased to enhance lung

TABLE 4 Effects of HS on blood biochemical indices in Dazu black goats.

Project	H	M	L
CHO (mmol/L)	2.39 ± 0.39	2.37 ± 0.59	2.16 ± 0.42
HDL-C (mmol/L)	1.46 ± 0.24	1.39 ± 0.26	1.33 ± 0.21
LDL-C (mmol/L)	0.74 ± 0.14	0.74 ± 0.27	0.60 ± 0.18
TG (mmol/L)	0.39 ± 0.09	0.38 ± 0.07	0.40 ± 0.09
NEFA (μmol/L)	97.53 ± 8.97[a]	90.75 ± 14.04[a]	76.46 ± 10.37[b]
T_3 (ng/mL)	5.85 ± 0.90	5.80 ± 1.29	6.43 ± 1.17
T_4 (nmol/L)	131.47 ± 20.1	142.11 ± 42.71	146.15 ± 34.24
COR (μg/dL)	247.69 ± 56.25[a]	208.22 ± 21.79[b]	160.12 ± 41.96[c]
INS (pmol/L)	21.29 ± 0.41[a]	22.06 ± 1.26[a]	31.57 ± 33.68[b]
GLU (mmol/L)	3.65 ± 0.26[a]	4.21 ± 0.69[b]	4.25 ± 0.64[b]
TP (mg/mL)	45.94 ± 3.93[a]	40.22 ± 5.09[b]	26.83 ± 4.70[c]
BUN (mmol/L)	6.23 ± 1.02[a]	6.00 ± 0.93[ab]	5.14 ± 0.98[b]

CHO, Cholesterol; HDL-C, High-density lipoprotein; LDL-C, Low density-lipoprotein; TG, Triglycerides; NEFA, Non-esterified fatty acid; T_3, Triiodothyronine; T_4, Thyroxin; COR, Cortisol; INS, Insulin; GLU, Blood glucose; TP, Total protein; BUN, Blood urea nitrogen.
[a–c]Demonstrate a statistically significant distinction, $p < 0.05$.

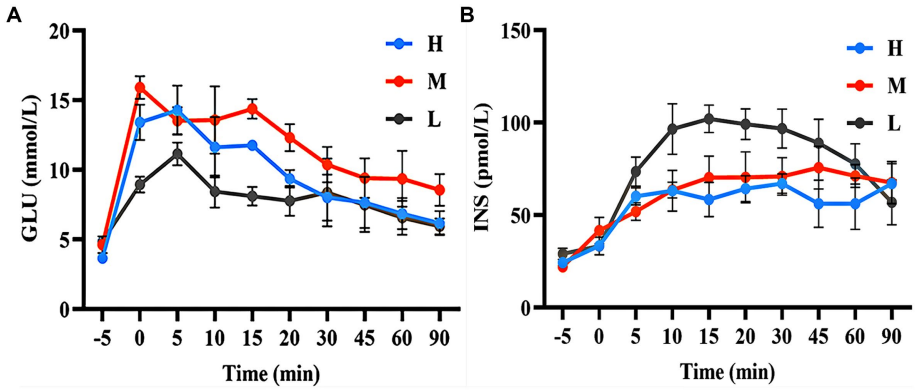

FIGURE 3
GLU concentration **(A)** and INS concentration **(B)** during the GTT.

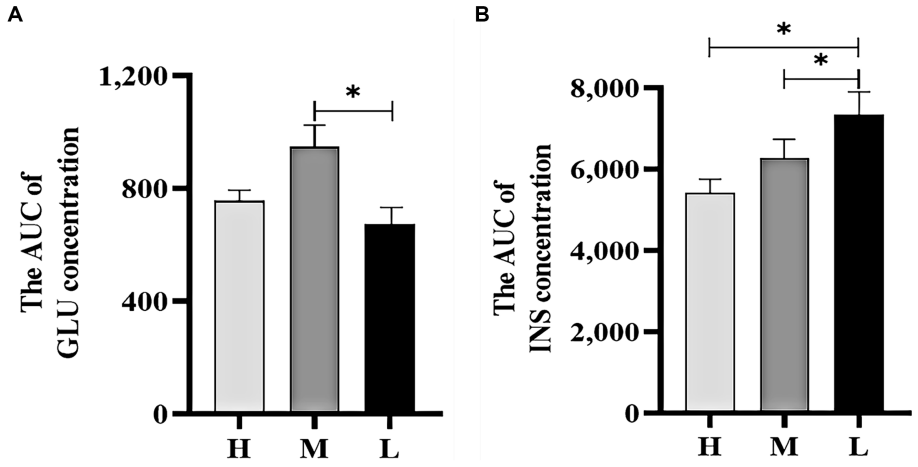

FIGURE 4
The AUC of GLU concentration **(A)** and INS concentration **(B)** during the GTT. *indicated significant difference ($p < 0.05$).

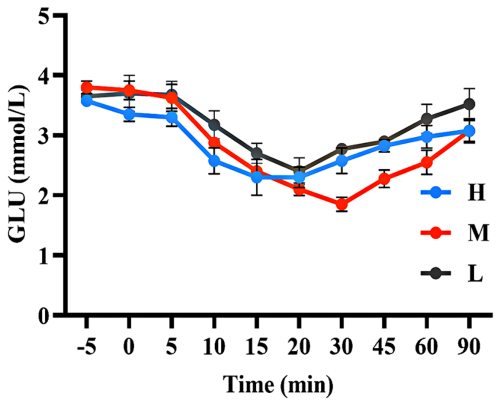

FIGURE 5
The GLU concentration during the ITT.

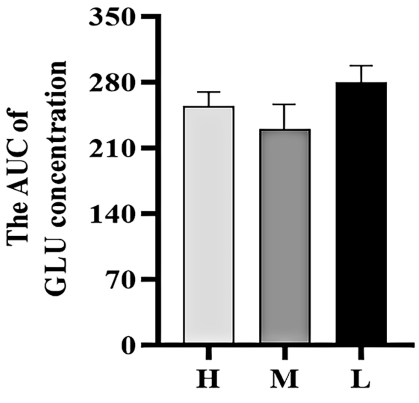

FIGURE 6
The AUC of GLU concentration during the ITT.

ventilation and dissipate heat (29). Under suitable environmental conditions, the RT of ewes fluctuates between 38.3°C and 39.0°C (30). Shilja et al. (31) found that the RR and RT of goats were significantly higher in the HS group than in the non-HS group (69.17 breaths/min and 39.08°C vs. 31.92 breaths/min and 38.70°C, respectively). Marai et al. (32) reported that the RR and RT of goats were significantly

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TABLE 5 Identified differential metabolites of the groups H, M, and L in positive and negative ion modes.

Metabolite	H/L					M/L				
	VIP	FC	*p*-value	Trend	ESI	VIP	FC	*p*-value	Trend	ESI
D-proline	2.63	2.12	2.68E-07	↑	+	2.46	2.27	2.3E-05	↑	+
D-mannose	1.84	3.00	4.66E-07	↑	+	1.40	2.54	0.0006	↑	+
Taurine	2.02	3.03	1.86E-06	↑	+	1.89	3.33	6.45E-05	↑	+
Creatinine	7.89	1.88	9.85E-06	↑	+	6.82	1.83	4.29E-06	↑	+
Choline	3.68	1.66	2.8E-05	↑	+	2.63	1.47	0.0009	↑	+
Indole-2-carboxylic acid	1.96	4.12	3.63E-05	↑	+	1.59	3.64	0.0003	↑	+
Phenylacetyl glycine	2.93	0.30	5.34E-05	↓	+	2.54	0.43	0.0002	↓	+
L-carnosine	1.88	2.04	5.49E-05	↑	+	1.81	2.34	0.0006	↑	+
Diethanolamine	1.59	236.01	6.45E-05	↑	+	1.51	254.49	0.0005	↑	+
Inosine	1.56	2.13	8.36E-05	↑	+	1.43	2.29	0.0046	↑	+
Triethanolamine	1.99	21.28	0.0002	↑	+	1.49	15.43	0.0044	↑	+
L-phenylalanine	2.51	2.05	0.0003	↑	+	2.42	2.17	8.89E-05	↑	+
L-arginine	1.85	2.09	0.0004	↑	+	1.51	2.15	0.0432	↑	+
Erucamide	7.34	0.36	0.0004	↓	+	6.55	0.49	0.0048	↓	+
N6,N6,N6-trimethyl-L-lysine	2.17	2.99	0.0005	↑	+	2.35	3.69	1.58E-05	↑	+
Alpha-tocopherol (vitamin E)	2.30	0.41	0.0005	↓	+	2.12	0.43	0.0027	↓	+
1-Palmitoyl-sn-glycero-3-phosphocholine	3.27	0.77	0.0006	↓	+	3.64	0.71	0.0359	↓	+
Betaine	2.11	2.20	0.0006	↑	+	2.15	2.58	0.0046	↑	+
L-NG-monomethylarginine	1.37	2.99	0.0006	↑	+	1.22	2.83	3.26E-05	↑	+
L-tryptophan	2.12	2.26	0.0008	↑	+	2.02	2.34	4.66E-05	↑	+
Creatine	2.74	0.85	0.0010	↓	+	3.63	0.80	0.0851	↓	+
DL-indole-3-lactic acid	2.22	2.21	0.0014	↑	+	2.20	2.29	6.25E-06	↑	+
Tyramine	1.64	2.05	0.0029	↑	+	1.74	2.31	0.0002	↑	+
L-carnitine	3.46	2.98	0.0044	↑	+	2.75	2.47	0.0004	↑	+
PC (16:0/16:0)	5.12	0.30	0.0059	↓	+	4.74	0.38	0.0169	↓	+
(3-Carboxypropyl) trimethylammonium cation	2.36	1.90	0.0060	↑	+	2.40	2.09	0.0008	↑	+
NG,NG-dimethyl-L-arginine	3.04	2.64	0.0073	↑	+	3.62	3.04	5.26E-05	↑	+
Hypoxanthine	1.83	1.97	0.0080	↑	+	1.28	1.58	0.0414	↑	+
L-leucine	1.68	1.59	0.0081	↑	+	1.84	1.85	0.0085	↑	+
L-isoleucine	1.23	1.39	0.0089	↑	+	1.44	1.64	0.0048	↑	+
N6-methyl-L-lysine	2.43	2.94	0.0108	↑	+	2.91	3.81	0.0011	↑	+
Trimethylamine N-oxide	1.64	5.54	0.0127	↑	+	1.86	7.60	0.0012	↑	+
1-Methylhistidine	2.89	2.52	0.0142	↑	+	1.27	1.95	0.0425	↑	+
L-histidine	1.01	2.46	0.0178	↑	+	1.01	2.85	0.0225	↑	+
L-citrulline	1.42	1.66	0.0241	↑	+	1.20	1.68	0.0139	↑	+
1,2-Dioleoyl-sn-glycero-3-phosphatidylcholine	9.41	0.20	0.0313	↓	+	5.09	0.37	0.0039	↓	+
Cyclohexylamine	3.90	2.69	0.0326	↑	+	3.92	2.69	0.0451	↑	+
L-histidine	1.31	2.28	0.0383	↑	+	1.34	2.45	0.0193	↑	+
Glycerophosphocholine	1.28	1.21	0.0418	↓	+	1.24	3.47	8.02E-06	↓	+
Thioetheramide-PC	3.54	0.38	0.0557	↓	+	3.71	0.34	0.0483	↓	+

(Continued)

TABLE 5 (Continued)

Metabolite	H/L					M/L				
	VIP	FC	*p*-value	Trend	ESI	VIP	FC	*p*-value	Trend	ESI
Acetyl carnitine	3.91	1.40	0.0687	↑	+	4.65	1.57	0.0338	↑	+
Hippuric acid	4.16	1.59	0.0002	↑	–	3.39	1.53	0.0178	↑	–
L-alanine	1.37	2.20	0.0003	↑	–	1.50	2.36	0.0001	↑	–
Indoxyl sulfate	5.83	0.44	0.0004	↓	–	5.68	0.50	0.0011	↓	–
Salicylic acid	1.99	2.16	0.0006	↑	–	1.53	1.78	0.0041	↑	–
Prostaglandin F3α	7.78	0.35	0.0007	↓	–	7.61	0.41	0.0042	↓	–
3,4-Dihydroxybenzoate (protocatechuic acid)	1.29	2.19	0.0011	↑	–	1.48	2.44	0.0001	↑	–
L-glutamate	1.33	2.53	0.0013	↑	–	1.51	3.32	0.0533	↑	–
15-Keto-PGE1	2.55	5.07	0.0030	↑	–	2.22	4.29	0.0056	↑	–
L-gluonic gamma-lactone	3.17	2.52	0.0032	↑	–	2.29	1.95	0.0914	↑	–
Pseudouridine	1.12	2.31	0.0033	↑	–	1.22	2.39	0.0016	↑	–
Thymidine	1.52	1.85	0.0060	↑	–	1.40	1.82	0.0022	↑	–
Arachidic acid	1.79	0.59	0.0113	↓	–	1.86	0.58	0.0137	↓	–
Allantoin	2.12	1.49	0.0120	↑	–	2.47	1.60	0.0180	↑	–
Taurolithocholic acid	1.93	2.73	0.0144	↑	–	1.83	2.70	0.0023	↑	–
Alpha-D-GLU	6.06	2.39	0.0152	↑	–	7.73	2.89	0.0015	↑	–
L-norleucine	1.83	1.73	0.0155	↑	–	2.56	2.05	0.0048	↑	–
Perindopril	3.99	0.42	0.0271	↓	–	3.53	0.50	0.0421	↓	–
D-allose	1.57	1.78	0.0337	↑	–	1.68	1.89	0.0134	↑	–
DL-lactate	3.57	1.73	0.0352	↑	–	1.15	1.69	0.0457	↑	–

↑, increase; ↓, decrease. A positive Fold Change (FC) Analysis value indicates an increase, while a negative value indicates a decrease.
VIP, Variable Importance for the Projection. $p < 0.05$ was considered to indicate significant difference.

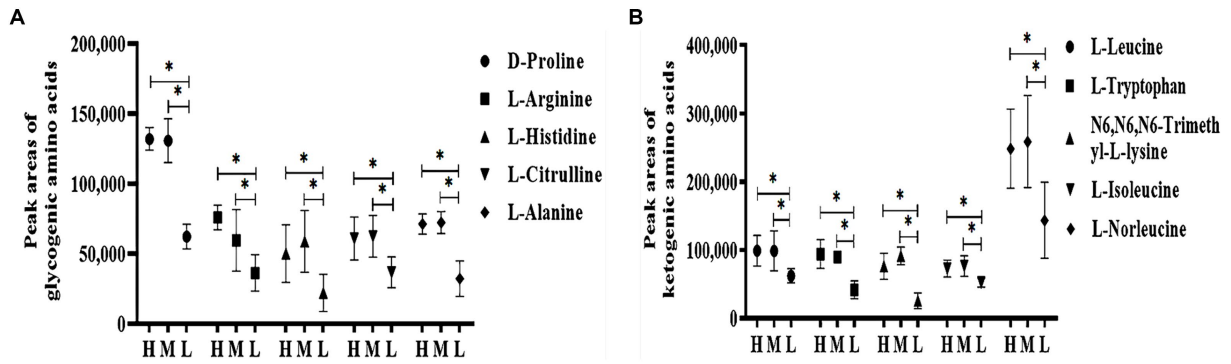

FIGURE 7
Effects of HS on peak areas of amino acid in Dazu black goats. **(A)** Glycogenic amino acids in the plasma; **(B)** Ketogenic amino acids in the plasma. *indicated significant difference ($p < 0.05$).

higher in the summer than the winter, but varied among different breeds, and physiological changes were greater in cold-adapted breeds during the summer than heat-adapted breeds. The results of the present study showed that the RR and RT of goats were significantly increased in response to HS, as RT increased to 39.8°C. Banerjee et al. (33) found that an increase in RT of ≤1°C decreased productivity and reproductive capacity.

4.2 Blood biochemistry

The hypothalamic–pituitary–adrenal axis of the neuroendocrine system is primarily involved in the stress response (34). HS-induced stimulation transmits nerve impulses through the cerebral cortex to the hypothalamus, which then releases hormones that promote the secretion of adrenocorticotropic

TABLE 6 KEGG pathways affected by HS in Dazu black goats.

KEGG level 1	KEGG level 2	KEGG level 3	Rich factor H/L	*p*	Rich factor M/L	*p*
Metabolism	Amino acid metabolism	Histidine metabolism	0.106383	7.27E-05	0.106383	8.61E-05
		Phenylalanine metabolism	0.069444	0.0006	0.069444	0.000652
		Arginine and proline metabolism	0.064935	0.0008	0.064935	0.000886
		Glycine, serine, and threonine metabolism	0.08	0.0012	0.08	0.001382
		Arginine biosynthesis	0.130435	0.0013	0.173913	6.47E-05
		Phenylalanine, tyrosine, and tryptophan biosynthesis	0.085714	0.0043	0.085714	0.004719
		Valine, leucine, and isoleucine biosynthesis	0.086957	0.019698	0.086957	0.021044
		Alanine, aspartate and glutamate metabolism	0.071429	0.028557	0.107143	0.00248
	Metabolism of other amino acids	Taurine and hypotaurine metabolism	0.136364	0.0011	0.181818	5.39E-05
		beta-Alanine metabolism	0.0625	0.036581	0.0625	0.039004
		D-Glutamine and D-glutamate metabolism			0.166667	0.005883
	Lipid metabolism	Glycerophospholipid metabolism	0.096154	0.0001	0.096154	0.000141
		Linoleic acid metabolism	0.107143	0.0022	0.071429	0.030475
		Secondary bile acid biosynthesis			0.055556	0.048326
		Primary bile acid biosynthesis			0.06383	0.010758
		Biosynthesis of unsaturated fatty acids	0.055556	0.014269		
	Carbohydrate metabolism Energy metabolism	Fructose and mannose metabolism	0.055556	0.014269	0.055556	0.015681
		Methane metabolism	0.035714	0.044956		
		Nitrogen metabolism			0.105263	0.014585
	Biosynthesis of other secondary metabolites	Glucosinolate biosynthesis	0.053333	0.0054	0.053333	0.006082
		Tropane, piperidine and pyridine alkaloid biosynthesis			0.044118	0.028777
	Nucleotide metabolism	Pyrimidine metabolism			0.045455	0.026639
Genetic Information Processing	Translation	Aminoacyl-tRNA biosynthesis	0.153846	2.14E-08	0.192308	4.31E-11
Environmental Information Processing	Membrane transport	ABC transporters	0.09375	1.6E-09	0.117188	6.53E-13
	Signal transduction	mTOR signaling pathway	0.333333	0.028205	0.333333	0.029203
		FoxO signaling pathway	0.2	0.046573	0.2	0.048204
		Two-component system			0.053571	0.017285

(Continued)

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TABLE 6 (Continued)

KEGG level 1	KEGG level 2	KEGG level 3	Rich factor H/L	*p*	Rich factor M/L	*p*
Cellular Processes	Transport and catabolism	Lysosome	0.25	0.037432	0.25	0.038749
Organismal Systems	Digestive system	Protein digestion and absorption	0.191489	3.3E-10	0.234043	4.06E-13
		Mineral absorption	0.172414	6.41E-06	0.206897	2.76E-07
		Bile secretion			0.028571	0.027977
	Nervous system	Retrograde endocannabinoid signaling	0.157895	0.0007	0.105263	0.014585
		Long-term depression	0.222222	0.0031		
		Glutamatergic synapse			0.25	0.00256
		GABAergic synapse			0.222222	0.00327
	Excretory system	Proximal tubule bicarbonate reclamation	0.117647	0.01098	0.176471	0.000556
Human Diseases	Cancers: Overview	Central carbon metabolism in cancer	0.216216	1.22E-09	0.243243	4.52E-11
		Choline metabolism in cancer	0.272727	0.0001	0.272727	0.000141
	Neurodegenerative diseases	Amyotrophic lateral sclerosis (ALS)	0.2	0.0038	0.2	0.004062
		Huntington disease	0.333333	0.028205	0.333333	0.029203
	Infectious diseases: Bacterial	Salmonella infection	0.25	0.037432	0.25	0.038749
	Infectious diseases: Parasitic	Amoebiasis	0.153846	0.006452		

$p < 0.05$ was considered to indicate significant difference.

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hormone (ACTH) and inhibit the production of thyroid hormone (TSH). ACTH and TSH act on the adrenal and thyroid glands, respectively, thus increasing the secretion of COR by the adrenal glands while decreasing the production of T_3 and T_4 by the thyroid gland. In response to HS, serum levels of COR are increased (35). COR is a common biomarker of the stress response and can help maintain the internal environment and reduce HS-induced damage (36, 37). However, excessive production of COR in response to long-term HS can damage immune-related organs by inducing the release of inflammatory factors and promoting the aggregation and adhesion of leukocytes, thereby triggering an inflammatory response. Exposure to excessive heat for more than 2 h will elevate serum COR levels (38). Przemyslaw et al. (39) reported that exposure to ET of 50°C increased serum COR levels of Merino rams by nearly 10 fold. In addition, HS-induced increases in serum levels of COR and epinephrine generally inhibit the production of INS and TG while promoting glycolysis, gluconeogenesis, lipolysis, and the production of NEFA (40, 41).

Low serum GLU can promote lipid mobilization (42), resulting in the release of NEFA into the blood for energy production. Therefore, increased serum levels of NEFA can conserve GLU (28). The decrease in serum levels of GLU with increased concentrations of NEFA in response to HS supports this view. HS was confirmed to decrease serum levels of GLU in cows (43), goats (44), and calves (45). The results of GTT and ITT showed that HS impaired GLU tolerance, but did not change INS tolerance and sensitivity, so it is speculated that HS may change blood GLU concentration by affecting INS secretion rather than sensitivity. In the context of HS, the increase in blood GLU concentration during the GTT can be attributed to the altered metabolic responses induced by the stress condition. HS can lead to the release of stress hormones such as COR, which can promote gluconeogenesis and glycogenolysis, consequently elevating blood GLU concentration (46). Additionally, HS may reduce INS secretion, further contributing to the hyperglycemic response observed during the GTT under HS conditions (47).

HS can promote the production of free radicals and subsequent oxidative damage and apoptosis (48, 49). Oxidation helps to maintain cellular integrity and provides energy. Antioxidation works in tandem with aerobic metabolism to combat free radical-induced tissue damage. Moreover, reduced production of antioxidants can promote oxidative stress (48) and increase secretion of inflammatory cytokines (50). Excessive oxidation induces the body to produce excessive inflammatory response and immune response, which further aggravates the damage of tissues, organs and systems. Reactive oxygen species (ROS) can increase protein degradation while reducing protein synthesis (48). Finocchiaro et al. (51) found that the protein content in ewe milk was negatively correlated with the THI during HS. In the present study, the TP increased significantly under HS, indicating that goat body protein degradation increased at this time. A large number of reactive oxygen species can damage the protein structure, DNA structure, cell membrane structure and various organelles of cells, thereby causing systemic inflammation, such as fatty liver, laminitis, metritis and mastitis, and reducing the yield and quality of milk and meat.

4.3 Blood metabolite

In this study, there were notable changes to metabolites of carbohydrate metabolism, gluconeogenesis, and glycolysis (e.g., alpha-D-GLU, D-allose, Phenylacetyl glycine, hippuric acid, glycogenic amino acids, and DL-lactate; Figure 8), indicating that HS influences the energy metabolism of Dazu black goats. The increased contents of alpha-D-GLU and D-allose in response to HS indicate decreased energy expenditure with increasing ET, which could reduce heat production in goats. Serum levels of glycogenic amino acids, including

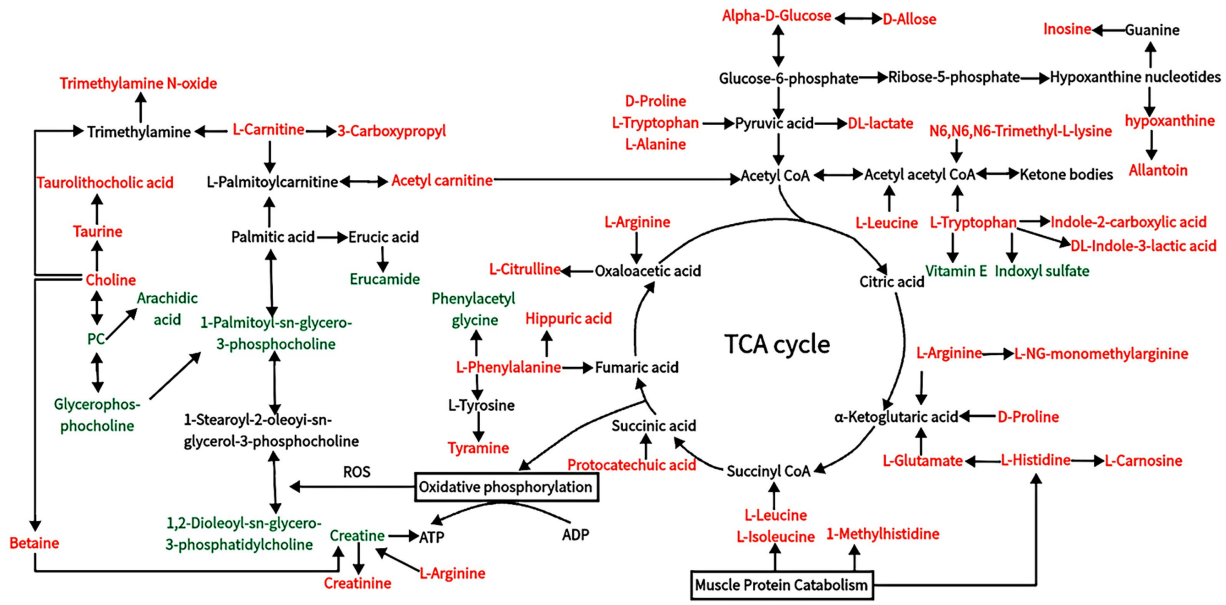

FIGURE 8
Changes in blood metabolic pathways of Dazu black goats under HS. The metabolites are colored according to the type of change in response to HS (black, no change; red, upregulation; green, downregulation).

D-proline, L-arginine, L-histidine, L-citrulline, and L-alanine, were increased in response to HS, indicating enhanced gluconeogenesis. In a state of HS, goats experience decreased DMI and energy supply, leading to insufficient nutrient intake, which results in the use of stored nutrients for energy, thereby increasing protein degradation and serum levels of amino acids. When the energy supply is low, glycogen reserves are limited, and carbohydrate transport occurs through gluconeogenesis (52). In addition, decreased production of TSH, T_3, and T_4 with increased production of ROS in response to HS will increase protein degradation and serum levels of amino acids. Guo et al. (53) found increased serum concentrations of total amino acids in cows in response to HS, especially glucogenic amino acids (alanine, aspartic acid, glutamic acid, and glycine). Cowley et al. (54) reported that low serum concentrations of GLU can increase the consumption of amino acids, thereby promoting gluconeogenesis in cows in response to HS. DL-lactate, the main metabolite of glycolysis, accumulated in the blood of Dazu black goats during HS. In addition to the amino acids involved in gluconeogenesis, those related to glycolysis are also increased by HS (55). L-alanine can regulate gluconeogenesis and glycolysis to ensure energy production when energy intake is insufficient (56). Increased serum levels of metabolites, such as alpha-D-GLU, glucogenic amino acids, and DL-lactate, indicate enhanced gluconeogenesis and glycolysis in response to HS to meet energy requirements. These changes were all observed in KEGG pathways (Table 6).

L-carnitine and acetyl carnitine are metabolites of fatty acids (57) and act as carriers of long-chain fatty acids, such as palmitoylcarnitine, across the mitochondrial inner membrane for fatty acid β-oxidation. Acetyl carnitine is produced in the mitochondrial matrix by carnitine and acetyl coenzyme A (58). Metabolites of acetylcarnitine can undergo fatty acid oxidation and enter the tricarboxylic acid cycle. Accumulation of L-carnitine indicates inhibition of β-oxidation in goats in a state of HS. However, L-carnitine can be beneficial by inhibiting aerobic oxidation of lipids, thereby reducing oxidative stress (59). The main application of vitamin E is as an antioxidant to protect polyunsaturated lipids from damage brought on by free radicals (60). In this study, the content of vitamin E was significantly decreased in goats in response to HS, indicating that HS may reduce the production of antioxidants. Therefore, Dazu black goats may resist cellular oxidative stress by reducing fatty acid β-oxidation rather than antioxidant regulation. Under physiological conditions, energy is produced by the complete oxidation of fatty acids in the kidney, myocardium, and other tissues. However, incomplete oxidation of fatty acids in liver cells forms ketone bodies. Notably, serum levels of ketogenic amino acids (L-leucine, L-tryptophan, L-lysine, L-isoleucine, and L-norleucine) were relatively increased in the HS group as compared to the non-HS group. Low serum GLU indicates insufficient intake of exogenous nutrients and cellular energy production. Intake of amino acids by liver cells increases production of ketone bodies, which then enter the circulation and are oxidized in extrahepatic tissues to supply energy. The L-leucine and L-isoleucine not only participate in ketogenesis but also play roles in immune regulation and protein metabolism (61, 62).

In addition to influencing the metabolism of liver cells, HS also significantly impacts the kidneys, which are crucial for physiological functions (63, 64). Under the conditions of HS and decreased feed intake, amino acid utilization is increased, leading to increased methylhistidine production as a marker of muscle fibrinolysis and increased liver urea synthesis (52). Abdelnour et al. (8) and Kamiya et al. (65) found that HS could break down histamine, as evidenced by increased serum concentrations of methylhistidine and urea. In the present study, serum levels of methylhistidine were significantly increased in response to HS, indicating increased fibrin catabolism. HS also increases serum concentrations of catecholamine and COR, leading to increased resistance in the kidneys and visceral vessels, resulting in renal ischemia and the timely excretion of metabolites (66). Furthermore, HS causes vasodilation and evaporative water loss, leading to lower blood pressure, followed by increased water retention, resulting in decreased renal clearance (67, 68). These two conditions are the main reasons for elevated serum concentrations of methylhistidine and urea. In addition, creatinine levels are significantly increased under HS conditions (8). The serum content of creatinine, a metabolite of creatine, is a reliable indicator of muscle tissue degradation (69). The serum concentration of creatinine is dependent on glomerular filtration and increases with impaired renal function. Although the creatine content was reduced under HS conditions due to decreased renal clearance, serum creatinine eventually increased. Citrulline is a product of muscle metabolism and is mainly filtered by the kidneys. Therefore, the serum citrulline concentration is considered a marker of renal function (70). An abnormally high serum citrulline level is an indicator of impaired renal function.

Serum levels of choline, betaine, taurine, and taurocholic acid were significantly increased, suggesting that Dazu black goats mainly recover from HS by producing a series of metabolites. Phospholipids, including glycerophospholipids and sphingosine phospholipids, are the main components of biofilms. In animals, phospholipids are hydrolyzed into glycerol, phosphate, choline, and ethanolamine by a series of phospholipases. Glycerophospholipids are major lipids in cell membranes and play important roles in cell signaling, G protein-coupled receptors, and ion transport (71). In the present study, serum levels of glycerophosphocholine, 1,2-dioleoyl-sn-glycero-3-phosphatidylcholine, and 1-palmitoyl-sn-glycero-3-phosphocholine were decreased in Dazu black goats in response to HS (Figure 8). Decreased levels of glycerophospholipid metabolites, such as hotline, indicate changes to the cell membrane structure and function of Dazu black goats. In addition, a significant decrease in glycerophospholipids alters the permeability and fluidity of cell membranes, which can serve as a defense mechanism to protect against oxidative damage caused by ROS (72, 73). Choline is a key precursor for the synthesis of acetylcholine (74), which can be easily oxidized to betaine (75), and plays an important regulatory role in maintaining cellular structural integrity and reducing oxidative stress (76). Heat shock proteins (HSPs) gradually restore proteins denatured by heat damage to normal states by refolding and preventing protein aggregation. Betaine can effectively improve the folding rate and decomposition rate of HSPs, thus enhancing their ability to withstand HS (77, 78). Choline, which reduces oxidative stress and thermal injury through a series of enzymatic reactions, produces glutathione and taurine (79, 80). Taurine can neutralize cholic acid in the liver to synthesize taurocholate, which promotes the absorption of lipids and fat-soluble vitamins. Chronic HS has been shown to influence purine metabolism, RNA transport, and down-regulate the metabolism of L-arginine and D-proline in dairy goats, while activating pathways associated with apoptosis and inhibiting pathways associated with tissue repair (81). In this study, serum levels of inosine and hypoxanthine were increased in response to HS, indicating that HS can induce apoptosis. Therefore,

the contents of choline, betaine, taurine, and taurocholic acid were increased to recover from heat injury, and the increased L-arginine and D-proline may be mainly used for energy metabolism.

5 Conclusion

HS decreased DMI and increased the RR and RT in Dazu black goats. HS changed blood hormone levels, increased protein degradation, increased plasma amino acid concentrations, elevated lipid levels, and impaired GLU tolerance. Gluconeogenesis, glycolysis, and ketogenic metabolism are increased under HS, thereby altering energy metabolic pathways in goats. Moreover, Dazu black goats primarily used an increase in amino acid metabolism as a source of energy in HS. In addition, in response to HS, a series of metabolites are produced to restore the heat damage of the organism.

Data availability statement

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

Ethics statement

The animal study was approved by the Institutional Animal Care and Use Committee of Southwest University (Chongqing, China). The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

LW: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Validation, Writing – original draft, Writing – review & editing. PZ: Data curation, Investigation, Validation, Writing – original draft. YD: Investigation, Validation, Writing – original draft. CW: Investigation, Validation, Writing – original draft. LZ: Writing – review & editing. LY: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Validation, Writing – original draft. FZ: Conceptualization, Funding acquisition, Project administration, Resources, Writing – review & editing. WH: Conceptualization, Data curation, Formal analysis, Funding acquisition, Project administration, Resources, Writing – review & editing.

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Conflict of interest

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

Publisher's note

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Effect of *Salvia sclarea* L. extract on growth performance, antioxidant capacity, and immune function in lambs

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The aim of this experiment is to explore the effects of *salvia sclarea* extract on the growth performance, apparent nutrient digestibility, antioxidant capacity, and immune function of lambs. Sixty female lambs (Chinese Merino sheep) aged 2 months and weighing 20 ± 2 kg were selected and randomly divided into five groups of twelve lambs in each. While the control group (CK) received only basal feed, the experimental group was supplemented with different concentrations of *salvia sclarea* extract in the basal feed at 0.04 mL/kg (group CL1), 0.08 mL/kg (group CL2), 0.12 mL/kg (group CL3), and 0.16 mL/kg (group CL4). The feeding period was 85 days, including 15 days of pre-feeding and 70 days of regular feeding. Body weight and feed intake were recorded during the test period, and blood was collected at the end of the test for the determination of immune and antioxidant indices. The results showed that the average daily gain and average daily feed intake of lambs were significantly increased in CL3 group compared to CK group ($p < 0.05$). Also, the apparent nutrient digestibility of crude protein and neutral detergent fiber was significantly increased ($p < 0.05$). The Dry matter, acid detergent fiber and Ether extract were not significantly different ($p > 0.05$). The serum levels of superoxide dismutase, catalase, glutathione peroxidase, and antioxidant capacity were significantly higher in the CL2, CL3, and CL4 groups compared to CK group, while malondialdehyde levels were significantly lower ($p < 0.05$). The serum levels of immune globulin A, immune globulin G, immune globulin M, interferon-γ, and interleukin-10 were significantly higher and the levels of tumor necrosis factor-α and interleukin-1β were significantly lower in the CL2, CL3, and CL4 groups ($p < 0.05$). In conclusion, the addition of *salvia sclarea* extract to the ration promotes growth performance and nutrient digestion in lambs. Improvement of immune response by increasing immunoglobulin and cytokine concentrations. And it enhances the antioxidant status by increasing the antioxidant enzyme activity in lambs.

Introduction: This study aimed to explore the effects of *Salvia sclarea* extract on the growth performance, apparent nutrient digestibility, antioxidant capacity, and immune function of the lambs.

Methods: Sixty female lambs (Chinese Merino sheep) aged 2 months and weighing 20 ± 2 kg were selected and randomly divided into five groups of 12 lambs each. The control group (CK) received only basal feed, whereas the experimental group was supplemented with different concentrations of *salvia sclarea* extract in the basal feed at 0.04, 0.08, 0.12, and 0.16 mL/kg (CL1, CL2, CL3, and CL4, respectively). The feeding period was 85 days, including 15 days of pre-feeding and 70 days of regular feeding. Body weight and feed intake were recorded during the test period, and blood was collected at the end of the test to determine immune and antioxidant indices.

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Results: The results showed that the average daily weight gain and feed intake of the lambs were significantly higher in the CL3 group than in the CK group ($p<0.05$). In addition, the apparent nutrient digestibility of crude protein and neutral detergent fiber increased significantly ($p<0.05$). The dry matter, acid detergent fiber, and ether extract were not significantly different ($p>0.05$). Serum levels of superoxide dismutase, catalase, and glutathione peroxidase and antioxidant capacity were significantly higher in the CL2, CL3, and CL4 groups than in the CK group, whereas malondialdehyde levels were significantly lower ($p<0.05$). The serum levels of immune globulin immune globulin A, immune globulin G, immune globulin M, interferon-γ, and interleukin-10 were significantly higher and the levels of tumor necrosis factor-α and interleukin-1β were significantly lower in the CL2, CL3, and CL4 groups ($p<0.05$).

Discussion: In conclusion, the addition of the *S. sclarea* extract to the diet promoted growth performance and nutrient digestion in lambs. Immune response was improved by increasing Ig and cytokine concentrations. It enhances antioxidant status by increasing antioxidant enzyme activity in lambs.

KEYWORDS

***Salvia sclarea* L. extract, lambs, growth performance, immune function, antioxidant capacity**

Introduction

Early postweaning stressors in lambs release glucocorticoids and coincide with a decrease in growth hormones, which suppress the immune system and reduce growth performance (1). A dietary change from milk to solid feed is considered a major stressor after weaning. The lack of a fully functional rumen in recently weaned ruminants reduces nutrient digestibility (2). Nutritional strategies have recently emerged. It has been proposed as a key factor in improving animal health and welfare as well as increasing livestock productivity (3). Dietary composition has long been recognized as an important factor in animal health, with significant effects on the acquired and innate immune systems, especially on inflammation (4). A number of compounds and products derived from plant-derived byproducts have been reported to show proinflammatory or anti-inflammatory effects and therapeutic responses and can trigger the expected response of the animal body to production parameters (5).

Salvia sclarea L. is a native Asian plant that is widely used in various fields including food, medicine, oils, and landscaping (6). It originated in Europe and is now primarily cultivated in France, Russia, and other countries. This plant was introduced into China in the early 1970s and cultivated in the Shaanxi and Henan provinces. In 2011, it was introduced into the Zhaosu area of Xinjiang, which has a cultivated area of approximately 1,000 ha per year. The plant produces 30 t of raw material and 60 kg of essential oil per ha (7). Previous phytochemical studies have identified and isolated various bioactive compounds, including flavonoids, volatile oils, fatty acids, triterpenes, and phenolic compounds, from *S. sclarea* frutescens (8). Owing to these biological components, pharmacological effects have been shown, including anti-inflammatory, antioxidant, and antibacterial effects (9). Previous studies have shown that dietary supplementation with *S. sclarea* improves meat quality without adversely affecting growth performance or carcass characteristics (10). However, few studies have investigated the effects of *S. sclarea* extract on lambs. Based on this, this study aimed to provide valuable scientific insights and powerful references for the rational application of *S. sclarea* extract in lamb production by adding different levels of *S. sclarea* extract to the growth performance, apparent nutrient digestibility, serum immunity, and antioxidant indices of lambs.

Materials and methods

All experimental procedures were performed in strict accordance with guidelines and were reviewed and approved by the Institutional Animal Bioethics Committee of Shihezi University (Xinjiang, China).

Experiment material

The *S. sclarea* extract (essential oil) used in the experiment appeared to be a light-yellow liquid. It was prepared by hydrodistillation of the flowers, leaves, and stems of *S. sclarea*. The active ingredients were linalyl acetate (54.79%) and linalool (30.22%), as detected using LC–MS. The purity of the *S. sclarea* extract was 85%. It was produced in the East Industrial Park of Zhaosu County, Xinjiang, China.

Experimental design, animals, and management

Sixty female lambs (Chinese Merino sheep) aged 2 months and weighing 20 ± 2 kg were selected and randomly divided into five groups of 12 lambs each. The control group (CK) received basal feed

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

Ingredient	Content	Nutrition level[b]	Content
Corn	27.2	ME/(MJ/Kg)	12.74
Soybean meal	14.9	CP	15.43
NaCl	0.5	DM	42.11
Alfalfa Hay	21.5	NDF	33.75
Silage Corn	35.5	ADF	3.23
Premix[a]	0.4	Ca	0.28
Total	100	P	0.23

[a]Premix is provided per kilogram of feed. Each kilogram of trace element premix contains 5 mg of $CuSO_4 \cdot 5H_2O$, 30 mg of $FeSO_4 \cdot 7H_2O$, 20 mg of $MnSO_4 \cdot 5H_2O$, 20 mg of $ZnSO_4 \cdot 7H_2O$, 20 mg of KI, 40 mg of Na_2SeO_3, and 50 mg of $CoCl \cdot 6H_2O$. VA 3000 IU, VD 400 IU, VE 90 IU.
[b]Metabolic energy is the calculated according to NRC (11), while other indicators are measured values.

alone, whereas the experimental group was supplemented with different gradients of *S. sclarea* extract in the basal feed at 0.04, 0.08, 0.12, and 0.16 mL/kg (CL1, CL2, CL3, and CL4, respectively). The feeding period was 85 days, including 15 days of pre-feeding and 70 days of normal feeding, and the sheep were fed daily at 06:00 h and 18:00 h. *Salvia sclarea* extract was precisely and uniformly mixed with the ration in strict accordance with the experimental design additive ratios. The sheep were housed in single-cage enclosures (1.5 × 1 × 1 m) with free access to water, and the sheds, water troughs, and troughs were cleaned and sterilized regularly to record daily feed intake and body weight. The composition and nutritional levels of the basal rations are listed in Table 1.

Sample collection and measurement methods

Growth performance

The lambs were weighed on the 1st and lower 70th days of the positive trial period and recorded as the initial body weight (IBW) and final body weight (FBW) of the test lambs, respectively, both on an empty stomach before the morning feeding. During the trial period, the amount of feed and leftovers were recorded in detail for each bureau pen per day, which was used as the basis for calculating the average daily feed intake (ADFI, ADFI = total feed intake/experimental days), average daily gain (ADG, ADFI = total feed intake/experimental days), and feed to weight ratio (F/G, F/G = ADFI/ADG) of the lambs.

Nutrient apparent digestibility

Ration and fecal samples were collected during the last 3 days of the positive trial period. Samples were collected using the partial collection method, in which the ewes in each group were placed in homemade collection bags at 09:00 h and feces were recovered at 17:00 h each day. A sample of 200 g of feces was added to 10 mL of 10% sulfuric acid for nitrogen fixation and stored at −20°C for measurement. The collected grain and manure samples were placed in an oven at 65°C for 48 h, weighed after 24 h of moisture return, and prepared as analytical samples by crushing through an 80 mesh sieve. The dry matter (DM), crude protein (CP), crude ash (Ash), ether extract (EE), Ca, and P contents were determined using the methods of AOAC (2010) (12). The content of neutral and acid detergent fibers (NDF and ADF, respectively) was determined according to the method described by Van Soest et al. (13). The apparent digestibility of nutrients in lambs was determined using the acid-insoluble ash (AIA) endogenous indicator method (14).

Serum biochemical and antioxidant indices

At the end of the experiment, 10 mL of blood was collected from the jugular vein of the sheep using a disposable syringe, and the serum was separated statically and stored frozen (−20°C) for subsequent experiments.

Serum immunity indicators, including antibodies: immunoglobulin G (IgG), immunoglobulin A (IgA) and immunoglobulin M (IgM), cytokines: tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), interleukin-2 (IL-2), were measured using enzyme-linked immunosorbent assay (ELISA) method according to the instructions of the kit, Interleukin-4 (IL-4), Interleukin-6 (IL-6), Interleukin-8 (IL-8) and Interleukin-10 (IL-10) and antioxidant indexes: total antioxidant capacity (T-AOC), superoxide dismutase (SOD), glutathione peroxidase (GSH-Px), catalase (CAT), and malondialdehyde (MDA). The kits were purchased from Nanjing Jianjian Bioengineering Institute. Each sample was tested using an enzyme-labeling analyzer to detect the corresponding absorbance and a standard curve was established according to the manufacturer's instructions to determine the concentration of the target factor.

Statistical analysis

All data were analyzed using one-way ANOVA, linear analysis, and quadratic correlation using the SPSS software (version 22.0; SPSS, Inc., Chicago, IL, United States). Duncan's method was used for multiple comparisons, and the experimental data were expressed as the mean and standard error of the mean (SEM). $p < 0.05$ indicated a significant difference, $p < 0.01$ indicated highly significant difference, and $0.05 \leq p < 0.10$ indicated a trend.

Results

Effect of *salvia sclarea* extract on the growth performance of lambs

No significant difference ($p > 0.05$) was observed in the initial weight of lambs in each group. The final weight of lambs in the CL4 group was significantly higher than that in the CK group ($p < 0.05$), and there was no significant difference ($p > 0.05$) among the CL2, CL3, and CL4 groups. Compared to the CK group, the ADG and ADFI of lambs in the CL3 and CL4 groups supplemented with *Salvia sclarea* extract were significantly higher, and the F/G was significantly lower ($p < 0.05$). The ADG of CL1, CL2, CL3, and CL4 increased by 7.97, 16.56, 23.31, and 34.35%, respectively, compared to that of the CK group (Table 2).

TABLE 2 Effect of *salvia sclarea* extract on the growth performance of lambs.

Items	Treatment					SEM	*p*-value		
	CK Group	CL1 Group	CL2 Group	CL3 Group	CL4 Group		Treat	Linear	Quadratic
Initial weight/kg	19.80	19.70	19.90	19.80	20.40	0.27	0.938	0.661	0.819
Final weight/kg	31.20^{b}	32.05^{ab}	33.29^{ab}	33.94^{ab}	35.78^{a}	1.76	0.014	0.471	0.007
ADG/(g/d)	163.00^{c}	176.00^{c}	190.00^{b}	201.00^{ab}	219.00^{a}	21.71	<0.01	<0.01	<0.01
ADFI/(kg/d)	1.27^{b}	1.31^{ab}	1.35^{ab}	1.40^{a}	1.44^{a}	0.068	0.021	0.043	0.014
F/G	7.79^{a}	7.44^{ab}	7.10^{b}	6.96^{b}	6.58^{c}	0.46	<0.01	<0.01	<0.01

The same superscript letters in peer data indicate no significant differences ($p > 0.05$), while different letters indicate significant differences ($p < 0.05$). CK, control group; CL1: 0.04 mL/kg; CL2: 0.08 mL/kg; CL3: 0.12 mL/kg; CL4: 0.16 mL/kg.

TABLE 3 Effect of *salvia sclarea* extract on apparent digestibility of lambs (%).

Items	Treatment					SEM	*p*-value		
	CK Group	CL1 Group	CL2 Group	CL3 Group	CL4 Group		Treat	Linear	Quadratic
DM	78.80	79.72	79.23	81.80	80.39	1.17	0.610	0.814	0.423
CP	70.20^{b}	72.05^{ab}	73.29^{ab}	74.94^{a}	73.78^{ab}	1.80	0.048	0.313	0.029
NDF	63.34^{b}	66.83^{a}	66.97^{a}	68.84^{a}	65.81^{ab}	2.01	0.043	0.102	0.241
ADF	51.27	53.42	54.13	54.49	53.67	1.25	0.647	0.428	0.460
EE	79.79	81.36	82.63	83.96	82.58	1.56	0.717	0.836	0.517

DM, dry matter; CP, crude protein; NDF, neutral detergent fibre; ADF, acid detergent fibre; EE, ether extract; CK, control group; CL1: 0.04 mL/kg; CL2: 0.08 mL/kg; CL3: 0.12 mL/kg; CL4: 0.16 mL/kg.

Effect of *salvia sclarea* extract on the apparent digestibility of lambs

The apparent digestibility of DM, ADF, and EE in the four experimental groups CL1, CL2, CL3 and CL4 with the addition of *S. sclarea* extract was higher than that in the CK group, but the difference was not significant ($p > 0.05$). The apparent digestibility of NDF in the CL2 group was significantly higher than that in the CK group ($p < 0.05$); however, there was no significant difference among the CL1, CL3, and CL4 groups ($p > 0.05$). The apparent digestibility of CP and NDF in the CL3 group was significantly higher than that in the CK group ($p < 0.05$); however, there was no significant difference between the CL1 and CL3 groups ($p > 0.05$) (Table 3).

Effect of *salvia sclarea* extract on serum antioxidant indices in lambs

Compared with the CK group, the levels of SOD, CAT, GSH-Px, and T-AOC in the serum of lambs in the CL2, CL3, and CL4 groups treated with *S. sclarea* extract were significantly higher, whereas the MDA content was significantly lower ($p < 0.05$). Moreover, with an increase in the amount of *S. sclarea* extract, the content of SOD, CAT, GSH-Px, and T-AOC in the four experimental groups first increased and then decreased, whereas the MDA content showed the opposite trend. The CL1 group with 0.04 mL/kg *S. sclarea* extract had significantly higher CAT and GSH-Px content than the CK group ($p < 0.05$), whereas the content of MDA, SOD, and T-AOC was not significantly affected ($p > 0.05$). The SOD content was significantly higher ($p < 0.05$) in the CL3 group with the addition of 0.12 mL/kg *S. sclarea* extract than in the other four groups (Figure 1).

Effect of *salvia sclarea* extract on serum immunity indices in lambs

Compared with the CK group, the content of IgA, IgG, IgM, IFN-γ, and IL-10 in the serum of lambs in the CL2, CL3, and CL4 groups was significantly higher ($p < 0.05$); that of TNF-α, and IL-1β was significantly lower ($p < 0.05$); and that of IL-2 and IL-6 showed no significant difference ($p > 0.05$). The IL-4 content decreased in the test groups CL1, CL2, CL3, and CL4 with the addition of *S. sclarea* extract; however, the difference between the CL2 and CK groups was significant ($p < 0.05$), whereas the rest were not significantly different ($p > 0.05$). The content of IgA, IgG, IgM, IFN-γ, and IL-10 in the serum of lambs in the CL3 group was higher than that in the other three test groups (Figure 2).

Discussion

Salvia sclarea has long been grown in China as a medicinal and food plant, and has attracted attention for its unique active substances (9). Studies have shown that the aromatic *S. sclarea* is rich in phenolic acids (caffeic and rosemarinic acids), flavonoids, and volatiles (monoterpenes and sesquiterpenes) (15), among other active ingredients that enhance the immunity and

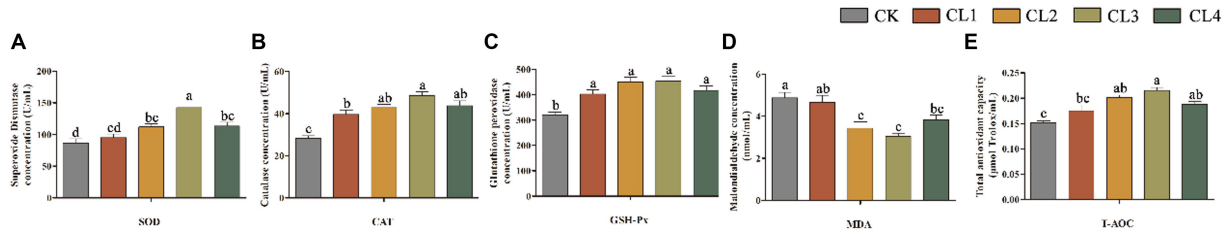

FIGURE 1
Effect of *salvia sclarea* extract on serum antioxidant indexes in lambs. **(A)** SOD; **(B)** CAT; **(C)** GSH-Px; **(D)** MDA; **(E)** T-AOC, CK, control group; CL1: 0.04 mL/kg; CL2: 0.08 mL/kg; CL3: 0.12 mL/kg; CL4: 0.16 mL/kg. Lower case letters in each bar chart indicate significant difference, the same letter indicates no significant difference ($p > 0.05$), and different lower case letters indicate significant difference ($p < 0.05$).

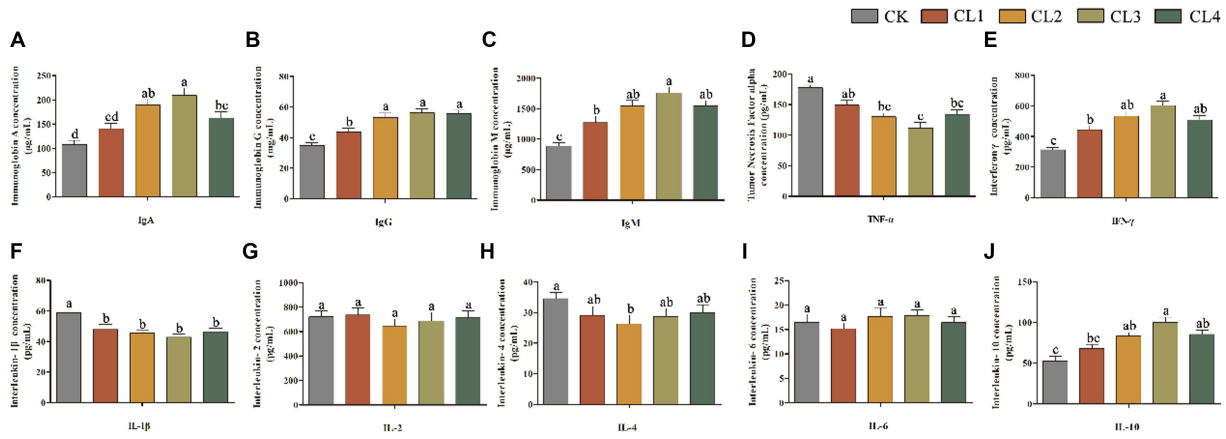

FIGURE 2
Effect of *salvia sclarea* extract on serum immunity indexes in lambs. **(A)** IgA; **(B)** IgG; **(C)** IgM; **(D)** TNF-α; **(E)** IFN-γ; **(F)** IL-1β; **(G)** IL-2; **(H)** IL-4; **(I)** IL-6; **(J)** IL-10, CK, control group; CL1: 0.04 mL/kg; CL2: 0.08 mL/kg; CL3: 0.12 mL/kg; CL4: 0.16 mL/kg. Lower case letters in each bar chart indicate significant difference, the same letter indicates no significant difference ($p > 0.05$), and different lower case letters indicate significant difference ($p < 0.05$).

antioxidant capacity of the animal and promote its growth and development (8). In this study, we found that the addition of *S. sclarea* frutescens extract to lamb rations had a positive effect on the growth performance of lambs by improving feed conversion efficiency and optimizing the nutrient utilization. Moreover, the feed intake of lambs increased with an increase in *S. sclarea* extract addition, and the feed intake significantly increased by 10.24% when the addition reached 0.12 mL/kg. This indicates that the unique aromatic odor due to the richness of terpenes, alkenes, and aromatic compounds improved the palatability of the feed and induced appetite in the lambs (16). Feed palatability can effectively increase the feed intake of lambs, increase their dietary intake, and improve their daily weight gain and feed utilization. Another study showed that phenolics in *S. sclarea* extracts could increase the activity of digestive enzymes by entering the intestinal tract (17). This reduces the number of harmful bacteria in the intestinal tract including *Escherichia coli*, *Staphylococcus aureus*, and *Bacillus subtilis* (18). Beneficial bacteria colonize the intestine, consume free oxygen, create a low-oxygen environment, and inhibit the growth of harmful aerobic bacteria and spoilage microorganisms. The joint action of the two reduces harmful bacteria on the consumption of nutrients to promote growth (19).

The maintenance of good production performance in animals is closely related to the digestion and metabolism of various nutrients in the body. Increasing nutrient digestibility can promote the absorption of nutrients such as DM and CP, thus promoting growth performance to a certain extent. The test group of lambs fed the *S. sclarea* extract had a higher apparent digestibility of CP and NDF than the CK group. No differences in the apparent digestibility of DM, EE, or NDF were found between the diets. In the present study, the concentrations of ingredients in the experimental TMR diet were similar. Therefore, these differences were attributed to the incorporation of the *S. sclarea* extract. There were some differences in the chemical compositions and bioactivities of the diets. The variable response of apparent digestibility in the diet treatment groups may be partly due to the higher CP of these lambs, as these factors positively influenced the degree of digestibility of the rations (20).

A positive correlation between oxidative stress and disease has been widely documented in animals. Antioxidant capacity reflects the body's ability to scavenge free radicals accumulated in cells and tissues and protect the structure and function of cell membranes from damage by peroxides (21). MDA is a product of the lipid peroxidation reaction, and its content reflects the degree of lipid peroxidation in the body, which in turn reflects the degree of cellular attack by free

radicals. The results of our study showed that the addition of *S. sclarea* extract to the ration increased serum CAT, SOD, GSH-Px activity, and T-AOC and decreased serum MDA content in lambs, with the best effect in the 0.12 mL/kg addition group. This indicated that the *S. sclarea* extract improved the performance and health of lambs by effectively reducing oxidative stress. Similar to the results of Deng et al. (22), the addition of *S. sclarea* to the diet of lambs significantly increased T-AOC activity and MDA content in the liver and improved the oxidative status of muscle and meat quality.

Li et al. (23) found that the dietary addition of *S. sclarea* extract increased serum SOD activity and decreased MDA content in the liver, spleen, and jejunal mucosa of piglets. The *S. sclarea* extract used in this study is rich in phenolic acids, flavonoids, and other bioactive components. Phenolic acids can combine with peroxyl radicals to reduce or eliminate free radicals. Phenolic hydroxyl groups can chelate transition metal ions to block biological oxidation (24). Terpenoids can increase the activity of antioxidant enzymes in animals and thus exert their antioxidant capacity (25). Flavonoids such as lignans, rosmarinic acid, and apigenin have powerful antioxidant properties (26). Based on this, we hypothesized that *S. sclarea* extract enhances serum CAT, SOD, and GSH-Px activities in lambs by competitively scavenging reactive oxygen species. Therefore, it has great potential as an antioxidant to prevent oxidative damage during livestock production.

Immunoglobulins are humoral immune effector molecules that play an important role in the immune system of young animals, and serum immunoglobulin levels can be used to evaluate the health status of an organism (27). IgA, IgM, and IgG, play a major role in the immune response. As a class of immunologically active molecules, they specifically bind to antigens and remove them via sedimentation and phagocytosis (28). They mediate immune and inflammatory responses when an animal is infested with bacteria and viruses and play an important role in the body's immune response and immunoregulation (29). In this study, the *S. sclarea* extract affected the immune response in lambs by inducing IgG and IgM production. These responses protect lambs from pathogenic and nonpathogenic immune attacks. Linoleic and linolenic acids are "essential fatty acids" converted into eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). Most studies have shown that EPA and DHA can clinically attenuate T cell immune-mediated inflammatory diseases. And α-linolenic acid enhances the immunity of the body (30). Previous studies demonstrated that essential fatty acids exert anti-inflammatory effects. When the animal body suffers from acute or chronic inflammation, as well as infection, it inhibits the production of prostaglandin 2 by secreting inflammatory factors and plays a role in regulating immune function (31). Liu et al. injected different doses of linolenic acid into the duodenum of dairy cows through a fistula and found that serum IgG levels increased significantly and prostaglandin 2 levels decreased in the 200 g/day dose group (32). Moreover, *S. sclarea* extract contains phenolics that increase leukocyte phagocytosis and can increase the release of IgA, IgM, and IgG. Regulating the expression of cytokines and increasing the expression of antibodies in the serum enhances the ability of the body to clear pathogens, thus strengthening its immune function (33).

As important components of cellular immunity, cytokines are critical for lymphocyte development and the subsequent functional activity of the peripheral immune system (34). Cytokines are produced by immune and non-immune cells and can be categorized into proinflammatory cytokines produced by a variety of immune cells (such as IL-1β, IL-6, and TNF-α) and anti-inflammatory cytokines (such as IL-4, IL-10) (35). Among them, IL-1β can induce cells to secrete inflammatory factors and inflammatory transmitters, triggering the body's inflammatory response (36). IL-6 promotes immune cell differentiation and enhances their functional activity. IL-1β secretes IL-2 via T cells, whereas IL-6 induces IL-2 production by activating the NF-κB signaling pathway through signal transduction. IL-2 is a core substance in the immunoregulatory network of the body, reflecting the initiation of the immune response and playing an important regulatory role in both cellular and humoral immunity (37). IL-10 is an important anti-inflammatory factor secreted by Tregs and is regulated by the immunomodulatory factor IL-4, which can be used to determine the level of immunity in lambs (38). In this study, the addition of *S. sclarea* extract to the ration decreased the levels of proinflammatory cytokines IL-1β and TNF-α and increased the serum anti-inflammatory cytokine IL-10, thereby modulating the immune response. Lignans, a constituent of *S. sclarea* extract, inhibited the production of TNF-α in mouse serum (39). Moreover, linolenic acid, an anti-inflammatory agent in a mouse model of colonic inflammation, had a protective effect against TNBS-induced colitis through the Th1/Th2/Th17 pathway with inflammation-reducing efficacy (40). In addition, a previous study demonstrated that dietary supplementation with phenolic compounds inhibited LPS-induced expression of proinflammatory cytokines IL-1β and IFN-γ through NF-κB and MAPK signaling pathways (41). These results demonstrate the potential of *S. sclarea* extracts to reduce the infection load and inflammatory responses *in vivo*.

Conclusion

In conclusion, the addition of 0.12 mL/kg *S. sclarea* extract to the diet improves the growth performance of lambs by increasing feed intake and nutrient digestibility. It also improves the health status of lambs by increasing their serum antioxidant capacity and immune function. *S. sclarea* extract has great potential as a feed additive in livestock production. It is also worth exploring its potential use in various animal production environments.

Data availability statement

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

Ethics statement

The animal studies were approved by the Institutional Animal Bioethics Committee of Shihezi University. The studies were conducted in accordance with the local legislation and institutional requirements. Written informed consent was obtained from the owners for the participation of their animals in this study.

Author contributions

XM: Writing – original draft. YN: Writing – review & editing. SN: Writing – review & editing. WZ: Writing – review & editing.

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Conflict of interest

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

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Effects of dietary supplementation with alkaline mineral complex on *in vitro* ruminal fermentation and bacterial composition

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Introduction: Dairy industry growth faces challenges in China due to inadequate forage, leading to high-concentrate diets and potential rumen issues. Buffering agents, like sodium bicarbonate, play a crucial role in stabilizing rumen pH. Alkaline Mineral Complex (AMC), a liquid additive with a pH of 14, shows promise in supporting dairy cow health and mitigating heat stress through ionization.

Methods: This experiment was aimed to study the effect of adding AMC to total mixed ration (TMR) on *in vitro* ruminal fermentation and bacterial composition. AMCat 1, 2, 4, and 8 mL/kg was added to the substrate (0.5 g TMR). Nutrient digestibility was measured after 48 h fermentation, and fermentation parameters and microbial composition were measured after 48 h fermentation.

Results and discussion: The results of the experiment indicated that: The different concentrations of AMC showed a significant impact on time taken for gas production to reach 1/2 of the total gas production (HT) parameters ($p < 0.05$). Linear pH increase occurs at 6 and 24 h with rising AMC concentration ($p < 0.05$), showing a quadratic trend at 12 h ($p < 0.05$). The optimal buffering effect on rumen acid-base balance was observed at a 2 mL/kg concentration of AMC. Microbial diversity analysis indicated that there was no significant change in α-diversity with different AMC concentrations ($p > 0.05$). The microbial level demonstrated no significant difference in species diversity of rumen fluid bacteria among the various AMC concentration treatment groups compared to the control group, further supporting that the advantage of adding AMC in stabilizing the rumen environment without altering the structure of the rumen microbiota. Besides, the addition of AMC significantly increased the concentrations of acetate, propionate, total fatty acids (TVFA), and NH3-N, suggesting that AMC contributed to enhancing the energy and nitrogen utilization efficiency in ruminants. Based on the above detection indicators, we recommend that the most favorable concentration is 2 mL/kg.

KEYWORDS

lactating ruminants, rumen preference parameters, additive concentration, subacute rumen acidosis, dairy cows

1 Introduction

The expansion of the dairy industry has been aided by the increasing demand for dairy products. However, the lack of high-quality forage in China makes it difficult to measure the nutritional requirement of lactating ruminants. Many ranches have decided to increase the proportion of concentrate feed to meet this requirement. High-concentrate diets have a

relatively low effective fiber content and can easily induce subacute rumen acidosis (SARA) in ruminants (1). SARA is a metabolic disorder in animals, and it is characterized by rumen fluid pH values that are consistently <5.8 and persist for more than 4 h after feed consumption (2, 3). This phenomenon, which the rumen is unable to effectively neutralize, is mainly caused by an excessive intake of highly fermentable carbohydrates. It severely impairs the lactation performance of dairy cows, leading to substantial financial losses for the pasture. Additionally, it can also cause other diseases such as mastitis, which endangers animal health. Hence, it is critical to address the adverse effects of high-concentrate diets and emphasize the importance of balancing the physiological wellbeing and productivity of lactating dairy cows.

A buffer is a type of compound or mixture that enhances the acid-base buffering capacity of a solution. In ruminant animal production, it is important to maintain the pH in the rumen at a stable level of 5.8–6.2 to support the activity of rumen microorganisms (4). To maintain the normal rumen fermentation performance of cow-fed high-concentrate diets, strongly alkaline and weakly acidic salts are typically used as buffering agents to prevent rumen acidosis and improve their productivity. Some buffering agents, commonly used in ruminants both domestically and internationally, include sodium bicarbonate, magnesium oxide, sodium acetate, sodium butyrate, calcium carbonate, and other minerals. Composite buffering agents have more efficient pH regulation ability than single buffering agents. Neiderfer et al. revealed that the supplementation of the daily diet of lactating cows with $CaCO_3$, MgO, and coated $NaHCO_3$ effectively maintained their rumen fluid pH (5). Similarly, Snyder et al. observed that the addition of $NaHCO_3$ and its composite buffering agent to the diet of lactating cows enhanced their milk production and milk fat percentage (6).

Alkaline mineral complex (AMC) is a colorless, tasteless, and non-toxic complex alkaline ion mixture with a pH of 14 (7–9). It is a liquid feed additive that helps cows maintain the acid-base balance of ruminal fluids, preserves the normal function of cellular ion pumps, and improves immunity. It activates immune cells by enhancing neuromuscular physiological information transmission and physiological regulatory functions, thereby alleviating heat stress in dairy cows. The ions generated by the ionization of AMC solution jointly regulate H^+ in the rumen. Despite its limited application in dairy cows, this composite buffering agent has a promising potential (9).

Therefore, the objective of this study was to investigate the effects of different concentrations of AMC on fermentation characteristics and bacterial composition *in vitro* to establish the optimal additive concentration for large-scale feeding applications in dairy herds.

2 Materials and methods

2.1 Animals and their feeding management

The rumen fluid was collected from three healthy, mid-lactating, and rumen-cannulated Holstein dairy cows with similar milk yield (26 ± 1.63 kg/d) from Zhongdi Dairy Holdings Co., Ltd. (Beijing, China). The dairy cows had *ad libitum* access to feed and water. The total mixed ratio (TMR) was fed to the cows three times daily (07:00, 14:00, and 19:00), and the cows were milked three times a day at 06:30, 13:30, and 18:30. All the animal procedures were approved by the Institutional Animal Care and Use Committee of China Agricultural University (approval number: AW61902202-1-4).

2.2 Experimental design

2.2.1 Fermentation substrates

All fermentation substrates (donor cows' TMR) were crushed and kept in the oven at 65°C for 48 h (10, 11). After drying, the samples were crushed and sieved through a 1 mm screen for subsequent fermentation processes, and the chemical composition was determined using the Association of Official Analytical Chemists (AOAC) methods (12). The ingredients and nutrient compositions of all the fermentation substrates are shown in Table 1.

2.2.2 AMC

The AMC used in this study was provided by Beijing Jinaer Biotechnology Co., Ltd. The AMC utilizes zinc oxide and germanium compounds as cell activators in combination with sodium and potassium compounds. The elements, such as Si, Ge, K, and Zn, in the alkaline solution remain in ionic and water-soluble states, thereby maintaining a weak alkaline internal environment for the animals. The composition and mineral ion contents are shown in Tables 2, 3, respectively.

2.2.3 Rumen fluid collection

The rumen fluid was collected 2 h after morning feeding. The collected rumen fluid was filtered through four layers of gauze and placed in a thermos for quick return to the laboratory. It was then transferred into a 4 L beaker filled in advance with CO_2 at 39°C in a water bath (11).

2.2.4 In vitro degradability

The test was divided into five groups with three replicates for measuring gas production and pH. The substrate degradation experiment was performed using five replicates per group. Except for the control group, AMC was added to each group at 1, 2, 4, and 8 mL/kg of the substrate.

For every *in vitro* gas production experiment, a total of 500 mg fermentation substrate, 25 mL of rumen fluid, and 50 mL of buffer (13) were added to a 120 mL anaerobic fermentation bottle. For the other fermentation bottles, 3 g samples from each treatment were individually placed into 250 mL glass bottles, which contained 150 mL of buffer solution and 75 mL of rumen fluid.

Each bottle was immediately sealed with butyl rubber stoppers and Hungate's screw caps after the addition of the experimental samples, and nitrogen was injected until oxygen was discharged.

The gas production bottles were placed inside a 39°C constant temperature incubator and were immediately connected to the corresponding gas channels of the AGRS-III system according

TABLE 1 Fermentation substrate composition and nutrients (dry matter basis, %).

Items[a]	Contents
Ingredients, % of DM	
Alfalfa hay	3.74
Alfalfa silage	1.72
Whole corn silage	32.31
Steam-flaked corn	14.86
Corn	10.18
Whole cottonseed	1.77
Extruded soybean meal	12.58
Soybean hull	10.72
DDGS	3.77
Fat powder	0.75
Corn gluten meal	2.56
Molasses	0.32
$NaHCO_3$	0.57
Premix	4.14
Total	100
Nutrient levels,% of DM	
NE_L (MJ/kg)[b]	7.28
Concentrate to forage ratio	47:53
CP	16.45
Ether extract	5.1
Ash	6.7
NDF	40.94
ADF	30.81

[a]EE, ether extract; CP, crude protein; NDF, neutral detergent fiber; ADF, acid detergent fiber.
[b]NE_L, net energy of lactation, these data in the diet are calculated by multiplying the net energy produced by each raw material and its proportion in the diet.

TABLE 2 The composition of alkaline mineral complex (AMC) water concentrate.

Ingredients	Chemical formula	Contents (mg/L)
Sodium metasilicate pentahydrate	$5H_2O{\cdot}Na_2SiO_3$	200
Potassium bicarbonate	KHCO3	100
Zinc oxide	ZnO	0.01
Bis-(carboxyethylgermanium) sesquioxide	Ge-132	0.001

TABLE 3 The mineral ion content of AMC water.

Ions	Calculated contents (mg/L)
$SiO3^{2-}$	179.25
Na^+	108.49
K^+	97.50
Zn^{2+}	0.02
Ge^{4+}	0.0005
HCO_3^-	152.50

to the pre-arranged inoculation order (14). Furthermore, the gas production (GP) was automatically recorded throughout the 48 h of fermentation. All the bottles were kept in a thermostatic incubator to ferment continuously for 6, 12, 24, 36, and 48 h. After 24 and 48 h of fermentation, the bottles were removed from the incubator. At every time point, the fermentation was halted by placing the bottles in a mixture of ice and water for 15 min.

2.3 Sample collection and measurement

The pH was measured five times at the five fermentation time points. After incubation, the contents of each bottle were filtered using a filter with 42 μm pores (sized 80 × 150 mm). As described in previous studies (12, 13), the volatile fatty acid concentration in the supernatant was determined using gas chromatography, and NH_3-N was measured using a spectrophotometer. The remaining samples were kept at −80°C. One sample was used for further microbial community analysis, while others were used to measure microbial crude protein (MCP).

2.4 DNA extraction and determination

Total microbial genomic DNA was extracted using a kit from MP Biomedicals, Solon, OH, USA, and the NanoDrop® ND-2000 spectrophotometer (Thermo Scientific Co., Ltd., Waltham, Massachusetts, USA) was used to assess the DNA purity and concentration. Additionally, DNA integrity was assessed using 1% agarose gel electrophoresis.

The V3-V4 region of the *16S rRNA* gene was amplified with forward primer 338F (5′-ACTCCTACGGGAGGCAGCAG-3′) and 806R (5′-GGACTACH VGGGTWTCTAAT-3′) through the polymerase chain reaction (PCR). For each sample, three PCR replicates were mixed, and 5 mL of the PCR product from each sample was detected using 2% agarose gel electrophoresis (15, 16). The PCR products were purified using an AxyPrep DNA Gel Extraction Kit (AP-GX-250, Axygen Biosciences, Union City, USA) and were quantified using a quantum fluorometer (E6150, Promega, WI, USA).

Finally, the amplicons were sequenced using a MiSeq pe300 platform (Illumina, Inc., San Diego, California, USA). Quality control (QC) and splicing of the original sequence and ASV representative sequences were clustered according to 97% similarity using UPARSE software (version7.0.1090,http://drive5.com/uparse/), and UCHIME software (version7.0, http://www.drive5.com/usearch/) was applied to eliminate the chimera (17).

The sequences containing more than 10% unknown nucleotides were excluded from the subsequent analysis. The paired-end clean tags were combined into raw tags using FLASH

v. 1.2.11 software, following the methodology outlined by Magoč and Salzberg (18). The merging process had a minimum overlap of 10 bp, and a mismatch rate of 0.1 was used to generate Fasta sequences.

The sequencing data were saved in the form of a FASTQ file. The sequences were subjected to ASV clustering at a 97% similarity threshold using UPARSE 7.1 (19), and the chimeras were removed. The taxonomy annotations of ASV of species were classified and annotated using the Ribosomal Database Project (RDP) (http://rdp.cme.msu.edu/) (17) against the Silva 16S rRNA gene database (v138) with a confidence threshold of 70%.

2.5 Calculation and analyses

The corresponding cumulative gas production (GP, mL/g, dry matter basis) was fitted non-linearly with each fermentation time using the exponential model described by France et al. (20) as follows:

$$GP_t = A[1 - e^{-c(t-lag)} \quad (1)$$

GP_t: where GP_t (mL) is the total gas production (mL/g dietary DM) over time t, A is the maximum gas production of the fermentation substrate at a gas production rate c (h^{-1}) (mL), and lag is the delay time of fermentation gas production (h).

For the AGPR, the average gas production rate is as follows:

$$AGPR = A \times \frac{c}{\log 2 + c \times lag} \quad (2)$$

where A, c, and lag are the same as those in Equation (1).

AGPR, Average gas production rate when half of the ideal maximum gas production is achieved (mL/h).

The test data obtained were preliminarily collated using Excel 2020 and analyzed using the mixed model in SAS 9.4 (21). The standard error (SEM) of the least-squares mean of each measurement indicator was determined using LSMENAS statements, and multiple comparisons were performed using Duncan's test. The minimum significant difference method was used for comparisons when the difference was significant ($p < 0.05$), and $0.05 < p < 0.1$ indicates that the data have a significant downward or upward trend.

The alpha diversity analysis at the ASV level was conducted using Mothur v1.30.1 (22) software. Differences in the α diversity index between different types were obtained using the Wilcoxon rank sum test.

In the beta diversity analysis, principal component analysis (PCA) based on the Bray-Curtis distance algorithm was used to test for discrepancies in microbial communities at the ASV level between different groups (23). The non-parametric Kruskal–Wallis rank sum test was used to detect the genera with significant differences in abundance between different groups, and the consistency of the differences in different genera was subjected to the Tukey–Kramer test in different subgroups between the groups. Additionally, hypothesis testing was performed to evaluate the genus abundance between multiple groups. These analyses revealed genus information that showed significant differences among the treatment groups.

The data were analyzed using the online platform Majorbio Cloud Platform (www.majorbio.com).

3 Results

3.1 Gas production kinetics parameters

Table 4 presents an overview of the different concentrations of AMC *in vitro* gas production kinetics parameters. Through data analysis, it was found that HT had a linear trend of growth at different concentrations of AMC ($p < 0.01$), whereas there was no significant difference between the AMC_1 and AMC_2 groups and the control group. This indicates that the addition of AMC at a concentration of 1 and 2 mL/kg did not affect the rumen fermentation efficiency.

3.2 Fermentation parameters

As shown in Table 5, with an increase in AMC concentrations, the pH of the rumen fluid increased linearly at 6 h and 24 h ($p < 0.05$), whereas the pH at 12 h showed a quadratic trend ($p < 0.05$). In addition, different concentrations of AMC had no significant effect on pH at 36 h and 48 h ($p > 0.05$). The data showed that AMC had a good buffering effect before 24 h, and this indicated a stabilizing effect on rumen pH. After 24 h, the rumen pH of both the control and treatment groups tended to stabilize.

Figure 1 shows the impact of the AMC concentration on the profiles of the fermentation parameters during the 48 h of fermentation. The data revealed a quadratic trend for all VFAs and TVFA at different concentrations of AMC, except for isobutyric acid ($p < 0.05$). With an increase in the concentration of AMC, the levels of acetate, propionate, butyrate, valerate, and isovaleric acid increased and subsequently decreased, with the highest value observed in AMC_3. The ratio of acetate to propionate showed an initial decrease, followed by an increase with increasing concentrations of AMC. The AMC_2 and AMC_3 groups tended to exhibit more propionic acid-type fermentation ($p < 0.05$). The influence of different AMC concentrations on MCP was not significant ($p > 0.05$).

3.3 *In vitro* degradability

Table 6 presents the effects of different AMC concentrations on nutrient degradability. The results showed that AMC has a negative effect in promoting ADF degradation during *in vitro* fermentation but has no significant effect on DM, NDF, or CP parameters. The acid detergent fiber (ADF) linearly increased with the concentration of AMC ($p < 0.05$).

TABLE 4 Effects of different concentrations of AMC on gas production kinetics parameters.

Items	Groups					SEM	*P*-value		
	CON	AMC_1	AMC_2	AMC_3	AMC_4		G	L	Q
GP_{48} (mL)	106.99	123.15	113.04	111.08	106.47	2.246	0.113	0.379	0.067
A (mL)	102.21	117.37	111.69	110.46	103.97	2.337	0.242	0.833	0.052
HT (h)	2.25^{c}	2.23^{c}	2.45^{bc}	2.61^{ab}	2.85^{a}	0.06	0.001	<0.001	0.238
AGPR (mL/h)	33.00	39.22	34.28	30.48	23.73	2.134	0.228	0.074	0.162

CON, no supplementation; AMC_1, the AMC concentration in the substrate is 1 mL/kg; AMC_2, the AMC concentration in the substrate is 2 mL/kg; AMC_3, the AMC concentration in the substrate is 4 mL/kg; AMC_4, the AMC concentration in the substrate is 8 mL/kg; G, group effect; L, linear effect; Q, quadratic effect; GP_{48}, the total gas production (mL/g dietary DM) over 48 h; A, the maximum gas production of the fermentation substrate at the gas production rate c (h^{-1}); HT, time taken for gas production to reach 1/2 of the total gas production; AGPR, average gas production rate when half of the ideal maximum gas production produced. In peer data, different lowercase letters on the shoulder indicate significant differences ($P > 0.05$), while the same or no letters indicate insignificant differences ($P > 0.05$).

TABLE 5 Effects of different concentrations of AMC on the pH *in vitro* fermentation.

Items	Groups					SEM	*P*-value		
	CON	AMC_1	AMC_2	AMC_3	AMC_4		G	L	Q
6 h	6.58^{c}	6.61^{bc}	6.65^{ab}	6.61^{bc}	6.68^{a}	0.012	0.024	0.005	0.964
12 h	6.52^{ab}	6.53^{a}	6.53^{a}	6.50^{b}	6.48^{c}	0.005	0.001	0.004	0.001
24 h	6.58^{b}	6.67^{a}	6.65^{a}	6.61^{ab}	6.67^{a}	0.010	0.004	0.047	0.089
36 h	6.49	6.50	6.54	6.51	6.52	0.009	0.500	0.269	0.433
48 h	6.65	6.63	6.67	6.64	6.67	0.011	0.671	0.518	0.585

CON, no supplementation; AMC_1, the AMC concentration in the substrate is 1 mL/kg;AMC_2, the AMC concentration in the substrate is 2 mL/kg; AMC_3, the AMC concentration in the substrate is 4 mL/kg; AMC_4, the AMC concentration in the substrate is 8 mL/kg; G, group effect; L, linear effect; Q, quadratic effect. In peer data, different lowercase letters on the shoulder indicate significant differences ($P > 0.05$), while the same or no letters indicate insignificant differences ($P > 0.05$).

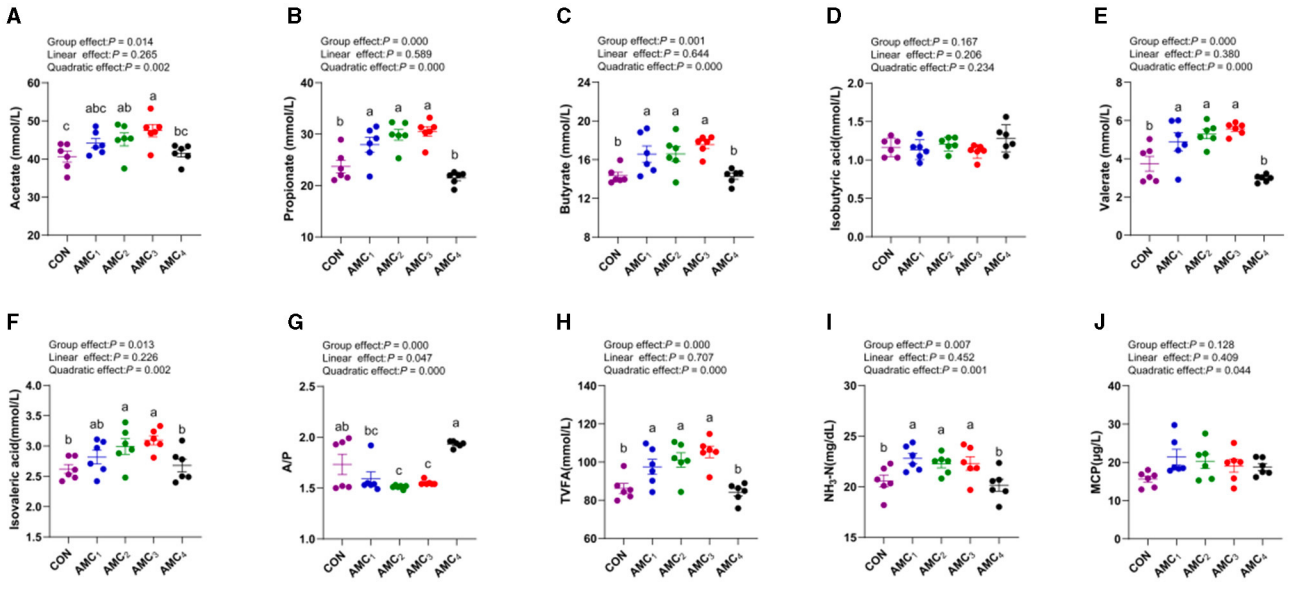

FIGURE 1
Effects of different concentrations of AMC on fermentation parameters. **(A)** Acetate content; **(B)** Propionate content; **(C)** Butyrate content; **(D)** Isbutyric content; **(E)** Valerate content; **(F)** Isovaleric acid content; **(G)** Acetate/Propionate; **(H)** TVFA-total volatile fatty acids; **(I)** NH_3-N:Ammonia nitrogen; **(J)** MCP:Microbial protein. CON, no supplementation; AMC_1, the AMC concentration in the substrate is 1 mL/kg; AMC_2, the AMC concentration in the substrate is 2 mL/kg; AMC_3, the AMC concentration in the substrate is 4 mL/kg; AMC_4, the AMC concentration in the substrate is 8 mL/kg; G, group effect; L, linear effect; Q, quadratic effect; A/P, the ratio of Acetate and Propionate; TVFA, total volatile acids; NH_3-N, ammoniacal nitrogen; MCP, microbial crude protein.

TABLE 6 Effects of different concentrations of AMC on the nutrient degradability *in vitro* fermentation.

Items	Groups					SEM	*P*-value		
	CON	AMC_1	AMC_2	AMC_3	AMC_4		G	L	Q
DM	66.53	68.72	67.98	67.19	66.52	0.391	0.281	0.572	0.083
NDF	54.39	52.32	51.43	52.44	49.91	0.663	0.314	0.066	0.856
ADF	22.30[a]	24.71[b]	25.27[b]	26.75[c]	26.86[c]	0.444	0.002	<0.001	0.215
CP	62.52	66.70	64.98	66.60	63.45	0.630	0.109	0.648	0.028

DM, dry matter; NDF, neutral detergent fiber; ADF, acid detergent fiber; CP, crude protein; CON, no supplementation; AMC_1, the AMC concentration in the substrate is 1 mL/kg; AMC_2, the AMC concentration in the substrate is 2 mL/kg; AMC_3, the AMC concentration in the substrate is 4 mL/kg; AMC_4, the AMC concentration in the substrate is 8 mL/kg; G, group effect; L, linear effect; Q, quadratic effect. In peer data, different lowercase letters on the shoulder indicate significant differences ($P > 0.05$), while the same or no letters indicate insignificant differences ($P > 0.05$).

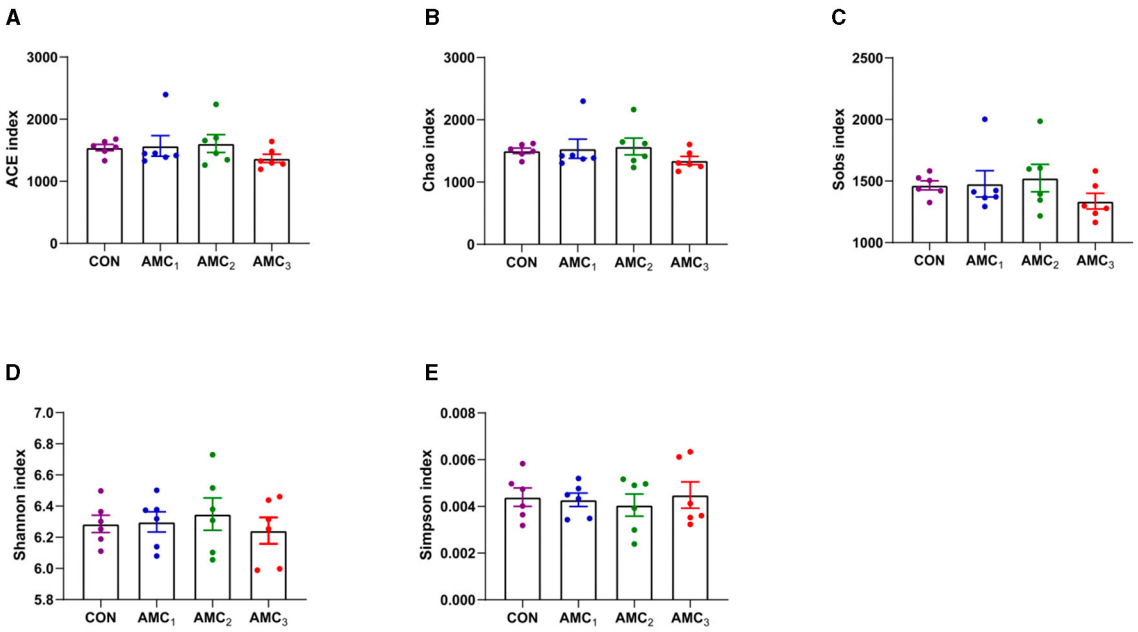

FIGURE 2
Effects of different concentrations of AMC on the nutrient degradability *in vitro* fermentation. **(A)** ACE diversity index; **(B)** Chao 1 diversity; **(C)** Sobs index; **(D)** Shannon diversity index; **(E)** Simpson index. CON, no supplementation; AMC_1, the AMC concentration in the substrate is 1 mL/kg; AMC_2, the AMC concentration in the substrate is 2 mL/kg; AMC_3, the AMC concentration in the substrate is 4 mL/kg.

3.4 Microbial diversity

Figure 2 shows the effects of different concentrations of AMC on the alpha diversity index *in vitro* fermentation. For the alpha diversity index, there was no significant difference between the treatment group and the CON group ($p > 0.05$).

In addition, there was no distinct separation between the different supplementation groups and the CON group in the PCoA plot based on Bray–Curtis staining (Figure 3, $p > 0.05$). These results indicated that there were no significant differences in the species diversity of rumen fluid bacteria among the different concentrations of AMC.

Figure 4 shows an overview of the genus composition of the microbiota. The abundances of *Prevotella*, *Rikenellaceae-RC9-gut-group*, and norank-f–F082 were found to be enriched in different groups.

Figure 5 shows the microbial composition at the genus level under the different AMC treatments. As shown in Figure 5B, the relative abundance of Bacteroidales-RF16 in the AMC_1 and AMC_3 groups was significantly lower than that in the CON group ($p < 0.05$). Furthermore, the relative abundance of Lachnospiraceae UCG-008 in the AMC_1 group was significantly lower than that in the CON group (Figure 5C, $p < 0.05$). Regarding Prevotellaceae-Ga6A1 and Lachnospira, different treatments revealed a significant impact on their abundance (Figure 5A, $p < 0.05$). However, the difference between the groups was not statistically significant (Figure 5D, $p > 0.05$). The relative abundance of the CON group was lower than that of the other treatment groups.

4 Discussion

In this study, we focused on one of the prerequisites for normal rumen fermentation, which is the normal pH range of rumen fluid (5.5–7.5) (24). By adding AMC at different concentrations during *in vitro* fermentation of the rumen fluid, we observed that the pH of the rumen fluid, when fermented *in vitro* with different concentrations of AMC, ranged from 5.8 to 7.0, which is consistent

with the optimal range of rumen pH. Notably, the addition of 1–2 mL/kg of AMC significantly elevated rumen pH at 6 and 24 h, suggesting that AMC had a significant buffering effect on rumen pH, particularly within the first 24 h of supplementation. Furthermore, the buffering effect of AMC stabilized after 24 h, as both the control and treatment groups exhibited a tendency toward stable rumen pH levels after this time point.

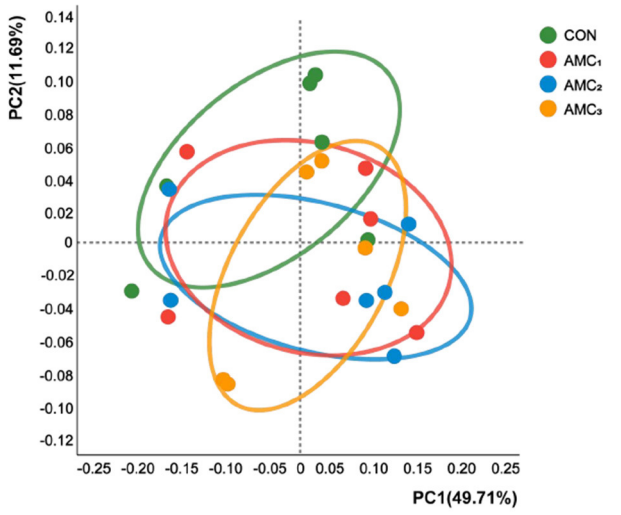

FIGURE 3
Principal coordinate analysis (PCoA) combined with permutational multivariate analysis of variance (PREANOVA) was calculated based on the ASV level and Bray–Curtis distances. CON, no supplementation; AMC_1, the AMC concentration in the substrate is 1 mL/kg; AMC_2, the AMC concentration in the substrate is 2 mL/kg; AMC_3, the AMC concentration in the substrate is 4 mL/kg.

GP_{48} and HT are important indicators of rumen fermentation capacity and nutrient digestibility. Through our experiments, we found that AMC had a significant impact on the HT, consistent with the results of the pH. Specifically, when the rumen microbiota metabolizes to produce excess hydrogen ions, AMC can neutralize these ions, maintaining the solution's pH at a relatively stable level. This provides a suitable environment for promoting microbial activity and gas production processes.

VFAs, the main products of rumen fermentation, serve as the main energy sources and raw materials for synthetic and milk fats. Acetic and butyric acids are mainly used for milk fat synthesis, whereas propionic acid serves as a precursor for glucose synthesis and can competitively consume hydrogen to reduce methane production (14). Propionic acid is rapidly oxidized in the liver to produce energy. Similar to other short-chain fatty acids, propionic acid is a product of intestinal microbial fermentation of fiber and other indigestible carbohydrates, which is crucial for maintaining intestinal health and function. Valeric acid, isovaleric acid, and isobutyric acid, collectively categorized as short-chain VFAs with four to five carbon atoms, are referred to as branched-chain VFAs (25). The results in the present study indicated that all VFAs in the fermentation broth, except isobutyric acid, showed a quadratic change with the increasing concentration of AMC. As the AMC concentration increased, the acetic, propionic, butyric, valeric, and isovaleric acid contents increased and then decreased, and the A/P ratio first decreased and then increased. Although the ammonia nitrogen concentration remained unchanged, the total VFA concentration increased in the group supplemented with bicarbonate, indicating that the addition of a combination of buffer altered the liquid turnover and the rumen fermentation mode (26), which was beneficial for providing energy for ruminants. This trend can be attributed to an increase in *Prevotellacea-Ga6A1*. Previous studies have shown that Prevotella metabolizes hemicellulose, pectin, and proteins, with acetic and formic acids

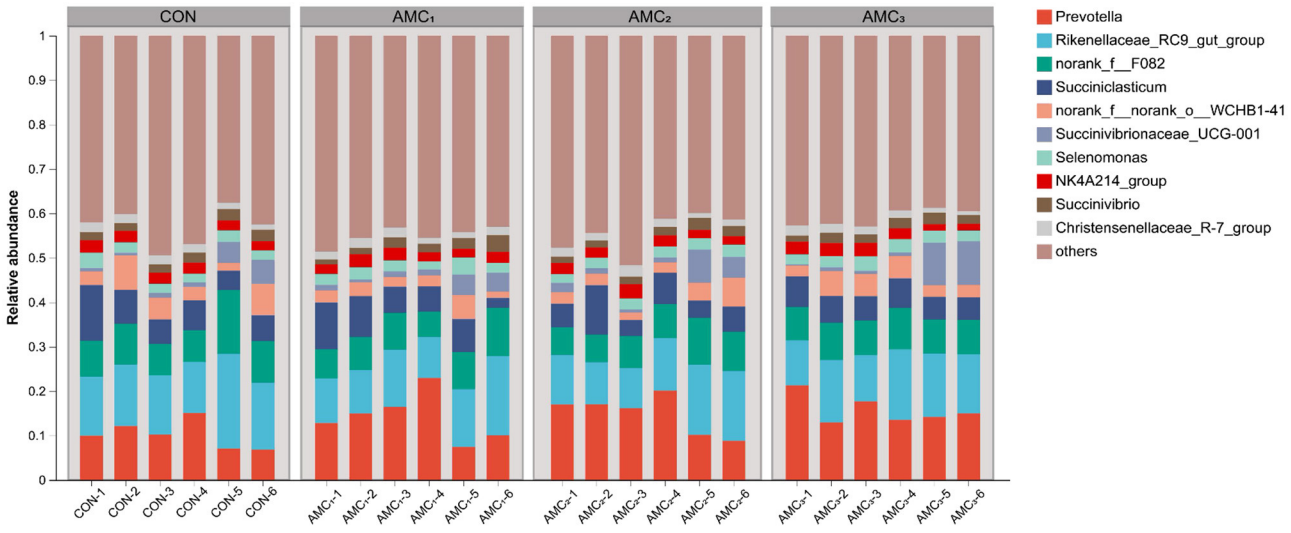

FIGURE 4
Genera composition of the microbiota from *in vitro* fermentation technique; CON, no supplementation; AMC_1, the AMC concentration in the substrate is 1 mL/kg; AMC_2, the AMC concentration in the substrate is 2 mL/kg; AMC_3, the AMC concentration in the substrate is 4 mL/kg.

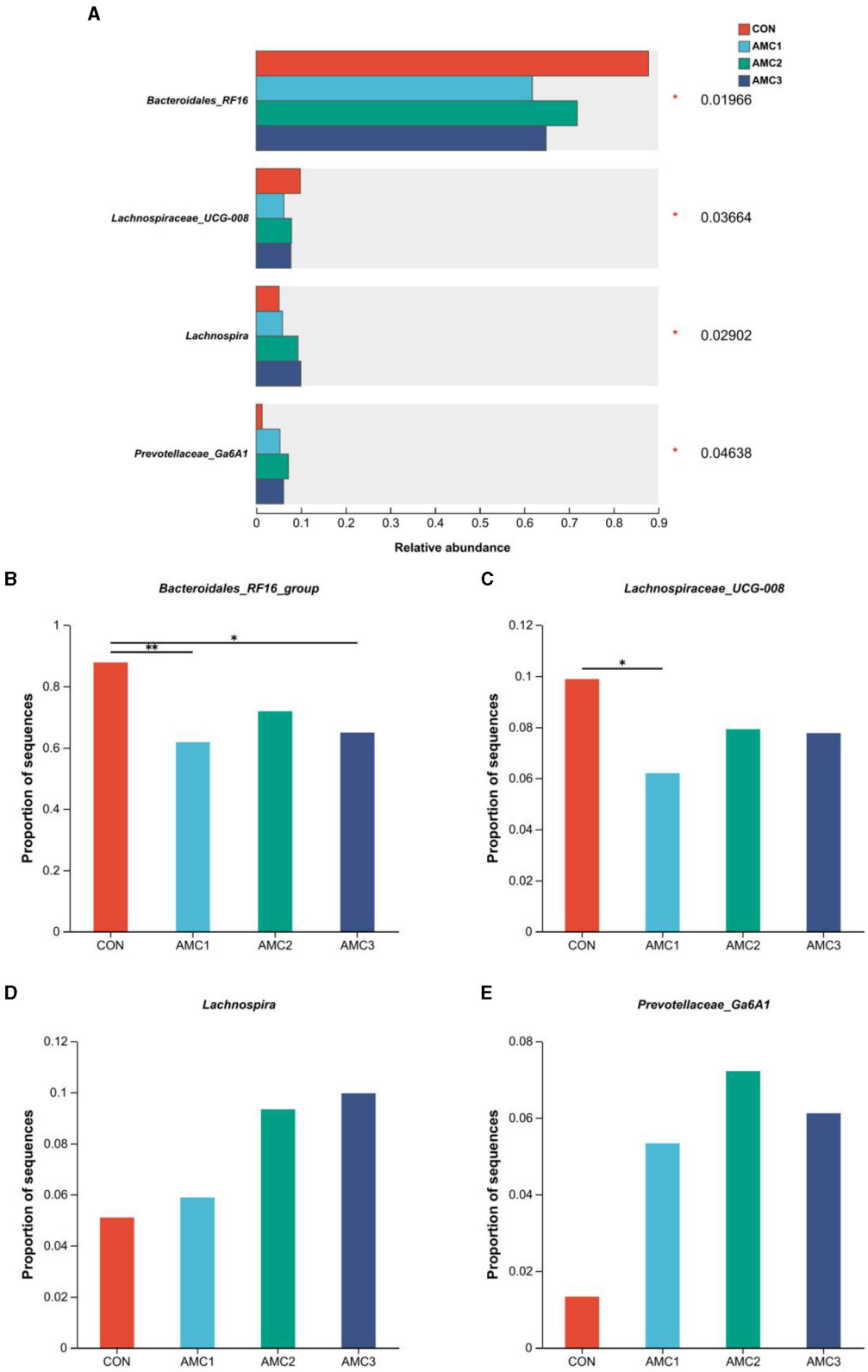

FIGURE 5
Bacterium with significant differences in species abundance at the genus level **(A)**, relative abundance and *p*-value cut-off were <0.05; Analysis of differences in bacteria between any two groups **(B–E)**: *0.01 < $p \leq 0.05$, **0.001 < $p \leq 0.01$, ***$p \leq 0.001$; CON, no supplementation; AMC_1, the AMC concentration in the substrate is 1 mL/kg; AMC_2, the AMC concentration in the substrate is 2 mL/kg; AMC_3, the AMC concentration in the substrate is 4 mL/kg.

being the main fermentation products (27). The increase in these short-chain fatty acids led to a trend toward propionic acid-type fermentation in the rumen, indicating that more propionic acid provides energy through gluconeogenesis, which is particularly important for maintaining the energy balance in ruminants. High levels of propionic acid can inhibit milk fat synthesis, but this needs to be validated via *in vitro* experiments. These findings support the significant role of buffering agents in maintaining energy balance and promoting rumen health in ruminants.

The competitive dynamics observed between fiber-degrading bacteria in the phylum Bacillota and the genus *Prevotella*, with an increased relative abundance of *Prevotellacea-Ga6A1*, correspond to our results and suggest an inhibitory effect on the growth of *Lachnospiraceae UCG-008*. *Lachnospira* can also degrade polysaccharides and fiber contents to produce acetic acid. The observed discrepancies in ADF and VFA may be attributed to an increase in *Lachnospira* abundance (28). This indicated that AMC supplementation promoted the growth of beneficial bacteria in rumen microorganisms and the reproduction and metabolism of acetic acid-producing bacteria. AMC facilitated the fermentation and decomposition of carbohydrates, thereby promoting the metabolism and absorption of nutrients by dairy cattle.

NH_3-N is produced by the fermentation of protein, non-protein nitrogen, and other nitrogenous compounds, and this can reflect the degree of rumen nitrogen metabolism (29). Inappropriate concentrations of NH_3-N affect animal health. The appropriate range of rumen NH_3-N concentration had been reported to be 6–30 mg/dL (30), and the result of the present study showed that the concentration of NH_3-N are all within a reasonable range. MCP is the predominant nitrogen source for dairy cattle, contributing 60–80% of the required protein. This reflects the microbial utilization of NH_3-N and indicates the abundance of microorganisms (31). Ample nitrogen sources, provision of VFAs as carbon scaffolds, and fermentation of organic matter have a collaborative effect on the synthesis efficiency and quantity of MCP (32). In this study, the addition of AMC significantly influenced the concentration of NH_3-N. Additionally, NH_3-N and MCP exhibited a quadratic trend with increasing AMC, indicating that AMC promoted rumen microorganisms to comprehensively utilize nutrients in the fermentation substrate.

The PCoA analysis revealed no significant differences in the microbial community structure between the treatment and control groups, suggesting the relatively mildness of the buffer. It is essential to emphasize that the subtle effects of the buffering agents do not imply a lack of impact on the microorganisms in all scenarios. These variations may be associated with the differences between individual samples, sample sizes, and the simulated environment of the *in vitro* experiments. It is imperative to conduct further *in vivo* experiments to validate the efficacy of this buffer.

Data availability statement

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

Ethics statement

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

Author contributions

SL: Methodology, Software, Visualization, Writing – original draft, Writing – review & editing. BX: Data curation, Formal Analysis, Methodology, Writing – review & editing. HJ: Formal Analysis, Methodology, Visualization, Writing – review & editing. SL: Funding acquisition, Writing – review & editing.

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Conflict of interest

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

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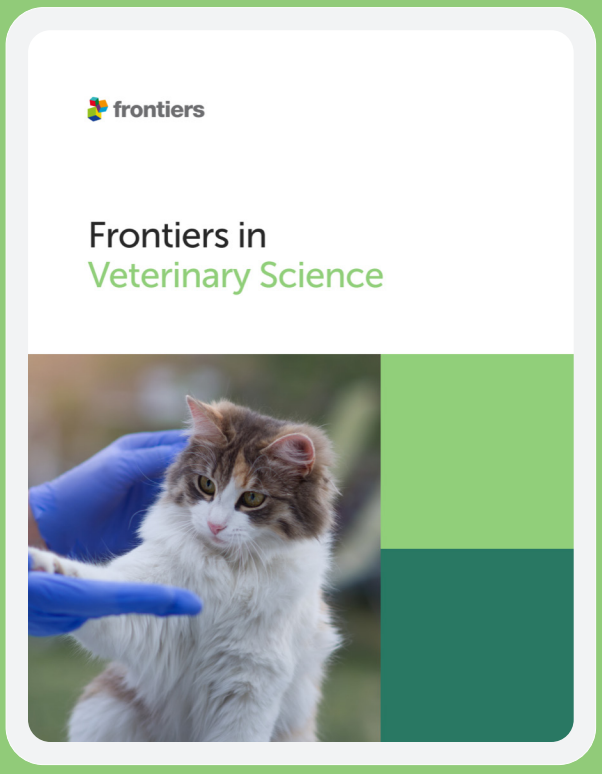

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